ABSTRACT

Title of Dissertation:	INVESTIGATION OF DISRUPTED INSULIN SIGNALING IN A SWINE MODEL
	Grace Irene Markley, Doctor of Philosophy, 2024
Dissertation directed by:	Professor, Chad Stahl PhD, Animal and Avian Sciences

Insulin is an anabolic hormone involved in glucose uptake and synthesis of fats, proteins, and glycogen. Domesticated livestock species such as swine require efficient insulin signaling to meet production demands across the world. Insulin signaling is tightly regulated and acts on metabolic tissues such as the liver, skeletal muscle, and adipose tissue. The most well characterized disruption of insulin signaling is insulin resistance that is often caused by obesity induced inflammation. However, insulin signaling can be disrupted via atypical mechanisms such as immune response to a pathogen and adaptor proteins. We aimed to evaluate the impact of pathogen exposure and intrinsic adaptor proteins on insulin signaling in pigs. The first study focuses on the impact of growth factor receptor bound protein 10 (GRB10) as an inhibitor of insulin signaling. In commercial swine, the presence of GRB10 has been linked to growth, reproduction, feed efficiency and lean muscle growth. While insulin induces glucose uptake in typical tissues, such as the liver and skeletal muscle, insulin also acts on other cell types including mesenchymal stem cells (MSC). MSC are adult multipotent stem cells that can self-renew and differentiate into multiple cell types including adipocytes. The process of

adipogenesis requires insulin signaling to synthesize new triglycerides and store them in lipid droplets. While GRB10 has been established as a regulator of insulin signaling, the role of GRB10 in swine MSC has yet to be firmly established. We generated GRB10 knockdown (GRB10-KD) MSC to evaluate the impact of GRB10 on insulin signaling and glucose uptake. We observed reduced glucose utilization under basal conditions and reduced insulin signaling when incubated with insulin over 48 hours. We also noticed a two-fold reduction in proliferation rate among GRB10-KD MSC. When differentiated into adipocytes, we observed an increase in transcript abundance with genes associated with insulin signaling and adipogenesis. GRB10 has the potential to regulate insulin signaling in swine MSC and contribute to overall growth and development. The second chapter focuses on the impact of lipopolysaccharide (LPS), an endotoxin produced by gram-negative bacteria, which can induce a severe, systemic immune response. In pigs, chronic LPS exposure has induced insulin resistance. However, the effects of acute exposure to LPS on insulin signaling and resistance have not been elucidated. We found that acute exposure to LPS in crossbred post-weaning pigs induced changes in insulin signaling and glucose metabolism. There was an LPS induced decrease in insulin two hours after injection which was paired with hyperglycemia. At 24 hours post LPS there was a marked insulin resistance indicated by hyperinsulinemia and hyperglycemia. We also noted that there were liver specific decreases in genes associated with glucose metabolism, insulin signaling and fatty acid metabolism. As well as reduction in protein abundance such as protein kinase B (AKT) and phosphoinositide-3 kinase (PI3K) in the liver after LPS administration. During an acute exposure to endotoxin, insulin signaling, and glucose metabolism is reduced in the liver. These results highlight that insulin signaling is a complex and dynamic process that can be controlled through a variety of mechanisms and swine can serve to model these disruptions.

INVESTIGATION OF DISRUPTED INSULIN SIGNALING IN A SWINE MODEL

by

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Dedication

This dissertation is dedicated to the people who have supported me throughout my education. To my lovely parents, Lisa and Joe, thank you for providing me with unwavering support and strength. To my sisters, Cora, Stephenie, and Allie thank you for your bottomless support and late-night encouragement. To my nieces and nephew, Anna, Ava, Alannah and Leo, thank you for providing motivation throughout the entire process.

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Chapter 1: Literature Review

Introduction

Swine are a dual-purpose species; they are an agricultural resource and are commonly used as a biomedical model in research. Despite not being consumed in certain areas or population centers, pork is one of the preferred meat products in the world. In the US, consumption of pork products ranks third amongst meat consumption, averaging 51 pounds per person, behind beef and chicken (1). Pigs share a variety of features with humans and therefore can be used to investigate disease pathologies, physiology, growth, and development (2). To continue to use swine models effectively and to meet production demands, we must enrich our understanding of pig physiology at an organismal, tissue and cellular level.

In today's intensive modern farming system, pigs are subjected to a variety of stressors including over-crowding, heat stress and pathogen exposure. Poor animal health due to any of these conditions can restrict swine production outcomes. Immune stress in an animal occurs when they are infected by a viral, bacterial, or fungal pathogen in livestock production (3). When an animal is under immune stress, there are behavioral and metabolic changes associated with the immune response. These signs include lethargy, loss of appetite, poor growth, and diarrhea (4). In pigs, bacterial infections are responsible for heavy economic losses, decreased growth rate, increased medical costs, morbidity, and mortality of animals in the herd (5). These physiologic changes because of infection result in poorer growth performance and less financial return for the producer (3).

The agricultural industry relies on efficient muscle accretion in pigs to generate food and revenue. Insulin is an anabolic hormone that is responsible for glucose uptake and nutrient utilization for growth and development (6). Insulin's control over glucose metabolism is limited by the rate of glucose uptake into liver, fat, and muscle (7). Proper insulin signaling is required to maintain daily homeostasis and disruptions in insulin's action can be devastating regardless of causative agent. Insulin signaling can be disrupted via metabolic state, pathogen exposure, development of disease, protein-protein interactions, genetic mutation, or autoimmune disorders. There is a large body of literature using rodent models to study typical causes of insulin resistance, obesity and diabetes. A pig model can be used to investigate both canonical and non-canonical disruptions in insulin signaling.

Swine Model

Animal models are commonly used to study human disease, pathology, or physiology. The most common animal models used in a laboratory setting are rodents, particularly mice models, due to their fully sequenced genome, low cost, ease of upkeep and quick reproduction rate. (8). Mouse models, however, have limitations in their ability to represent human physiology as they are classified as herbivores, have a well-developed cecum (9), exhibit pathophysiological differences, lack some clinical signs associated with gastrointestinal disease (10), practice coprophagy, and have a smaller genome (2). Pigs have specific behavioral characteristics and husbandry requirements for research in both agricultural and biomedical settings (11) but are still used frequently in a wide variety of studies. The pig has been utilized as a model for research in a variety of areas including cardiovascular physiology, obesity, metabolic syndrome,

dermatology, gastroenteritis, drug development, and nutrition (12). The immune system of a pig has been shown to have similar anatomy, organization and responses compared to the human immune system (13). Pigs and humans have a high chromosome structure homology, comparable genome sizes, and a high sequence homology, with 60% of the pig genome sharing homology with humans, compared to only 40% for rodents (14). Like humans, pigs can display pathophysiological signs of disease such as intestinal inflammation and corresponding clinical signs including diarrhea and decreased weight gain if exposed to a stressor (2).

There are pig models using both miniature pigs and commercial swine for obesity and cardiovascular disease research, most commonly utilizing administration of a high fat western diet (15) among other methods. The porcine model can exhibit at least three of the clinical signs of metabolic syndrome and is considered the ideal non-primate model for metabolic syndrome research (16). The young pig has been used for studying many nutritional and metabolic processes such as parenteral nutrition, viral and bacterial infection, and amino acid metabolism (17). Previous studies have shown that newborn piglets given the bacteria Bordetella pertussis, the causative agent of whooping cough, show clinical signs that mirror infant illness (18). Pig models are vital to our understanding of a variety of physiological processes including insulin sensitivity.

The Insulin Signaling Cascade

Insulin is an anabolic peptide hormone that is required for nutrient utilization. Appropriate insulin action in target tissues is necessary for glucose utilization, lipid, and carbohydrate metabolism (6). The secretion of insulin is tightly regulated by the pancreas in

response to hyperglycemia. When secreted, insulin acts to transport glucose from the bloodstream into target tissues to maintain euglycemia. Once glucose has entered the target tissue, it can be stored or utilized as needed based on the metabolic needs of the tissue and system. Tissues do no utilize glucose at the same rate, skeletal muscle is responsible for 80-85% of glucose disposal while adipose tissue is only responsible for less than 5% of glucose uptake after a meal (19).

Insulin is a dipeptide hormone which contains A and B chains linked by disulphide bridges. The stimulus for insulin secretion is glucose entry into the beta cells of the pancreas where the glucose is converted to glucose-6-phosphate by glucokinase. This conversion results in the generation of ATP and the subsequent closure of K^+/ATP dependent channels to depolarize the membrane to activate voltage dependent calcium channels that increase intracellular calcium concentration to trigger insulin secretion (20). Synthesis of insulin occurs in the islets of Langerhans in the pancreatic β-cells and insulin is initially synthesized as a preprohormone, pre-proinsulin. Pre-proinsulin is generated in the rough endoplasmic reticulum by combining a signal peptide, the A/B chains, and a connecting (C) peptide. Pre-proinsulin is converted into proinsulin via the removal of the signal peptide and stored in vesicles. Proinsulin is cleaved to generate insulin and c-peptide which are release into circulation (21). Once in circulation, insulin can travel to target tissue and initiate an enzyme cascade to stimulate glucose uptake.

Insulin signaling is conducted via a cascade of tyrosine kinases that act sequentially to relay information downstream. The first step of insulin signaling occurs when insulin binds to the insulin receptor (INSR) or another receptor tyrosine kinase. Insulin can interact with other

membrane receptors such as insulin-like growth factor-1 (IGF1) and insulin receptor-related receptor (IRR). Insulin binds to INSR via the alpha extracellular subunit which triggers auto-phosphorylation of the beta intracellular subunit for activation (22). Once enabled, INSR can phosphorylate the associated insulin receptor substrate (IRS) proteins. Of the IRS proteins, insulin receptor substrate-1 (IRS1) is responsible for glucose uptake and utilization (23). When IRS1 is phosphorylated, it can recognize and phosphorylate phosphatidylinositol-3-kinase (PI3K). PI3K is an enzyme that is vital in the metabolic actions of insulin. PI3K catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PI(2,4)P2) into phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P3). As PI(3,4,5)P3 accumulates in the cytoplasm, phosphoinositide-dependent kinase 1 (PDK1) is recruited to the plasma membrane which then phosphorylates protein kinase B (AKT). Once AKT is activated, the conversion of PI(3,4,5)P3 is terminated by phosphatase and TENsin homolog deleted on chromosome 10 (PTEN) (24). AKT is one of the major effectors of insulin signaling and mediates the many functions of insulin. During insulin mediated glucose uptake, AKT stimulates the translocation of glucose transporter-4 (Glut4) to the plasma membrane to allow entry of glucose to the cells. Once in the cells, glucose can be used to synthesize glycogen, lipids, proteins or enter directly into glycolysis (Figure 1) (25). Appropriate and efficient action of insulin is crucial to utilize nutrients properly and is subjected to a variety of regulations and disruptions via adaptor proteins, other hormones, disease, and inflammatory states.

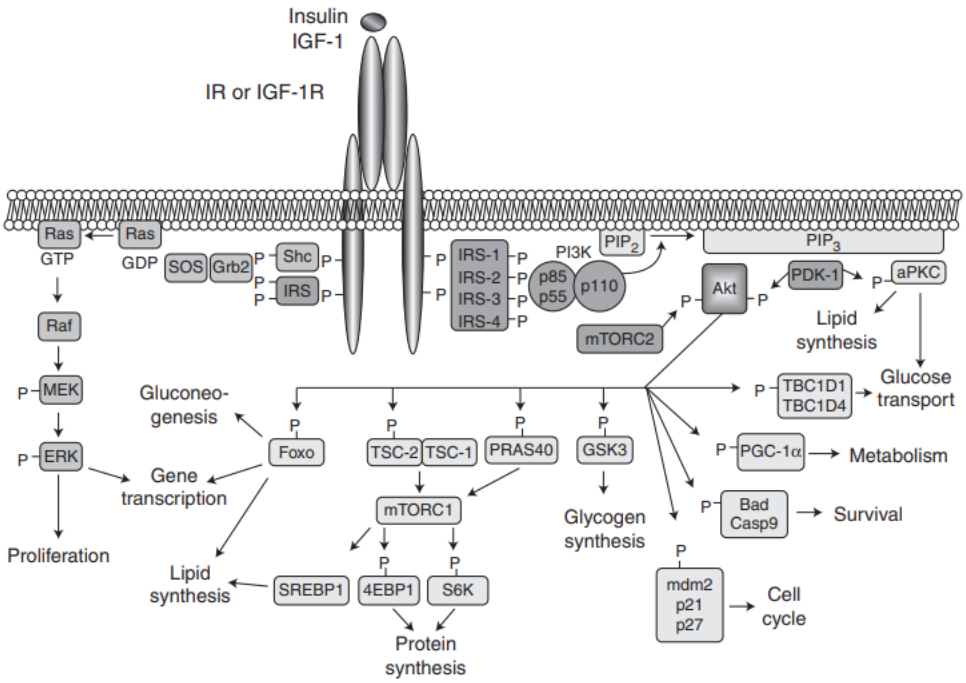


Figure 1-1 – Illustration of the insulin signaling cascade and subsequent downstream receptor tyrosine kinases. Insulin binding to the insulin receptor allows for downstream activation of IRS1, PI3K, and AKT sequentially. AKT is a major mediator of insulin action and can initiate glycogen synthesis, protein synthesis, lipid synthesis and glucose transport into the cell. Adapted from Boucher et. Al 2014

Insulin inhibition via GRB10

A family of adaptor proteins known as growth hormone receptor bound proteins (GRB) mediate the actions of other proteins via binding to one another, despite lacking catalytic functions (26). Growth factor receptor bound protein 10 (GRB10) is member of a family of this adaptor protein family which includes GRB7 and GRB14 that bind to and interact with mitogenic receptor tyrosine kinases such as INSR, epidermal growth factor receptor (EGFR) and IGFR (27). Genome wide association studies have found that there is a strong linkage of GRB10 to the development of insulin resistance and obesity (49)**.** An overexpression of GRB10 is linked to roughly 10% of Silver-Russel Syndrome cases where affected individuals display severe

growth retardation (28)**.** GRB10 has been established as an inhibitor of insulin signaling as it binds to INSR, to halt the insulin signaling cascade. (29). The inhibitory action of GRB10 is accomplished via conserved domains on the amino acid sequence. The src-homology 2 (SH2) domain and a pleckstrin homology domain (PH) which are on the outside of the between the PH and SH2 (BPS) domain. GRB10 will utilize the three domains differently to bind to IGFR and INSR. The GRB10 BPS domain is responsible for GRB10 binding with IGFR, an interaction which is stabilized by the SH2 domain. On the contrary, when GRB10 binds INSR with the SH2 domain, and the BPS domain is used for stabilization (30). Under no insulin stimulation, GRB10 is inactivated and forms tetramers using the BPS, SH2 and PH domains which are converted into active monomers upon stimulation (31).

Under normal physiologic conditions, the binding of insulin results in the phosphorylation of downstream enzymes IRS1, PI3K, AKT sequentially to initiate Glut4 translocation to the plasma membrane (6). In addition, once AKT is phosphorylated, AKT can then activate mammalian target of rapamycin (mTORC1) by removing its inhibitor complex tuberous sclerosis complex 1/2 (TSC1/2) (32). When AKT is activated, it phosphorylates TSC2 which is then able to inhibit TSC1. The TSC complex acts as a GTPase-activating protein (GAP) for the GTPase RAS homologue enriched in brain (RHEB). When the TSC complex is inhibited via AKT mediated phosphorylation, RHEB-GTP binds to the catalytic domain of mTOR to allow for activation of mTORC1 (33). mTORC1 consists of subunits regulatory associated protein of mTOR (RAPTOR) and mammalian lethal with SEC thirteen 8 (mLST8). mTORC1 is activated by nutrient intake, energy status and other growth factors, such as insulin. The active mTORC1

phosphorylates and stimulates GRB10 to bind to the intracellular subunit of the INSR to provide negative feedback on insulin signaling (Figure 2) (32, 34).

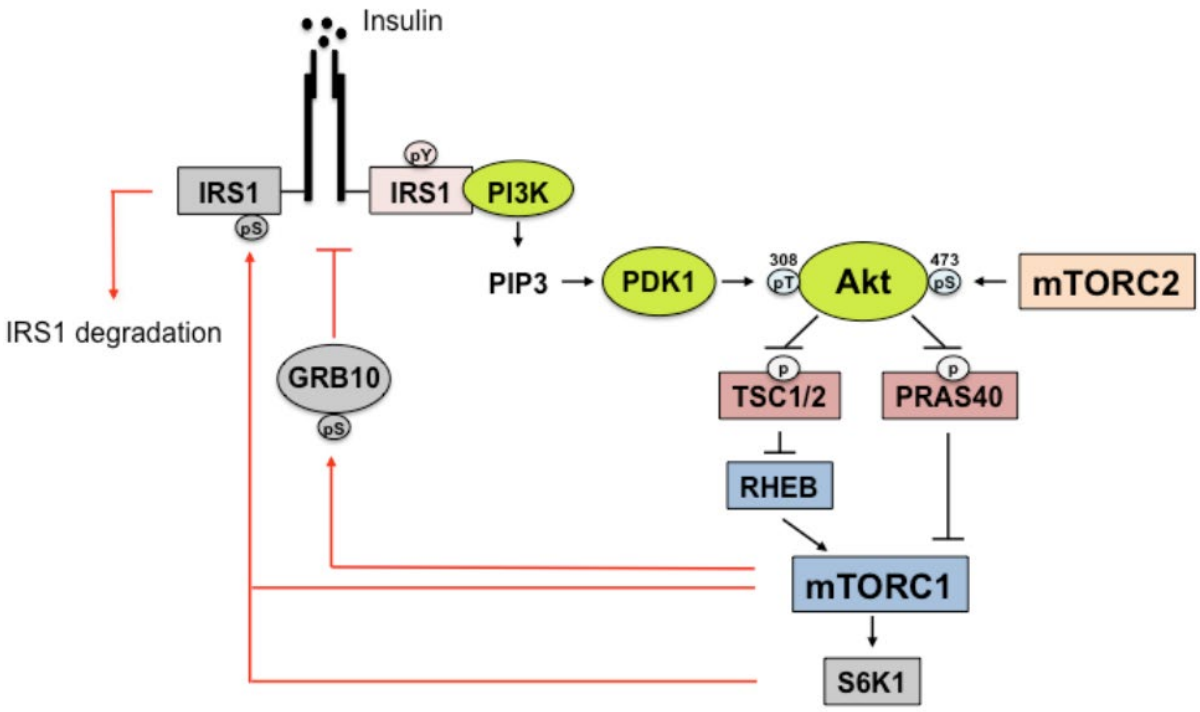


Figure 1-2 – Illustration of GRB10 inhibition of insulin signaling via mTORC1 activation. Insulin binds to the insulin receptor which causes the receptor to bind to IRS1. IRS1 activates PI3K who in turn activates Akt. Akt is a major effector of insulin signaling and activates mTORC1 via TSC complex inhibition. mTORC1 once activates, stabilizes, and phosphorylates GRB10 to blunt insulin signaling. Adapted from Yu 2011

The functional role of GRB10 is not fully elucidated and is somewhat contradictory. GRB10 and its functional SH2 domain was initially discovered as a binding partner EGFR during bacterial screening (35). A yeast hybrid screen associated with the SH2 domain of GRB10 as a binding partner of Ret tyrosine kinase receptor (36). Yet another yeast experiment demonstrated that the expression level of GRB10 as well as the presence of the SH2 domain have indicated GRB10 as a coactivator for AKT in yeast (37). In mice fibroblasts, there is an inhibitory effect of GRB10 in IGF1 mediated cell growth (29). While the inhibitory effect on the

insulin receptor has been noted in yeast (38), humans and mice (39) via disrupting the association of IRS1 with INSR. In mice where GRB10 is ablated, the mice had higher levels of phosphorylated Akt and increased glucose uptake in skeletal muscle (28). Transgenic mice overexpressing GRB10 show stunted growth, decreased muscle mass, increased adiposity, and insulin resistance (40). GRB10 is also under the action of mTORC1 in adipose tissue as GRB10 is a regulator of adiposity and energy expenditure under mTORC1 action (41). The role of GRB10 as a regulator of insulin signaling is complex and can vary depending on the system.

Insulin modulation via immune stimulation

Insulin resistance is the reduction of insulin's capacity to appropriately regulate glucose metabolism. Any metabolic dysfunction that results in inflammation such as pregnancy, obesity, hibernation, and sepsis can induce insulin resistance (42). Prolonged insulin resistance can lead to the development of metabolic syndrome which includes type two diabetes, hyperglycemia, atherogenic dyslipidemia and hypertension (43). When insulin resistant, metabolic tissues such as skeletal muscle, liver and white adipose tissue become less sensitive to insulin (44). The molecular mechanism of insulin resistance is not fully understood but the strength of the insulin signaling is reduced via modifications to its receptor or related proteins. If IRS1 is phosphorylated on an alternate serine, IRS1 can be degraded to reduce insulin signaling strength (45). Serine phosphorylation reduces IRS1 proteins capability to attract PI3K reducing the kinases activation potential (46). The enhanced serine phosphorylation of IRS1 can occur in response to cellular inflammation. Inflammatory mediators such as tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6) are released in response to the c-JUN N-terminal kinase (JNK) or IκB kinase (IKK) complex and can cause serine phosphorylation of IRS1 and result in insulin

resistance (47). These inflammatory mediators can be released due to internal stressors such as obesity or external factors such as pathogen exposure.

Obesity causes excess lipid accumulation in adipocytes which leads to activation of JNK and nuclear factor kappa beta (NFκB) signaling pathways due to mechanical stress. NF-κB is a transcription factor that is involved in physiological processes such as inflammation and immune responses (49)**.** NfκB is inactive when bound to its inhibitor IκBα under normal, non-inflammatory conditions. After stimulated with obesity mediated inflammation, IKK is activated to trigger the degradation of IκBα to expose the nuclear localization sequence of NF-κB. Once in the nucleus, NFκB is responsible for upregulation or pro-inflammatory mediators (50). JNK regulates inflammatory cytokine production, cell division and cell death to enhance the inflammatory response and the pathogenesis of obesity and insulin resistance (51). JNK inhibits insulin signaling because of endoplasmic reticulum stress causing serine phosphorylation of IRS1 (52). While the presence of JNK in metabolic tissues can enhance insulin resistance, JNK can worsen insulin resistance via inhibition of secretion directly from the pancreatic beta cells via its pro-inflammatory mediators (53). The activation of these signaling pathways increases the production of proinflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin-6 (IL6) (54).

Circulating plasma TNFα levels are increased in both obese and diabetic subjects (40), which associates TNFα levels with insulin resistance and inflammation. The cytokine IL6 plays a role in inflammation and regulating T and B cell function (55). Increased plasma IL6 has been associated with insulin resistance and glucose intolerance (56) in humans and impaired insulin

signaling in hepatocytes (57). IL6 secreted from adipose tissue reduces the expression of Glut4 and IRS1 to inhibit glucose uptake and insulin responsiveness (52). Secretion of IL6 and TNFα from inflamed adipose tissues have a direct linear relationship (59) and further compound insulin resistance. While obesity mediated inflammation can lead to insulin resistance, immune stimulation via pathogen exposure can also modulate the insulin signaling cascade.

Lipopolysaccharide (LPS) is an endotoxin released by gram negative bacteria that can initiate a severe systemic immune response (59). LPS is a pathogen-associated molecular patterns (PAMPs) in gram negative bacteria and works via the toll-like receptor (TLR4) to initiate an inflammatory response (60). TLR4 belongs to the pattern recognition receptor family and plays a role in the innate immune system to detect microbial infections (61). TLR4 initiates the proinflammatory signaling from LPS via recruitment of NFκB, interferons and cytokines which results in inflammation and potentially sepsis (59). Once an animal is exposed to LPS, the LPS-binding protein (LBP) forms a complex with LPS which can interact with the cell surface protein CD14. CD14 binds to LBP and transfers the LBP-LPS complex to TLR4 and its co receptor myeloid differentiation protein-2 (MD-2) (62). The LPS is now bound to TLR4-CD14-MD2 complex and allows for the activation of TLR4 via oligomerization to recruit downstream proteins. The LPS mediated TLR4 activation results in the recruitment of TIR domain containing adaptor protein (TIRAP) to the cell membrane. The presence of TIRAP will recruit MyD88 and follow the MyD88-dependent pathway (63). When activated by LPS, MyD88 recruits and activates IL-1 receptor associated kinase-4 (IRAK-4) and TNFα receptor associated factor 6 (TRAF6) which create a complex that can then disassociate and recruit TAK1, a mitogen activated protein kinase kinase kinase (MAPKKK) family member. TAK1 phosphorylates IKK

complex to induce phosphorylation and degradation of IκBα. This exposes the nuclear localization sequence of NFKB and allows for its translocation to the nucleus and initiation transcription of inflammatory genes including TNFα, IL-6, IL1 and IFNγ (Figure 3) (64, 65).

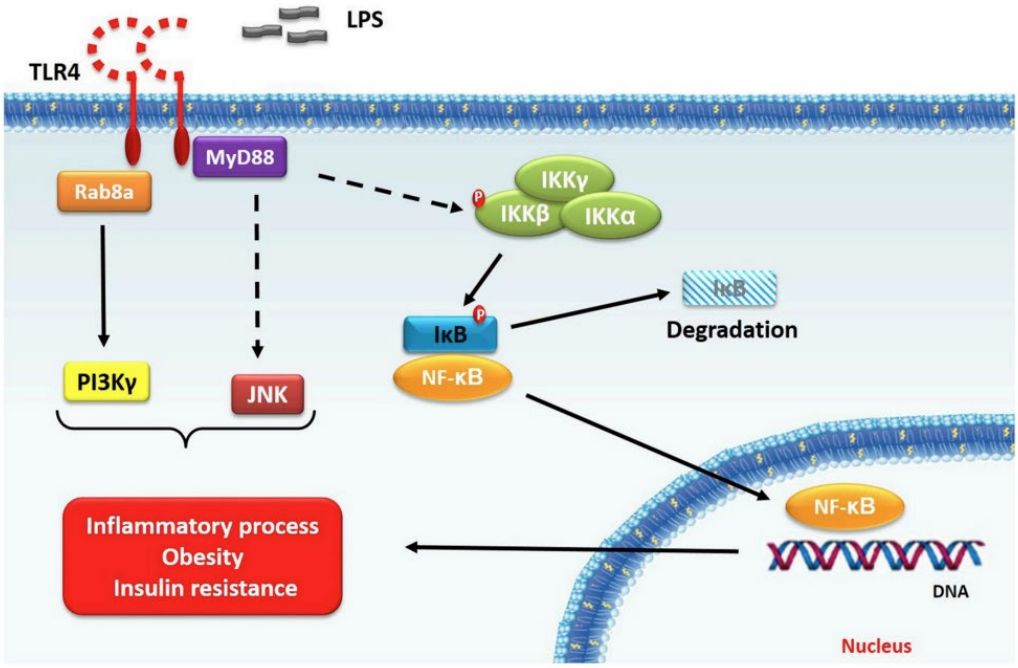


Figure 1-3 – LPS mediated activation of inflammatory cytokines and proteins. LPS is detected by TLR4 and the signal is transmitted through the MyD88 dependent pathway removing the inhibition of NFκB allowing for inflammation to increase.

When an individual is exposed to LPS, the endotoxin causes severe systemic inflammation which disrupts normal physiology. Metabolic endotoxemia is a leaky gut induced increase in LPS levels associated with low grade inflammation which can lead to the development of insulin resistance, cardiovascular and metabolic disorders. Metabolic endotoxemia triggered by exposure to LPS or a high fat diet in mice, dysregulates the immune system, increases serum LPS and aids in the development of insulin resistance, obesity, and later diabetes (66). Physiological metabolic changes due to LPS exposure have been noted in a variety of models including human, mice, rat, dairy cows, and pigs (5). Rats when administered a large

dose of LPS exhibited an initial increase in glucose levels one hour after injection with a subsequent hypoglycemia and hyperinsulinemia five hours after injection (67). This was accompanied by a reduction of hepatic glucose production as seen in diminished glucose-6-phosphatase activity (G6P). A septic shock rat model found that LPS administration reduced hepatic gluconeogenesis (68). LPS administration in rats increases enzymes related to glycogenolysis and gluconeogenesis, hepatic glycogen phosphorylase activity and phosphoenolpyruvate respectively (69). LPS injection led to hyperglycemia, insulin resistance and increase in inducible nitric oxide synthase (iNOS) (70). LPS mediated alterations in insulin signaling have also been noted in large animal models.

In a large animal model, LPS administration has been noted to alter metabolism in a variety of ways. In dairy cows, plasma insulin concentrations increased after intra-mammary LPS exposure (71). The same group was able to uncover that in lactating cows, LPS administration altered hepatic metabolic capacity (72). LPS administration in pigs' results in severe hemodynamic, clinical signs and behavioral changes (5). Chronic administration of LPS in post-pubertal gilts resulted in an acute, marked, insulin resistance after 24 hours which resolved at 72 hours. At the time of sample collection, insulin mediated genes such as *INSR*, *IRS1* and *AKT* were not statistically different (73). An LPS-euglycemic clamp was used to determine the glucose requirement of an activated immune system in growing pigs to be approximately 1.1g/kg $BW^{0.75}$/h (74). This study also reported an increase in insulin concentration four hours post LPS injection that increased through the end of the clamp experiment. Administration of LPS has been shown alter glucose metabolism in *longissimus dorsi* (LD) muscle, reduce skeletal muscle mitochondrial metabolism, and suppression of hepatic

glucose production in male growing pigs after six hours (75). Another study in male growing pigs administered intramuscular LPS had, after six hours, impacted hepatic triglyceride metabolism by decreasing de novo lipogenesis via the action of glucocorticoid receptor (76). Neonatal piglets infused with LPS had reduced protein synthesis in muscles and yet increased protein synthesis in the liver, spleen, and kidney (77).

Alterations in insulin signaling have also been demonstrated during inflammatory states caused by other pathogens. Nursey pigs exposed to porcine epidemic diarrhea virus (PEDV) had increased insulin for five days post infection (78), highlighting that immune stimulation from non-bacterial agents can induce insulin resistance. The effect of LPS on insulin resistance is also exacerbated by increased temperatures, high ambient temperatures in conjunction with LPS administration further increased insulin serum concentration compared to their thermoneutral counterparts (79). Previous literature has demonstrated that LPS administration induces systemic alterations in insulin signaling and sensitivity. However, there is potential for insulin signaling related changes on a cellular level.

Mesenchymal stem cells as a model for GRB10

Stem cells have the capability to self-renew and differentiate into a variety of lineages. Embryonic stem cells are harvested from the blastocyst stage of a human embryo (80). While embryonic stem cells are more pluripotent than some of their adult counterparts, the use of embryonic stem cells in research is quite controversial. Adult stem cells, however, provide a multipotent alternative to cellular research. Mostly found in bone marrow, mesenchymal stem cells (MSC) represent an adult stem cell that are capable of self-renewal through division, in

vitro growth, and multiple lineage differentiation (81). MSC were first isolated from bone marrow and splenic cells that were harvested from guinea pigs (82). MSC are non-hematopoietic and can differentiate into osteogenic, chondrogenic, adipogenic and myogenic cell types (Figure 4) (53). For stem cells to be considered MSC, they must be plastic adherent and express CD105, CD73 and CD90 and lack expression of CD45 and CD34 and be able to differentiate into different lineages (83). The use of human MSC has been established as a model for human adipogenesis as they are a renewable source of precursor that can differentiate into adipocytes under appropriate hormonal stimuli (87). MSCs are also capable of influencing the immune system and can be used to treat inflammatory diseases (84). MSC can proliferate readily and maintain their differentiation potential (both in vivo and in vitro) which makes MSCs an ideal candidate for cell-based therapies in humans (85). In recent studies, human MSC derived from extracellular vesicles have the potential to alleviate type II diabetes and relieve β-cell destruction by reversing peripheral insulin resistance (75). In addition, MSC have been shown undergo structural and functional changes associated with obesity (86). Both studies highlight that MSC are sensitive to environmental factors and can be used to study a variety of illnesses and mechanisms.

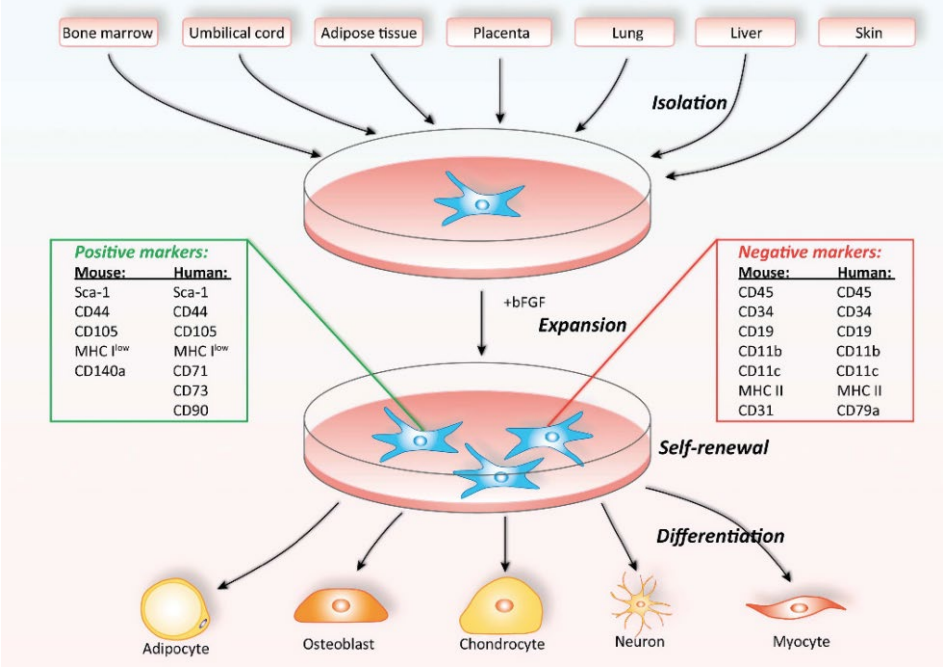


Figure 1-4 – Diagram illustrating the potential sources of mesenchymal stem cells, cell surface markers and adherent cell culture conditions. The image also details the self-renewal of MSC and the possible differentiation avenues. Adapted from Chen 2016

Porcine MSC from bone marrow can be used as an animal model for human MSC due to similarities in isolation, growth, and proliferation for basic research and cell engineering (88). Purification and differentiation methods used in human MSC research have been adopted for porcine MSC analysis (88). Swine MSCs have observed changes in proliferation and differentiation potential in response to both in vivo and in vitro experimentation. MSCs derived from obese pigs with an increased expression of TNFα, have increased adipogenic potential and an inclination towards senescence (89) indicating micro-environmental changes will impact MSC function. Dietary changes in calcium and phosphorous concentration in nursery pigs induced a significant impact on MSC characteristics (90, 91) Given the experimental potential of MSC, there is an increased interest in genetic manipulation and tissue engineering of MSC from different species and source tissues.

Genetic modification of MSC requires the delivery of novel DNA oligonucleotides into the cells to be incorporated into the host genome. The methods for gene delivery include transfection of a novel DNA oligonucleotide or plasmid and infection of cells via viral constructs such as adenovirus or retrovirus (92). Transfection with viral vectors is more efficient yet there is potential for mutations that can be tumorigenic, as seen in a clinical trial where hematopoietic stem cells modified with retrovirus were transplanted into patients, of which four developed leukemia (93). Human MSCs have not demonstrated similar behavior in clinical trials (94) but the use of viral vectors adds uncertainty and unnecessary risk. Nonviral nucleic acid delivery methods such as transfection can be safer and more flexible but are less efficient and more cytotoxic to human MSC (95). A new gene delivery method for difficult to transfect primary cells using Nucleofector™ technology developed by Amaxa biosystems. This system modifies the standard electroporation method to use proprietary electrical parameters and cells specific solutions. The new system allows for the delivery of novel DNA and genome editing components, directly into the nucleus of cells and has a reported efficiency of up to 90%. A study by Haleem-smith et. Al (92) transfected human derived MSC using the Nucleofection™ system and demonstrated 86% efficiency and 50% cell viability. Post transfection event, human-MSC were able to successfully differentiate into chondrocytes. Pig MSC isolated from bone marrow can be genetically modified using viral vectors and used for the generation of somatic-cell nuclear transfer embryos (85). Using genome editing in MSC, we can elucidate the actions proteins of interest under a variety of circumstances.

The differentiation of porcine MSC into adipocytes has been well documented. To undergo adipogenesis, MSC lose their stem-ness, transition into preadipocytes and then commit to terminal differentiation, with developing lipid droplets which will fill the entire cell. In the early stage of differentiation, genetic markers change along the differentiation process (88). For MSC to differentiate into adipocytes, they must respond to extracellular signals including insulin. As we have established, insulin is a tightly regulated hormone that has a lot of metabolic responsibilities. However, the role of GRB10 in insulin signaling of porcine MSC has yet to be established. Imprinted genes, such as GRB10, have been implicated in normal development and a potential role in regulating adult stem cell fate (96). Investigating the role of GRB10 in MSC has the potential to provide insights into cellular growth, nutrient utilization, lipid accumulation and insulin resistance.

Conclusion

The hormone insulin is a growth promoter that is responsible for a variety of metabolic processes such as glucose uptake, glycogen synthesis, lipogenesis, and protein synthesis through a signaling cascade of enzymes. Proper insulin action is essential for the overall health and wellness of vertebrate species. Insulin signaling can be disrupted via a variety of mechanisms resulting in reduced insulin sensitivity and subsequent alterations in metabolism. Insulin action can be disturbed via adaptor proteins such as GRB10 and an acute immune response after exposure to an endotoxin, such as LPS. For our domesticated livestock species, pigs require efficient nutrient utilization via insulin to accumulate skeletal muscle for production purposes. When exposed to a pathogen such as LPS, there is the potential for disrupted insulin signaling that can have downstream effects on body composition which ultimately leads to poor growth

rate and financial hardship on producers. The effects of altered insulin signaling can be investigated on a cellular level as well as insulin on a holistic scale.

On a cellular level, swine MSCs have the potential to differentiate into adipocytes and require appropriate insulin stimulation to begin adipogenesis. Insulin is a common additive to cell culture media to induce differentiation into adipocytes of MSC in vitro. Therefore, MSC are an insulin responsive cell type that can be subjected to experimental manipulation. However, the actions of insulin related proteins have yet to be fully characterized in human MSC, let alone swine MSC. GRB10 is a known inhibitor of insulin signaling that has the potential to reduce or enhance the adipogenic potential of pig MSC depending on its absence or presence respectively. Using gene delivery techniques, we can alter the expression of GRB10 in porcine MSC to evaluate the role of GRB10 on insulin signaling. This research presents an opportunity to elucidate the effects of reduced insulin sensitivity in a variety of conditions and improve our understanding of swine metabolism for production purposes and to enhance the model that swine MSC presents.

References

1. Davis, C. and Lin, B. H. (2005) Factors Affecting U.S. Pork Consumption. *Livestock, Dairy, Poultry Outlook*
2. Gonzalez, L. M., Moeser, A. J., and Blikslager, A. T. (2015) Porcine models of digestive disease: The future of large animal translational research. *Translational Research* **166**, 12–27
3. Song, C., Jiang, J., Han, X., Yu, G., and Pang, Y. (2014) Effect of immunological stress to neuroendocrine and gene expression in different swine breeds. *Mol Biol Rep* **41**, 3569–3576
4. Weekers, F., Giulietti, A. P., Michalaki, M., Coopmans, W., Van Herck, E., Mathieu, C., and Van Den Berghe, G. (2003) Metabolic, Endocrine, and Immune Effects of Stress Hyperglycemia in a Rabbit Model of Prolonged Critical Illness. *Endocrinology* **144**, 5329–5338
5. Wyns, H., Plessers, E., De Backer, P., Meyer, E., and Croubels, S. (2015) In vivo porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. *Vet Immunol Immunopathol* **166**, 58–69
6. Bevan, P. (2001) Insulin signalling. *J Cell Sci* **114**, 1429–1430
7. Saltiel, A. R. (2021) Insulin signaling in health and disease. *Journal of Clinical Investigation* **131**
8. Low, M. J. (2012) Mouse models in gastroenterology research. *Gastroenterology* **143**, 1410–1412
9. Houpt, K. A., Houpt, T. R., and Pondb, W. G. (1979) The Pig as a Model for the Study of Obesity and of Control of Food Intake: A Review. *BIOLOGY AND MEDICINE* **52**
10. Guilbault, C., Saeed, Z., Downey, G. P., and Radzioch, D. (2007) Cystic fibrosis mouse models. *Am J Respir Cell Mol Biol* **36**, 1–7
11. Swindle, M. M., Makin, A., Herron, A. J., Clubb, F. J., and Frazier, K. S. (2012) Swine as Models in Biomedical Research and Toxicology Testing. *Vet Pathol* **49**, 344–356

12. Miller, E. R. and Ullrey, D. E. (1987) THE PIG AS A MODEL FOR HUMAN NUTRITION. *Ann. Rev. Nutr* **7**

13. Mair, K. H., Sedlak, C., Käser, T., Pasternak, A., Levast, B., Gerner, W., Saalmüller, A., Summerfield, A., Gerdts, V., Wilson, H. L., and Meurens, F. (2014) The porcine innate immune system: An update. *Dev Comp Immunol* **45**, 321–343

14. Thomas, J. W., Touchman, J. W., Blakesley, R. W., Bouffard, G. G., Beckstrom-Sternberg, S. M., Margulies, E. H., Blanchette, M., Siepel, A. C., Thomas, P. J., McDowell, J. C., Maskeri, B., Hansen, N. F., Schwartz, M. S., Weber, R. J., Kent, W. J., Karolchik, D., Bruen, T. C., Bevan, R., Cutler, D. J., Schwartz, S., Elnitski, L., Idol, J. R., Prasad, A. B., Lee-Lin, S.-Q., Maduro, V. V. B., Summers, T. J., Portnoy, M. E., Dietrich, N. L., Akhter, N., Ayele, K., Benjamin, B., Cariaga, K., Brinkley, C. P., Brooks, S. Y., Granite, S., Guan, X., Gupta, J., Haghighi, P., Ho, S.-L., Huang, M. C., Karlins, E., Laric, P. L., Legaspi, R., Lim, M. J., Maduro, Q. L., Masiello, C. A., Mastrian, S. D., McCloskey, J. C., Pearson, R., Stantripop, S., Tiongson, E. E., Tran, J. T., Tsurgeon, C., Vogt, J. L., Walker, M. A., Wetherby, K. D., Wiggins, L. S., Young, A. C., Zhang, L.-H., Osoegawa, K., Zhu, B., Zhao, B., Shu, C. L., De Jong, P. J., Lawrence, C. E., Smit, A. F., Chakravarti, A., Haussler, D., Green, P., Miller, W., and Green, E. D. (2003) Comparative analyses of multi-species sequences from targeted genomic regions. *Nature* **424**, 788–793

15. Litten-Brown, J. C., Corson, A. M., and Clarke, L. (2010) Porcine models for the metabolic syndrome, digestive and bone disorders: A general overview. *Animal* **4**, 899–920

16. Spurlock, M. E. and Gabler, N. K. (2008) The Development of Porcine Models of Obesity and the Metabolic Syndrome 1,2. *The Journal of Nutrition Symposium: Animal Models in Nutrition Research J. Nutr* **138**

17. Baker, D. H. (2008) Animal Models in Nutrition Research 1,2. *The Journal of Nutrition Symposium: Animal Models in Nutrition Research J. Nutr* **138**

18. Elahi, S., Brownlie, R., Korzeniowski, J., Buchanan, R., O'Connor, B., Peppler, M. S., Halperin, S. A., Lee, S. F., Babiuk, L. A., and Gerdts, V. (2005) Infection of newborn piglets with Bordetella pertussis: A new model for pertussis. *Infect Immun* **73**, 3636–3645

19. DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., and Felber, J. P. (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* **30**, 1000–1007

20. Soria, B., Quesada, I., Ropero, A. B., Pertusa, J. A., Martín, F., and Nadal, A. (2004) Novel players in pancreatic islet signaling: from membrane receptors to nuclear channels. *Diabetes* **53 Suppl 1**, S86-91

21. Weiss, M., Steiner, D. F., and Philipson, L. H. (2014) Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships. MDText.com, INC, South Dartmouth (MA)

22. Wilcox, G. (2005) Insulin and Insulin Resistance. *Clin Biochem Rev* **26**

23. Kido, Y., Nakae, J., and Accili, D. (2001) Clinical review 125: The insulin receptor and its cellular targets. *J Clin Endocrinol Metab* **86**, 972–979

24. Saltiel, A. R. (2021) Insulin signaling in health and disease. *Journal of Clinical Investigation* **131**

25. Boucher, J., Kleinridders, A., and Ronald Kahn, C. (2014) Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harb Perspect Biol* **6**

26. Han, D. C., Shen, T. L., and Guan, J. L. (2001) The Grb7 family proteins: structure, interactions with other signaling molecules and potential cellular functions. *Oncogene* **20**, 6315–6321

27. Holt, L. J. and Siddle, K. (2005) Grb10 and Grb14: enigmatic regulators of insulin action-and more? *Biochem. J* **388**

28. Hitchins, M. P., Monk, D., Bell, G. M., Ali, Z., Preece, M. A., Stanier, P., and Moore, G. E. (2001) Maternal repression of the human GRB10 gene in the developing central nervous system; evaluation of the role for GRB10 in Silver-Russell syndrome. *Eur J Hum Genet* **9**, 82–90

29. Morrione, A., Valentinis, B., Resnicoff, M., Xu, S.-Q., and Baserga, R. (1997) The Role of mGrb10 in Insulin-like Growth Factor I-mediated Growth*.

30. He, W., Rose, D. W., Olefsky, J. M., and Gustafson, T. A. (1998) Grb10 Interacts Differentially with the Insulin Receptor, Insulin-like Growth Factor I Receptor, and Epidermal Growth Factor Receptor via the Grb10 Src Homology 2 (SH2) Domain and a Second Novel Domain Located between the Pleckstrin Homology and SH2 Domains*.

31. Dong, L. Q., Porter, S., Hu, D., and Liu, F. (1998) Inhibition of hGrb10 Binding to the Insulin Receptor by Functional Domain-mediated Oligomerization*.

32. Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villén, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P., and Blenis, J. (2011) Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science (1979)* **332**, 1322–1326

33. Shimobayashi, M. and Hall, M. N. (2014) Making new contacts: The mTOR network in metabolism and signalling crosstalk. *Nat Rev Mol Cell Biol* **15**, 155–162

34. Hsu, P. P., Kang, S. A., Rameseder, J., Zhang, Y., Ottina, K. A., Lim, D., Peterson, T. R., Choi, Y., Gray, N. S., Yaffe, M. B., Marto, J. A., and Sabatini, D. M. (2011) The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science (1979)* **332**, 1317–1322

35. Ooi, J., Yajnik, V., Immanuel, D., Gordon, M., Moskow, J. J., Buchberg, A. M., and Margolis, B. (1995) The cloning of Grb10 reveals a new family of SH2 domain proteins. *Oncogene* **10**, 1621–1630

36. Pandey, A., Duan, H., Fiore, P. Di, Dixit, V. M., Santoro, M., Carlomagno, F., Romano, A., Bottaro, D. P., Dathan, N. A., Grieco, M., Fusco, A., Vecchio, G., Matˇoškova, M. M., Kraus, B., and Difiore, P. (1995) The Ret Receptor Protein Tyrosine Kinase Associates with the SH2-containing Adapter Protein Grb10* Ret is a receptor protein tyrosine kinase that has been implicated in the development of the enteric nervous, endocrine, and renal systems. Mutations associated with multiple endocrine neoplasia types 2A and 2B (MEN 2A and 2B) have been shown to activate the in-trinsic kinase and transforming ability of ret (.

37. Jahn, T., Seipel, P., Urschel, S., Peschel, C., and Duyster, J. (2002) Role for the Adaptor Protein Grb10 in the Activation of Akt. *Mol Cell Biol* **22**, 979–991

38. Liu, F. and Roth, R. A. (1995) Grb-IR: A SH2-domain-containing protein that binds to the insulin receptor and inhibits its function (phosphatidylinositol 3-kinase/yeast two-hybrid system/insulin-receptor substrate 1). **92**

39. Wick, K. L. R., Werner, E. D., Langlais, P., Ramos, F. J., Dong, L. Q., Shoelson, S. E., and Liu, F. (2003) Grb10 inhibits insulin-stimulated insulin receptor substrate (IRS)-phosphatidylinositol 3-kinase/Akt signaling pathway by disrupting the association of IRS-1/IRS-2 with the insulin receptor. *Journal of Biological Chemistry* **278**, 8460–8467

40. Shiura, H., Miyoshi, N., Konishi, A., Wakisaka-Saito, N., Suzuki, R., Muguruma, K., Kohda, T., Wakana, S., Yokoyama, M., Ishino, F., and Kaneko-Ishino, T. (2005) Meg1/Grb10 overexpression causes postnatal growth retardation and insulin resistance via negative modulation of the IGF1R and IR cascades. *Biochem Biophys Res Commun* **329**, 909–916

41. Liu, M., Bai, J., He, S., Villarreal, R., Hu, D., Zhang, C., Yang, X., Liang, H., Slaga, T. J., Yu, Y., Zhou, Z., Blenis, J., Scherer, P. E., Dong, L. Q., and Liu, F. (2014) Grb10 promotes lipolysis and thermogenesis by phosphorylation-dependent feedback inhibition of mTORC1. *Cell Metab* **19**, 967–980

42. Odegaard, J. I. and Chawla, A. (2013) Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis. *Science (1979)* **339**, 172–177

43. Ragheb, R. and M. Medhat, A. (2011) Mechanisms of Fatty Acid-Induced Insulin Resistance in Muscle and Liver. *J Diabetes Metab* **02**

44. Chawla, A., Nguyen, K. D., and Goh, Y. P. S. (2011) Macrophage-mediated inflammation in metabolic disease. *Nat Rev Immunol* **11**, 738–749

45. Pederson, T. M., Kramer, D. L., and Rondinone, C. M. (2001) Original Articles Serine/Threonine Phosphorylation of IRS-1 Triggers Its Degradation Possible Regulation by Tyrosine Phosphorylation. *24 DIABETES* **50**

46. Yu, C., Chen, Y., Cline, G. W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J. K., Cushman, S. W., Cooney, G. J., Atcheson, B., White, M. F., Kraegen, E. W., and Shulman, G. I. (2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *Journal of Biological Chemistry* **277**, 50230–50236

47. Dali-Youcef, N., Mecili, M., Ricci, R., and Andrès, E. (2013) Metabolic inflammation: Connecting obesity and insulin resistance. *Ann Med* **45**, 242–253

48. Sohani, Z. N., Anand, S. S., Robiou-Du-pont, S., Morrison, K. M., McDonald, S. D., Atkinson, S. A., Teo, K. K., and Meyre, D. (2016) Risk alleles in/near ADCY5, ADRA2A, CDKAL1,

CDKN2A/B, GRB10, and TCF7L2 elevate Plasma glucose levels at birth and in early childhood: Results from the FAMILY study. *PLoS One* **11**

49. Sakurai, H., Suzuki, S., Kawasaki, N., Nakano, H., Okazaki, T., Chino, A., Doi, T., and Saiki, I. (2003) Tumor necrosis factor-α-induced IKK phosphorylation of NF-κB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *Journal of Biological Chemistry* **278**, 36916–36923

50. Lee, B. C. and Lee, J. (2014) Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. *Biochim Biophys Acta Mol Basis Dis* **1842**, 446–462

51. Han, M. S., Jung, D. Y., Morel, C., Lakhani, S. A., Kim, J. K., Flavell, R. A., and Davis, R. J. (2013) JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation. *Science (1979)* **339**, 218–222

52. Chen, L., Chen, R., Wang, H., and Liang, F. (2015) Mechanisms Linking Inflammation to Insulin Resistance. *Int J Endocrinol* **2015**

53. Lanuza-Masdeu, J., Isabel Arévalo, M., Vila, C., Barberà, A., Gomis, R., and Caelles, C. (2013) In vivo jnk activation in pancreatic β-cells leads to glucose intolerance caused by insulin resistance in pancreas. *Diabetes* **62**, 2308–2317

54. Sharma, M., Vikram, N. K., Misra, A., Bhatt, S. P., Tarique, M., Parray, H. A., Pandey, R. M., and Luthra, K. (2013) Assessment of 11-b hydroxysteroid dehydrogenase (11-bHSD1) 4478T>G and tumor necrosis factor-a (TNF-a)-308 G>A polymorphisms with obesity and insulin resistance in Asian Indians in North India. *Mol Biol Rep* **40**, 6261–6270

55. Fried, S. K., Bunkin, D. A., and Greenberg, A. S. (1998) Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6: Depot Difference and Regulation by Glucocorticoid*.

56. Bugianesi, E., McCullough, A. J., and Marchesini, G. (2005) Insulin resistance: A metabolic pathway to chronic liver disease. *Hepatology* **42**, 987–1000

57. Senn, J. J., Klover, P. J., Nowak, I. A., and Mooney, R. A. (2002) Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes* **51**, 3391–3399

58. Fernández-Real, J. M. and Ricart, W. (2003) Insulin resistance and chronic cardiovascular inflammatory syndrome. *Endocr Rev* **24**, 278–301

59. Mohammad, S. and Thiemermann, C. (2020) Role of Metabolic Endotoxemia in Systemic Inflammation and Potential Interventions. *Front Immunol* **11**, 594150

60. Rhee, S. H. (2014) Lipopolysaccharide: Basic Biochemistry, Intracellular Signaling, and Physiological Impacts in the Gut. *Intest Res* **12**, 90

61. Jang, J. H., Shin, H. W., Lee, J. M., Lee, H. W., Kim, E. C., and Park, S. H. (2015) An Overview of Pathogen Recognition Receptors for Innate Immunity in Dental Pulp. *Mediators Inflamm* **2015**

62. Park, B. S. and Lee, J. O. (2013) Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med* **45**

63. Lu, Y. C., Yeh, W. C., and Ohashi, P. S. (2008) LPS/TLR4 signal transduction pathway. *Cytokine* **42**, 145–151

64. Kawasaki, T. and Kawai, T. (2014) Toll-like receptor signaling pathways. *Front Immunol* **5**

65. Cavalcante-Silva, L. H. A., Galvão, J. G. F. M., da Silva, J. S. de F., de Sales-Neto, J. M., and Rodrigues-Mascarenhas, S. (2015) Obesity-Driven Gut Microbiota Inflammatory Pathways to Metabolic Syndrome. *Front Physiol* **6**, 341

66. Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Chabo, C., Waget, A., Delmée, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrières, J., Tanti, J. F., Gibson, G. R., Casteilla, L., Delzenne, N. M., Alessi, M. C., and Burcelin, R. (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56**, 1761–1772

67. Maitra, S. R., Gestring, M. L., El-Maghrabi, M. R., Lang, C. H., and Henry, M. C. (1999) Endotoxin-induced alterations in hepatic glucose-6-phosphatase activity and gene expression. *Mol Cell Biochem* **196**, 79–83

68. Tanaka, H., Nishikawa, Y., Fukushima, T., Taniguchi, A., Fujita, Y., Tsuda, K., Inagaki, N., and Hosokawa, M. (2018) Lipopolysaccharide inhibits hepatic gluconeogenesis in rats: The role of immune cells. *J Diabetes Investig* **9**, 494–504

69. Caton, P. W., Nayuni, N. K., Murch, O., and Corder, R. (2009) Endotoxin induced hyperlactatemia and hypoglycemia is linked to decreased mitochondrial phosphoenolpyruvate carboxykinase. *Life Sci* **84**, 738–744

70. Sugita, H., Kaneki, M., Tokunaga, E., Sugita, M., Koike, C., Yasuhara, S., Tompkins, R. G., Jeevendra Martyn, J. A., and Tomp-kins, R. G. (2002) Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance.

71. Waldron, M. R., Kulick, A. E., Bell, A. W., and Overton, T. R. (2006) Acute experimental mastitis is not causal toward the development of energy-related metabolic disorders in early postpartum dairy cows. *J Dairy Sci* **89**, 596–610

72. Waldron, M. R., Nishida, T., Nonnecke, B. J., and Overton, T. R. (2003) Effect of lipopolysaccharide on indices of peripheral and hepatic metabolism in lactating cows. *J Dairy Sci* **86**, 3447–3459

73. Bidne, K. L., Kvidera, S. S., Ross, J. W., Baumgard, L. H., and Keating, A. F. (2018) Impact of repeated lipopolysaccharide administration on ovarian signaling during the follicular phase of the estrous cycle in post-pubertal pigs. *J Anim Sci* **96**, 3622–3634

74. Kvidera, S. K., Horst, E. A., Mayorga, E. J., Sanz-Fernandez, M. V., Abuajamieh, M., and Baumgard, L. H. (2017) Estimating glucose requirements of an activated immune system in growing pigs. *J Anim Sci* **95**, 5020–5029

75. Sun, H., Huang, Y., Yin, C., Guo, J., Zhao, R., and Yang, X. (2016) Lipopolysaccharide markedly changes glucose metabolism and mitochondrial function in the longissimus muscle of pigs. *Animal* **10**, 1204–1212

76. Liu, Z., Liu, W., Huang, Y., Guo, J., Zhao, R., and Yang, X. (2015) Lipopolysaccharide significantly influences the hepatic triglyceride metabolism in growing pigs. *Lipids Health Dis* **14**

77. Orellana, R. A., Kimball, S. R., Nguyen, H. V., Bush, J. A., Suryawan, A., Thivierge, M. C., Jefferson, L. S., and Davis, T. A. (2004) Regulation of Muscle Protein Synthesis in Neonatal Pigs during Prolonged Endotoxemia. *Pediatr Res* **55**, 442–449

78. Curry, S. M., Burrough, E. R., Schwartz, K. J., Yoon, K. J., Lonergan, S. M., and Gabler, N. K. (2018) Porcine epidemic diarrhea virus reduces feed efficiency in nursery pigs. *J Anim Sci* **96**, 85–97

79. Campos, P. H. R. F., Merlot, E., Renaudeau, D., Noblet, J., and Le Floc'h, N. (2019) Postprandial insulin and nutrient concentrations in lipopolysaccharide-challenged growing pigs reared in thermoneutral and high ambient temperatures. *J Anim Sci* **97**, 3354–3368

80. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147

81. Owen, M. and Friedenstein, A. J. (1988) Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* **136**, 42–60

82. Friedenstein, A. J., Chailakhjan, R. K., and Lalykina, K. S. (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* **3**, 393–403

83. Secunda, R., Vennila, R., Mohanashankar, A. M., Rajasundari, M., Jeswanth, S., and Surendran, R. (2015) Isolation, expansion and characterisation of mesenchymal stem cells from human bone marrow, adipose tissue, umbilical cord blood and matrix: a comparative study. *Cytotechnology* **67**, 793–807

84. Calle, A., Barrajón-Masa, C., Gómez-Fidalgo, E., Martín-Lluch, M., Cruz-Vigo, P., Sánchez-Sánchez, R., and Ramírez, M. Á. (2018) Iberian pig mesenchymal stem/stromal cells from dermal skin, abdominal and subcutaneous adipose tissues, and peripheral blood: In vitro characterization and migratory properties in inflammation. *Stem Cell Res Ther* **9**

85. Bosch, P., Pratt, S. L., and Stice, S. L. (2006) Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. *Biol Reprod* **74**, 46–57

86. Meng, Y., Eirin, A., Zhu, X. Y., Tang, H., Chanana, P., Lerman, A., van Wijnen, A. J., and Lerman, L. O. (2018) Obesity-induced mitochondrial dysfunction in porcine adipose tissue-derived mesenchymal stem cells. *J Cell Physiol* **233**, 5926–5936

87. Janderová, L., McNeil, M., Murrell, A. N., Mynatt, R. L., and Smith, S. R. (2003) Human mesenchymal stem cells as an in vitro model for human adipogenesis. *Obes Res* **11**, 65–74

88. Ringe, J., Kaps, C., Schmitt, B., Büscher, K., Bartel, J., Smolian, H., Schultz, O., Burmester, G. R., Häupl, T., and Sittinger, M. (2002) Porcine mesenchymal stem cells: Induction of distinct mesenchymal cell lineages. *Cell Tissue Res* **307**, 321–327

89. Zhu, X.-Y., Ma, S., Eirin, A., Woollard, J. R., Hickson, L. J., Sun, D., Lerman, A., and Lerman, L. O. (2016) Functional Plasticity of Adipose-Derived Stromal Cells During Development of Obesity. *Stem Cells Transl Med* **5**, 893–900

90. Zhang, W., Kroscher, K. A., Murray, R. L., Gagliardi, R., Guiltinan, C., Rhoads, R. P., and Stahl, C. H. (2020) Dietary calcium and phosphorus amounts affect development and tissue-specific stem cell characteristics in neonatal pigs. *Journal of Nutrition* **150**, 1086–1092

91. Mahajan, A. and Stahl, C. H. (2009) Dihydroxy-cholecalciferol stimulates adipocytic differentiation of porcine mesenchymal stem cells. *Journal of Nutritional Biochemistry* **20**, 512–520

92. Haleem-Smith, H., Derfoul, A., Okafor, C., Tuli, R., Olsen, D., Hall, D. J., and Tuan, R. S. (2005) High-Efficiency Transfection of Human MSCs 9 MOLECULAR BIOTECHNOLOGY Abstract Optimization of High-Efficiency Transfection of Adult Human Mesenchymal Stem Cells In Vitro.

93. Oggu, G. S., Sasikumar, S., Reddy, N., Ella, K. K. R., Rao, C. M., and Bokara, K. K. (2017) Gene Delivery Approaches for Mesenchymal Stem Cell Therapy: Strategies to Increase Efficiency and Specificity. *Stem Cell Rev Rep* **13**, 725–740

94. Barkholt, L., Flory, E., Jekerle, V., Lucas-Samuel, S., Ahnert, P., Bisset, L., Büscher, D., Fibbe, W., Foussat, A., Kwa, M., Lantz, O., Mačiulaitis, R., Palomäki, T., Schneider, C. K., Sensebé, L., Tachdjian, G., Tarte, K., Tosca, L., and Salmikangas, P. (2013) Risk of tumorigenicity in mesenchymal stromal cell-based therapies--bridging scientific observations and regulatory viewpoints. *Cytotherapy* **15**, 753–759

95. Hamann, A., Nguyen, A., and Pannier, A. K. (2019) Nucleic acid delivery to mesenchymal stem cells: A review of nonviral methods and applications. *J Biol Eng* **13**

96. Berg, J. S., Lin, K. K., Sonnet, C., Boles, N. C., Weksberg, D. C., Nguyen, H., Holt, L. J., Rickwood, D., Daly, R. J., and Goodell, M. A. (2011) Imprinted genes that regulate early mammalian growth are coexpressed in somatic stem cells. *PLoS One* **6**

Chapter 2: Impact of GRB10 on insulin signaling in swine MSC

Introduction

Energy metabolism is tightly controlled at all levels, from single cells to an entire organism. Glucose is a key nutrient that is regulated by the dual action of hormones glucagon and insulin. Glucagon is secreted from the pancreatic alpha cells to increase glucose levels while insulin is secreted from the beta cells of the pancreas to lower glucose levels. Appropriate insulin action is required for the system to maintain homeostasis and process nutrients efficiently. Tissues can become unresponsive to insulin's action, otherwise known as insulin resistance. Insulin resistance can be caused by a variety of factors including lifestyle choices, autoimmune disease, pathogen exposure and adaptor proteins (1). Growth factor receptor bound protein 10 (GRB10) is an adaptor protein that lacks inherit catalytic activity and can inhibit insulin signaling (2).

Insulin signaling is a well conserved process a variety of species and is responsible for anabolic processes such as lipogenesis, glycogenesis, glucose disposal and synthesis of new skeletal muscle. The hormone insulin binds to and activates its own receptor in response to post-prandial increase in glucose. The insulin receptor (INSR) is a receptor tyrosine kinase that has autophosphorylation capability to activate itself and the next downstream enzyme, insulin receptor substrate 1 (IRS1). Once IRS1 is activated, it can proceed to switch on the next enzyme, phosphoinositide-3 kinase (PI3K). The activation of PI3K is crucial to insulin action as it activates protein kinase B (AKT) which is a major mediator of insulin signaling (3). An activated AKT can aid in the anabolic functions of insulin and activate downstream effectors such as

mammalian target of rapamycin (mTOR), glycogen synthase kinase 3 (GSK3) and translocation of glucose transporter 4 (glut4) to the plasma membrane to pull glucose from blood into tissues (4).

GRB10 aids in the regulation of insulin signaling via binding to INSR to induce a conformational change that prevents the insulin signaling cascade from progressing (2). GRB10 is a substrate of the mammalian target of rapamycin complex 1 (mTORC1) and aids in the regulation of adipose tissue functions such as lipolysis, thermogenesis, and energy expenditure (5). mTORC1 will phosphorylate GRB10 allowing for inhibition of receptor tyrosine kinases such as INSR, insulin like growth factor receptor (IGFR) and epidermal growth factor receptor (EGFR) signaling (6). The Src homology 2 (SH2) region was identified as the domain of the GRB10 protein binds to the phosphorylated INSR in Chinese hamster ovarian (CHO) cells to inhibit signal transduction (53). In human studies, GRB10 limits the access of IRS1 to INSR preventing downstream activation of the IRS1/PI3K/AKT pathway (7). The complex formed when GRB10 binds to INSR inhibits downstream phosphorylation. (8). GRB10 has also been implicated in other processes as recent studies have shown that GRB10 participates in regulating the incidence and development of cancer via moderating cellular metabolism, growth, proliferation, and apoptosis (9). GRB10 has been documented as an imprinted gene and when the maternal inheritance is abolished, mice have increased birth weights (10). Mice lacking GRB10 exhibit hypertrophy of skeletal muscle with increased fiber number as adults resulting from increased glucose metabolism (11). Ablation of GRB10 in mice caused increased myogenesis and glucose uptake of skeletal muscles (12). Taken together, these studies highlight the role of GRB10 as a negative regulator of insulin signaling.

While GRB10 has been characterized in insulin responsive metabolic tissues such as liver, muscle and adipose, (13) there are still tissues and cell types that have been neglected. Mesenchymal stem cells (MSC) are adult multipotent stem cells derived from bone marrow that can differentiate into chondrocytes, myocytes, osteocytes, and adipocytes (14–16). Commitment to adipogenesis is a multi-step process that begins in vitro incubation with insulin and other compounds that increase glucose uptake, triglyceride synthesis and increase abundance of transcription factors such as peroxisome proliferation-activated receptor gamma (PPARγ) and sterol regulatory binding protein 1c (SREBP1c) that initiate adipogenesis (17). The differentiation capabilities of MSC have been well documented (18) however, there is less information available on the presence and role of GRB10 in MSC insulin signaling. Porcine MSC can be purified, expanded, and undergo differentiation like that of human MSC making them an excellent model system (19). Pigs have been documented as a model for human research due to their similarities in the various organ systems, nutritional requirements, anatomy, and physiology (20). In swine, *GRB10* has been established as a candidate gene in pathways related to growth (21) and muscle deposition (22). Investigation of GRB10 in swine can provide useful information on nutrient utilization and regulation of insulin signaling. In this study we attempted to characterize the role of GRB10 in swine MSC utilizing genome editing technology.

Materials and Methods

Mesenchymal stem cell isolation and culture

MSC used in this study were isolated and utilized in a previous study in our lab (23) from an 18-day-old female piglet designated as a control. Briefly, MSCs were isolated from the bone

marrow as described previously (24). All MSCs isolations were verified to be >95% negative for CD45 and >95% positive for CD105/CD44. After in vitro experiments were completed, the cells were stored in liquid nitrogen until the time of the outlined experiment. To prepare for transfection, MSCs were cultured in growth media. The growth media (GM) consisted of high glucose DMEM (Gibco) supplemented with 10% Fetal bovine serum (Gibco), 1% penicillin/streptomycin (Gibco), 0.1% gentamicin (Gibco). Cells were cultured in a 37°C incubator with 5% CO2 and the media was changed every three days until cells were at 80% confluence.

Nucleofection of MSC and clonal expansion

On the day of transfection, the media was refreshed two hours before the nucleofection occurred. The cells were harvested using 0.05% Trypsin EDTA (Gibco) and 400,000 total cells were pelleted in a microcentrifuge tube for nucleofection. Cells were resuspended in the Lonza buffer P1 (Lonza) and then transferred to the Lonza 4D cuvette for nucleofection. Each nucleofection reaction contained the ribonucleoprotein complex (RNP) and the Grb10-KD oligonucleotide or GFP plasmid (Table 1). The RNP consisted of 20 μM Alt-R S.p. Cas9 nuclease V3 (Integrated DNA Technologies) and 60 μM of the GRB10-KD small guide RNA (sgRNA). The Grb10-KD system contained 50 μM of the HDR oligo. As a transfection control, both WT and KD cells were incubated with 1ug of pmaxGFP vector (Lonza). After transfection, cells rested at room temperature for 10 minutes. After incubation, growth media was added to each cuvette and transferred to a pre-equilibrated 6- well dish. The media was changed within twenty-four hours after the transfection and changed every 2-3 days until 80% confluence. The

day after the transfection, the GFP containing wells were visualized using Zeiss AxioObserver (Carl Zeiss, AG, Germany) to verify electroporation success.

Table 2-1: Sequences for genome editing via CRISPR/Cas9 and colony screening

Item	Sequence (5'-3')
GRB10-KD HDR Oligonucleotide	C*C*C*TCCGTTTTCCCGTGTCCTAGAATTTCTTCCCCGAACAGATGGTTACCTaGGTGCCAGCAGTCCAA CGGCAGTCAGACCCAGCTTTTGCAGGTACT*G*G
sgRNA	TCCCCGAACAGATGGTTACC
TracRNA	TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT CGGAGCTTT
GRB10-KD Screen Primer	F: TGAGACACAACAGCAGCGACGC
	R: AACCCGTGGCAGTACCTTGCAG

** in sequence indicates a phosphorothiorate bond. Arrow indicates point mutation on HDR oligonucleotide*

Once cells reached confluence, they were detached with trypsin and seeded for colony formation at a density of 50 cells/mL. Cells were incubated in growth media as described above until colonies formed. The colonies were picked up using cloning cylinders (VWR international) coated with vacuum grease (VWR international) to create a seal around the colony. The colony was harvested from the plate using trypsin and carefully transferred to a 24-well plate. Media was changed in the 24 well plate the next day and growth checked daily until 80% confluence was reached. This process was repeated in both 60mm dish and then in a 10cm dish. Once cells were at the desired confluency in a 10cm dish, the cells were harvested using trypsin and centrifuged to pellet the cells. After resuspension in growth media, cells were allocated for genomic DNA collection, RNA extraction or cryopreservation.

Colony screening for GRB10-KD modification and *GRB10* expression

Genomic DNA (gDNA) was extracted from the cell pellet using the PureLink Genomic DNA mini kit (Invitrogen) according to the manufacturer's instructions. The only deviation from the manufacturer's instructions was to increase the length of incubation with RNase A,

Proteinase K and the Purelink Genomic Lysis/Binding Buffer at 55°C from 10 minutes to 60 minutes. Isolated gDNA was quantified using the Synergy HTX microplate reader using the Gen 5.0 v3.0 software (BioTek Instruments, Winnoski, VT) and stored at -20°C for colony screening. Grb10-KD was verified via PCR followed by a restriction enzyme digestion. Screening PCR was completed using Q5 High-Fidelity DNA Polymerase (New England Biolabs). Briefly, using iCycler Thermal cycler (BioRad) 100ng template, Q5 reaction buffer, Q5 High GC enhancer, 200 µM dNTPs, 500nM forward and reverse primer (Table 1-1) The thermocycling conditions were as follows: Initial denaturation 98°C for 30s then 35 cycles of denaturation 98°C for 10s, anneal 66°C for 30s and extension at 72°C for 1:45 minutes. The final extension was performed at 72C for 2:00 minutes. Successful PCR reaction was confirmed via agarose gel electrophoresis and DNA was visualized using SYBR Safe DNA Gel Stain (Invitrogen). The PCR products were then purified using Monarch PCR & DNA Cleanup (New England Biolabs) following the manufacturer's instructions. Purified PCR products were sent to Azenta for sanger sequencing. A total of 1ug of purified PCR product was then digested via the restriction enzyme AVRII (NEB Catalog R0174S) for 1 hour at 37°C.

Potential colonies were lysed using RLT lysis buffer from the Qiagen RNAeasy kit (Qiagen, Hilden, Germany) and stored in -80°C until RNA extraction. Total RNA was extracted per manufacturer's instructions which included the optional DNAase digestion step. Total RNA was quantified using the Synergy HTX microplate reader using the Gen 5.0 v2.0 software (BioTek instruments, Winooski, VT). RNA was reverse transcribed with the SuperScript IV First-Strand Synthesis System (Invitrogen) and treated with RNAse H to remove residual RNA. The resulting cDNA was quantified using the Quant-iT OliGreen ssDNA Reagent (Invitrogen)

and kit following manufacturer's instructions and detected using the same Synergy HTX microplate reader. A total of 20ng cDNA was used for single RT-qPCR using Bio-Rad's CFX96 Touch Real-Time PCR Detection System with iTaq Universal SYBR Green Supermix. Primers were obtained from Integrated DNA Technologies (Table 2-1). Genes of interest were normalized to the reference gene, RPL4, using the $2^{\Delta\Delta CT}$ method. The single RT-qPCR reactions were amplified at 60°C for 30s containing 300 nM forward and reverse primers.

Proliferation assessment using 5-ethynyl-2′-deoxyuridine

WT and GRB10-KD cells were proliferated and cultured as described above until time of experiment. Cells were plated at $1x10^4$ in a 24 well plate to assess proliferation rate using the Click-it EdU Imaging Kit (Invitrogen). The following day, GM was removed and replaced with GM containing 10 μM EdU and cells were incubated for 24 and 48 hours. After the incubation period, cells were fixed in 4% paraformaldehyde and stained according to manufacturer's instructions. Briefly, cells were blocked in 3% bovine serum albumin in PBS and then permeabilized in 0.1% Triton-X 100 in PBS. The Click-it reaction cocktail was then added to each well and incubated for 30 minutes covered. After washing with PBS, 5ug/mL Hoescht was added as a counter stain. Cells were visualized and imaged on a Zeiss AxioObserver Z.1 and analyzed using ZenPro image analysis software (Carl Zeiss AG, Oberkochen, Germany).

In vitro assessment of insulin uptake

After verification, the frozen cells were thawed quickly in a 37°C bath and seeded into a 10cm dish in growth media. The cells were cultured at 37°C, 5% CO2 with media changes every 2-3 days until confluent. Once confluent, the cells were passaged into multiple 10cm dishes and

incubated until ready for seeding and in vitro experiments. When the cells are confluent, they are detached and seeded into a 6 well plate for insulin uptake experiment. The cells were seeded at a density of 6,250 cells/cm^2 into individual wells. The cells were incubated in GM at 37°C, 5% CO2 with media changes every 2-3 days until 80-90% confluent. Once confluent, the media was removed, and the cells rinsed twice with PBS. The media was replaced with DMEM containing 10nM insulin without antibiotics and FBS. The cells were returned to the incubator and protein and cell culture supernatant was collected 6 hours, 18 hours, 24 hours, and 48 hours post insulin addition. The cell culture supernatant was pipetted from the well and placed into a microcentrifuge tube on ice until centrifugation. The cell culture supernatant was centrifuged at 1200 rpm for 10 minutes at 4°C to pellet cellular debris. The cell culture supernatant was aliquoted and stored at -80°C until further analysis.

After the cell culture supernatant was collected, the remaining media was removed, and the cells were rinsed twice with ice cold PBS. After rinsing, the ice-cold RIPA (Invitrogen) containing a 1:100 dilution of Halt Protease Inhibitor Cocktail (Invitrogen) is added to each well and a cell scraper was used to completely detach cells. The cells were pelleted by centrifugation for 20 mins at 16,000G at 4°C after which the supernatant was removed and aliquoted for later use. The concentration of insulin in the cell culture supernatant was determined by an insulin sandwich ELISA (Invitrogen). Briefly, cell culture supernatants were diluted 1:5 in the provided assay diluent and incubated at room temperature for 2.5 hours at room temperature with gentle shaking. The wells were washed with the provided buffer four times before a biotinylated conjugate was added to each well and then incubated for 1 hour at room temperature with gentle shaking. The wells were then rinsed again as described above before a streptavidin HRP

antibody was added to each well and incubated with gentle shaking for 45 minutes. The wells were then washed and the TMB substrate was added to each well and incubated covered for 30 minutes at room temperature with gentle shaking. The reaction was terminated by adding the provided stop solution to each well and the plate was read using Synergy HTX microplate reader using the Gen 5.0 v2.0 software (BioTek instruments, Winooski, VT) at 450nM. A standard curve was generated using GraphPad Prism using a four-parameter algorithm.

Glucose uptake using Promega Pro-Glow

WT and GRB10-KD cells were proliferated and cultured as described above until time of experiment. Cells were plated at $3x10^4$ in a 96 well plate and media was changed every 2-3 days until the well reached 90% confluency. Glucose uptake was analyzed using the Glucose Uptake-Glo Assay (Promega, Madison WI) following manufacturer instructions. Briefly, cells were rinsed twice with PBS and then incubated with low glucose media (Gibco) containing no antibiotics, no FBS and either no insulin, 10 μM or 20 μM concentrations of insulin for one hour. After the incubation, 1mM two-deoxy glucose (2DG) diluted in PBS was added to each well for 10 minutes. The uptake of 2DG was then halted using the provided stop buffer and neutralized. A small portion of the lysate was collected at this time for protein quantification via Pierce Bradford (Invitrogen). A detection reagent was added to the wells to quantify the concentration of 2-deoxyglucose-6-phosphate (2DG6P) accumulated in each well after 30 minutes of incubation. Luminescence was recorded using Synergy HTX microplate reader using the Gen 5.0 v2.0 software (BioTek instruments, Winooski, VT.)

Differentiation of MSC into adipocytes.

Cells were plated at $2x10^4$ cells/mL into 24 well plates and GM was replaced every 2-3 days until 80-90% confluency was achieved. Once confluent, GM was replaced with differentiation media and changed every three days for 18 days. The differentiation media on day 0, 6, 12, and 18 consisted of growth media containing 500 µM isobutyl methylxanthine (IBMX) (Sigma), 1 µM dexamethasone (Sigma), 10 µM insulin (Sigma) and 200 µM indomethacin (Sigma). The differentiation media on days 3, 9, 15 consisted of growth media plus 10 µM insulin. RNA was collected on days 0, 3, 6, 12 and 18 of differentiation as described previously (23–25). Cell lysate was collected and processed into RNA using the RNAeasy Mini kit (RNAeasy, Qiagen) following the manufacturer's instructions, including the option DNAse incubation step. On day 18, cells underwent functional staining to assess lipid accumulation. Briefly, cells were fixed for 60 minutes in 4% paraformaldehyde and then stained with oil-red-O (ORO). A stock solution of 0.3% ORO was made in a 70% isopropanol and sterile filtered. The stock solution was then diluted in water at a ratio of two parts ORO and three parts waters. After fixation, cells were rinsed in PBS twice then incubated with 70% isopropanol for 10 minutes. Cells were incubated with ORO working solution for 30 minutes and then rinsed until clear.

Total RNA was quantified, reverse transcribed and quantified using the procedures and kits as described in the previous section. 20 ng of cDNA was used for both single and multiplex qPCR using Bio-Rad's CFX96 Touch Real-Time PCR Detection System with iTaq Universal SYBR Green Supermix or iQ Multiplex Powermix respectively. Primers and probes were obtained from Integrated DNA Technologies (Table 2). Genes of interest were normalized to the reference gene, RPL4, using the $2^{\Delta\Delta CT}$ method. The single RT-qPCR reactions were amplified at

60°C for 30s containing 300 nM forward and reverse primers. The multiplex RT-qPCR reactions were amplified for 45s at 60°C. The multiplex reactions required varying primer:probe ratios between the housekeeping gene and the genes of interest. Amplification of RPL4 utilized equal amounts of primer and probe while the genes of interest was a 2:1 ratio.

Table 2-2: Primers and probe nucleotide sequences for gene expression analysis via RT-qPCR

Primer Name	Gene ID		Primer Sequence (5'-3')	Probe
GRB10	100188977	F	TCAGGCTCCTCAAGTACGGA	
		R	GCCCAGAAAATCCATCGG	
ACC	397324	F	CCGTAGAAATCAAATTCCGCAG	
		R	CCTTCAGCTTGCTCTCCAG	
FASN	397561	F	CTCAACTTCCGAGACGTC ATG	
		R	ACCATTCCCATCACGCG	
PPARy	397671	F	AGATGATGACCAAGA AGCCC	
		R	GCAGAAGACGTCCAG GATG	
SREBP1	396572	F	GCTGACCGACATGCAAGACA	
		R	GAGCTGGCATCAGGACTGG	
RPL4	100038029	F	ACTTCCTTTGGTGGTTGAAGA	/CGY5/ACCCAAGGA/TAO/GGCTGTTCTGCTTCT/3IAbRQSp/
		R	CTCATTCGCTGAGAGGCATAA	
PI3K	396979	F	TCTCATGGTGGGAGGTGTAAG	/5HEX/CGGGTCAGT/ZEN/GAACACAGAATCTTACAACT/3IABkFQ/
		R	AGTCAAGCTGTGGTGAATCTAA	
AKT	100126861	F	CCGAGAAGAACGTGGTGTATC	/56-FAM/TTGTCCAGC/ZEN/ATGAGGTTCTCCAGC/3IABkFQ/
		R	CCGAAGTCAGTGATCTTGATGT	
INSR	396755	F	CAGAAAGTGTGCCCGACCAT	/5TEXRD-XN/CGATGTTTTGGGCAACTGCTCTGA/3IAbRQSp/
		R	ATCTGCCGTCCAGGTAGAAGT	

Western blot analysis of proteins in MSC

Cells were plated in GM and allowed to proliferate until 80% confluence. Cells were rinsed with ice cold PBS and incubated with RIPA buffer (Invitrogen) containing a Halt Protease/phosphatase Inhibitor Cocktail (Invitrogen). Cell lysate was collected and incubated on ice for 30 minutes with vortexing every 10 minutes. Cell lysate was centrifuged at 16,000g for 20 minutes at 4°C. Supernatant was removed and stored at -20°C until further use. Protein was quantified using the Pierce BCA assay (Invitrogen) and 10ug of protein was loaded into a gel (Biorad) and separated via polyacrylamide electrophoresis. The gel was transferred to a PVDF membrane using the Biorad Turbo Transfer system. The blots were blocked for one hour at room temperature in blocking buffer containing 5% non-fat milk in tris buffered saline with 0.1% tween (TBST). The blots were incubated overnight at 4°C with primary antibodies AKT (Cell Signaling Technology, 1:1000) or PI3K (Proteintech 1:1000) and β-actin (Cell Signaling

Technology, 1:1000) loading control in blocking buffer. Blots were rinsed three times in TBST before incubation with secondary antibodies Goat-Anti Rabbit (Cell signaling Technology, 1:1,000) or Goat-Anti Mouse (Novus Biologicals, 1:20,000). Gels were visualized using the SignalFire Elite ECL reagent (Cell signaling technology) chemiluminescent system in the Biorad Chemidoc XRS.

Statistics

Gene expression of *GRB10* in MSC was examined using a student's t-test. Impact of GRB10 modification on proliferation rate was analyzed using a student's t-test. The impact of GRB10 on glucose and insulin uptake was analyzed using an ordinary two-way ANOVA with Bonferroni's multiple comparison test with a single pooled variance. Gene expression data were analyzed using a two-way ANOVA/mixed effects model and Bonferroni's multiple comparison test with a single pooled variance. A minimum of four technical replicates were performed for all comparisons; a probability of $p<0.05$ was significant and $p<0.10$ was considered to be a trend. All statistical procedures were analyzed using GraphPad Prism 10 (GraphPad Software, INC, La Jolla, CA). Data are reported as mean ± SEM.

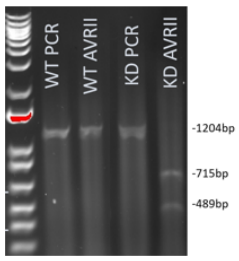
Results

Swine MSC exhibits genetic modification of GRB10.

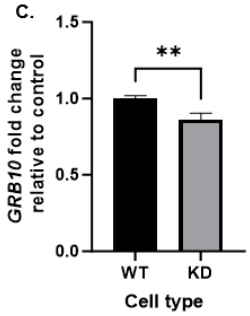
The GRB10 screening primers generated a PCR product that is 1204 base pairs long. If the editing was successful, the product should be able to be digested by AVRII to create two fragments that are 489 bp and 715 bp. The screening products are shown alongside the restriction enzyme digest in Figure 2-1A. The sanger sequencing results of the PCR product displayed the

point mutation that generated the new restriction site (Figure 2-1B). The successful CRISPR-Cas mediated HDR event introduced a single nucleotide addition to generate a premature stop codon and a AVRII digestion site. To assess expression levels of *GRB10* in genetically modified MSC, we utilized RT-qPCR to detect transcript abundance. There is a significant decrease ($p<0.05$) in *GRB10* transcript abundance in GRB10-KD MSC (Figure 2-1C).

A.



C.



B.

Sample	DNA (5'-3')	Translation
WT MSC	AATTTCTTCCCCGAACAGATGGTTACCTGGTGCCAGCAGTC CAACGGCAGTCAGACCCAGCTTTTGCAGG	NFFPEQMVTWCQQSNGSQTQLLQ
GRB10-KD MSC	AATTTCTTCCCCGAACAGATGGTTACCTAGGTGCCAGCAGT CCAACGGCAGTCAGACCCAGCTTTTGCAGG	NFFPEQMVT*VPAVQRQSDPAFA

Figure 2-1: Screening, sequencing, and expression of GRB10 KD MSC. (A) Agarose gel depicting entire screening product (WT PCR and KD PCR) adjacent to the digested PCR product (WT AVRII and KD AVRII). (B) Sanger Sequencing results for WT PCR and KD PCR products from panel A with the corresponding translation. Underlined segment is new AVRII site and * indicates new stop codon (C) Relative *GRB10* expression of WT and KD MSC values are means ± SEM; n=8 technical replicates; ** indicates significance of $p<0.01$

Proliferation potential is reduced post GRB10 modification

As insulin plays a role in nutrient sensing and cell growth, we aimed to assess the impact of GRB10 modification on proliferation rate. As new daughter cells are produced, they incorporated EdU into their DNA. The EdU can then be visualized via fluorescent dye at 24 (Figure 2-2A) and 48 (Figure 2-2B) hours post plating, while Hoeschst was used as a nuclear counter stain. The GRB10-KD mesenchymal stem cells had reduced ($p<0.001$) in vitro

proliferation rates compared to their wild type counterparts at 24 hours and 48 hours post seeding as indicated by a reduction in the percentage of GFP positive cells (Fig 2-2B and 2-D).

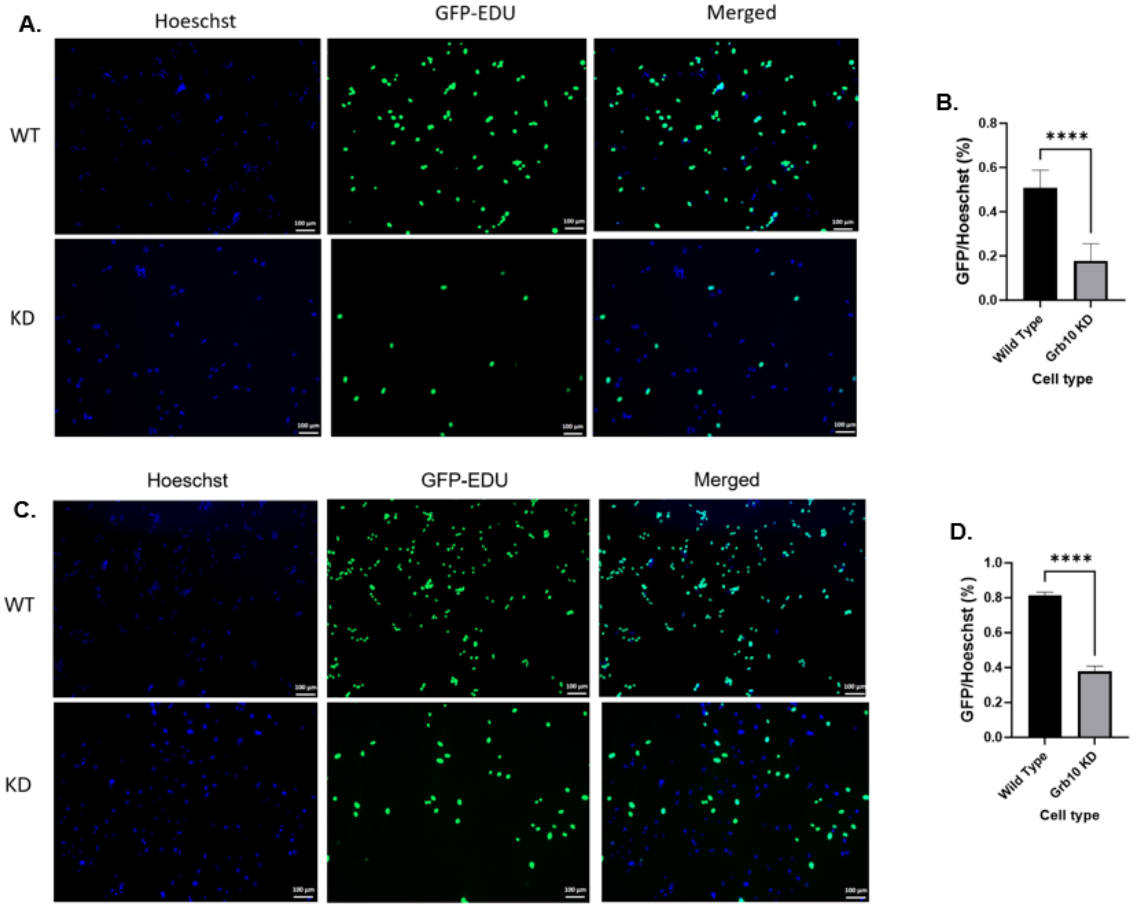


Figure 2-2: Proliferation assessment of GRB10 WT and GRB10-KD mesenchymal stem cells in vitro using immunostaining. (A) Fluorescent images of proliferation rate after 24 hours. (B) Bar graph representing percentage of GFP positive cells after 24 hours of proliferation. (C) Fluorescent images of proliferation rate after 28 hours. (D) Bar graph representing percentage of GFP positive cells after 48 hours of proliferation. Both bar graphs data are represented as mean ± SEM, n=32 images. **** indicates a p value of <0.0001.

Altering GRB10 impacts insulin usage and glucose uptake

Insulin is required for cells to use glucose appropriately, in altering the expression of GRB10 there is potential for differences in how insulin and glucose are utilized. When 2DG is used in cell culture, it can be consumed in the same manner as glucose and be processed into

2DG6P by hexokinase. Due to the lack of a hydroxyl group, the 2DG6P cannot pass through glycolysis and will accumulate in the cell. The accumulated 2DG6P detected via a luciferase reaction is indicative of how much 2DG was transported into the cell. The amount of 2DG6P reflects how much glucose the cell would have utilized over the same period. There is a decrease ($p<0.5$) of 2DG6P in GRB10-KD MSC when no insulin is added to media. At increasing concentrations of insulin, 10 µM and 20 µM, there is no difference in 2DG uptake (Figure 2-3A). When insulin binds to its receptor, it is internalized and removed from the surrounding environment. To evaluate the insulin usage between WT and GRB10-KD cells, the remaining amount of insulin in the cell culture supernatant was evaluated over a period of 48 hours. After six and 12 hours of insulin stimulation, there was no difference in insulin concentration in the cell culture supernatant in GRB10-KD cells. At the 24-hour mark and 48-hour mark, there was a significant increase in insulin concentration in cell culture supernatant in GRB10 KD cells ($p<0.05$) (Figure 2-3B)

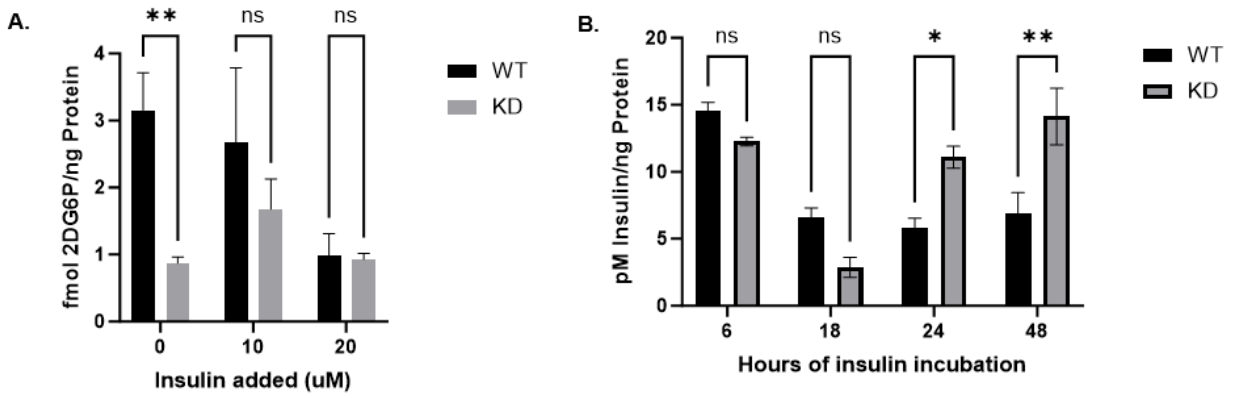


Figure 2-3: Comparison of glucose uptake and insulin utilization in WT and GRB10-KD MSC. (A) Concentration of 2DG6P after stimulation with insulin at varying concentrations normalized to amount of protein in each well. (B) Insulin in cell culture supernatant after incubation in serum free media with 1mM insulin added normalized to amount of protein in each well. All bar graphs are presented as mean ± SEM. * indicates $p<0.05$ and ** indicates $p<0.01$)

Differentiation into adipocytes is impacted by GRB10

A characteristic of MSC is the potential to differentiate into other cell lineages such as adipocytes. WT and GRB10-KD MSC were differentiated into adipocytes over 18 days and functional staining of neutral lipids was completed using Oil-Red-O (Figure 2-4A). Over the course of differentiation, there are many transcriptional changes that occur in cells regarding genes associated with insulin signaling and lipogenesis. In response to GRB10 modification, the insulin receptor (*INSR*) expression was greater across differentiation time points with a trend for increase at day 0 ($p<0.10$) and increased at day 3, 6, 12, and 18 (Figure 2-4B) in GRB10-KD cells. A downstream effector of insulin signaling, phospoinositide-3-kinase (*PI3K*) expression was not altered at day 0 and was increased ($p<0.05$) across days 3, 6, and 12 of differentiation with a trend for increase at day 18 (Figure 2-4C) in GRB10-KD cells. There was no difference in expression of protein kinase B (*AKT*), a major effector of insulin signaling, across differentiation time points between both cell types (Figure 2-4D). Transcription factors responsible for initiating adipogenesis, peroxisome proliferator receptor gamma (*PPARy*) and sterol-element binding protein 1c (*SREBP1c*) had changes in expression in GRB10-KD cells across differentiation time points. There was a trend for increased *PPARy* expression at day 0 ($p<0.10$) and a significant increase at days 6 and 12 ($p<0.05$) of differentiation time points (Figure 2-4E). Expression of *SREBP1c* was increased ($p<0.05$) at day 0 and 12 of differentiation (Figure 2-4F). Acetyl CoA Carboxylase (*ACC*) and fatty acid synthase (*FAS*) are the final two steps in lipogenesis. Expression of *ACC*, the rate limiting step of lipogenesis, was increased at day 12 of differentiation ($p<0.05$) and there was a trend ($p<0.10$) for an increase on day 0 of differentiation (Figure 2-4G) in GRB10-KD cells. The final step in lipogenesis, *FAS* was significantly increased ($p<0.05$) in GRB10-KD MSC on day 12 of differentiation (Figure 2-4H).

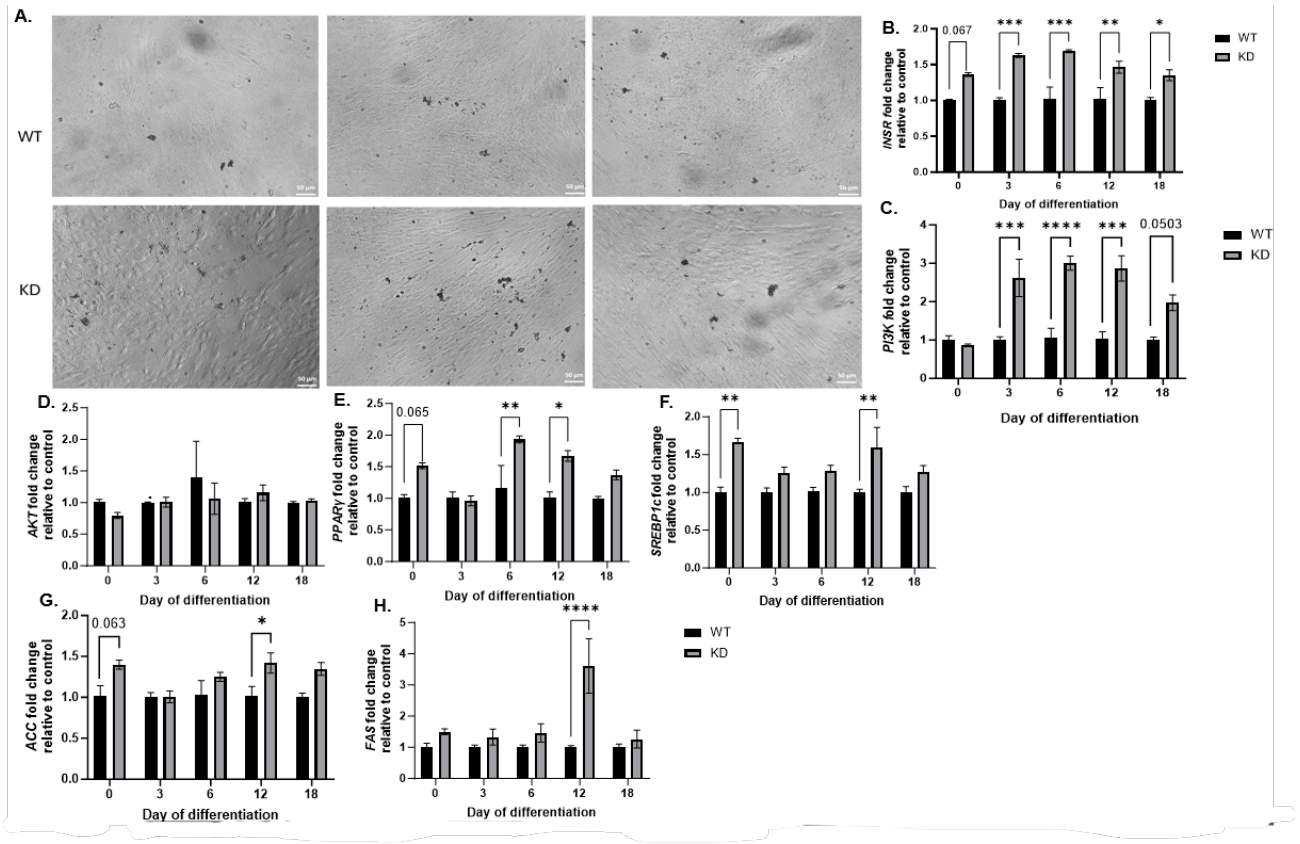


Figure 2-4: Impact of GRB10 modification on differentiation to adipocytes. (A) Functional staining of adipocytes with Oil-Red-O at day 18 of adipogenesis. (B) Gene expression of *INSR* during adipogenesis (C) Gene expression of *PI3K.* (D) Gene expression of *AKT.* (E) Gene expression of *PPARy.* (F) Gene expression of *SREBP1c.* (G) Gene expression of *ACC.* (H) Gene expression of *FAS.* All bar graphs are presented as mean ± SEM. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$; indicates $p<0.0001$

GRB10 modification alters expression of insulin related proteins.

Relative expression of insulin signaling proteins was assessed using western blot analysis. The serine/tyrosine kinase, AKT displayed a twofold reduction ($p<0.05$) in abundance in GRB10-KD MSC compared to the WT control (Figure 2-5A-B). A downstream effector of insulin signaling, PI3K displayed increased abundance ($p<0.05$) in GRB10-KD MSC (Figure 2-5C-D).

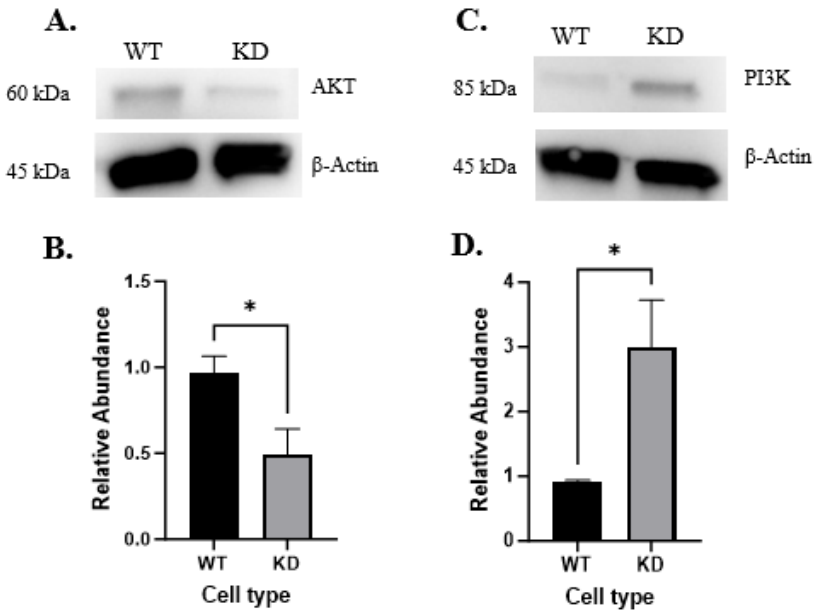


Figure 2-5: Expression of insulin related proteins in GRB10 WT and KD MSC (A) Western blot of AKT expression in WT and GRB10-KD MSC. (B) Graphical representation of abundance of AKT in MSC. (C) Western blot of PI3K expression in WT and GRB10 KD MSC. (D) Graphical representation of abundance of PI3K in MSC. Four technical replicates were completed for each western blot analysis. All bar graphs represent mean ± SEM. * indicates $p<0.05$

Discussion

GRB10 has been well characterized as a regulator of mitogenic and metabolic pathways including insulin signaling (2). GRB10 is an imprinted adaptor protein that lacks inherit enzymatic function but can mediate protein interactions via protein binding motifs (26). The dosage of GRB10 upon maternal inheritance can account for approximately 10% of Silver-Russel syndrome's severe growth retardation (10). GRB10 has been shown to regulate insulin signaling via binding to the intracellular domain of the insulin receptor using its own Src homology-2 (SH2) domain (27). However, the type of regulation is varied depending on the model system and context (28). GRB10 inhibits the function of both insulin and IGF1 receptors

to negatively regulate growth in transgenic mice and induce insulin resistance (29). There is a growing body of previous work in conventional models that establishes the role of GRB10 in metabolism. In swine there is an established quantitative trait locus (QTL) for reproduction, growth, development, and energy metabolism in swine that contains GRB10 (21). However, there is still much to be elucidated on the role of GRB10 in a swine system (30). Little information is available on the role of GRB10 in porcine MSC.

While the current literature surrounding MSC is centered around cellular therapies for the treatment of various diseases and injuries (31). This data establishes that porcine MSC can be successfully edited via CRISPR/Cas9 genetic engineering to model disrupted insulin signaling. Previous studies have shown that porcine bone marrow derived MSC have undergone transfections via human adenovirus and plasmids (36). Our data contributes to how the CRSIPR/Cas system is being used to investigate improving MSC physiology. Other studies have engineered MSC to have increased stemness, angiogenic potential and reduction in apoptosis (33). Mirroring our findings of potential for increased adipogenesis, MSCs have been modified to enhance their osteogenic differentiation potential (34). Genomic editing in MSC has proven to be potential therapy for Type 1 Diabetes (35) which highlights how genetically modified MSC can be beneficial to the study of disrupted insulin signaling. These data further establishes that swine primary cells can be isolated, cultured, differentiated and modified in order to understand mechanisms of insulin disruption.

GRB10 functions in cell division via interactions with the epidermal growth factor receptor (EGFR) (37). Our study noted heavy reduction in proliferation rates when swine MSC

underwent genetic editing. A similar phenomenon has been noted in swine studies as a micro-RNA (miR-222) has been shown to silence the *GRB10* gene via inactivation of the PI3K/AKT pathway in porcine Sertoli cells resulting in reduced proliferation (38). This finding is also supported in GRB10 knockdown in primary bone marrow cells leading to reduced proliferation rate (39). In oncology studies of gastric cancer, knockdown of GRB10 inhibits proliferation and migration ability of the cancerous cells (40). However, this is contradicted by studies using murine hematopoietic stem cells where knock out of GRB10 was shown to increase hematopoietic stem cell regeneration and proliferation (41). Overexpression of GRB10 in animal and cell culture models has also been shown to impact proliferation rates. Mouse GRB10 overexpression inhibited IGF1 induced proliferation and colony formation in vitro (42). While the overexpression of GRB10 in mouse immune cells resulted in increased proliferation and survival in culture (43).

In cultured fibroblasts, elevated GRB10 regulates MAP kinase induced mitogenesis (65). There is evidence for a mechanism for the impact of GRB10 on proliferation as GRB10 binds to neural precursor cell expressed developmentally downregulated 4-1 (NEDD4) to promote internalization, ubiquitination, and degradation of targets under ligand activation to negatively regulate cell proliferation (44, 45). As an E3 ligase, NEDD4 inhibits protein kinase complex formation to degrade the mitogen-activated protein (MAPK) or downstream substrates (46). The MAPKs aid in the regulation of cellular programs such as cell proliferation, differentiation, motility, and general survival (47). This interaction between GRB10/NEDD4/MAPK has been demonstrated in rat adipocytes with an overexpression of GRB10 exhibited reduced insulin activation of pathway via NEDD4 interactions with GRB10(48).

We aimed to characterize the impact of GRB10 modification on insulin signaling in swine MSC. When incubated with insulin, there was a reduction in insulin sensitivity after 24 and 48 hours of culture as indicated by increased insulin in the cell culture supernatant. Once we established that insulin signaling is disrupted between our cell types, we noted a reduction in glucose uptake under basal culture conditions which was abolished with increasing amounts of insulin. This finding of apparent inhibited insulin signaling in GRB10-KD MSC is contradicted by studies in human myotubes where GRB10 knockdown enhanced insulin induced PI3K and AKT signaling and glucose uptake (49). In animal models where GRB10 peripheral knockout mice had increased body weight, improved glucose tolerance and insulin sensitivity implicating GRB10 as a negative regulator of insulin signaling (50). Our findings appear to surprisingly correlate with different GRB10 modeling systems entirely. In 3T3-L1 adipocytes overexpression of GRB10 caused diminished insulin stimulated tyrosine phosphorylation of IRS and reduced insulin signaling and adipogenesis in these cells (7). As GRB10 was initially identified as a substrate of EGFR in a yeast hybrid screen (6), it is plausible that the INSR is not the preferred receptor in swine MSC. It is clear the GRB10 does impact insulin signaling and glucose uptake, however, the result will vary based on experimental conditions.

Insulin is required in MSC culture systems to successfully induce adipogenesis and increase the rate of fatty acid synthesis in MSC (51). We aimed to differentiate WT and GRB10-KD MSC into adipocytes and evaluate transcriptional changes over the course of the differentiation period. We noted an increase in various insulin signaling genes such as *INSR* and *PI3K* over the differentiation period. Other studies noted no changes in *INSR* expression in

GRB10 KO systems but an increase in INSR protein abundance (52). While siRNA-mediated knockdown of GRB10 negatively regulated abundance of INSR (53) . There was also an increase in transcription factors (*PPARy* and *SREBP1c)* and adipogenic genes (*ACC* and *FASN)* in our study. Commitment to adipogenesis first requires activation of both PPARy and SREBP1c. As adipogenesis progresses, ACC and FASN are two enzymes that are crucial to de novo lipogenesis and later lipid accumulation in droplets in MSC (51). An increase in the transcripts of these key transcription factors and enzymes can indicate increased adipogenic potential of GRB10-KD MSC.

Typical activation of the insulin signaling cascade results in the activation of downstream kinases such as PI3K and AKT. We noted an increase in PI3K abundance and decrease in AKT abundance in GRB10-KD MSC. Under normal physiologic conditions mTORC1 phosphorylates and stabilizes GRB10 leading to inhibition of pathways downstream of PI3K and MAPK (54). The changes noted in PI3K have been observed in mice studies where GRB10 was able to provide a functional link between INSR and PI3K activation highlighting the regulatory role of GRB10 in the metabolic response to insulin(55). Studies in carcinoma cells highlighted that a knockdown of GRB10 reduces AKT phosphorylation via interaction with PI3K (56). Our findings do not match those in skeletal muscle where the loss of GRB10 resulted in enhanced AKT and MAPK activation under insulin action (50). We were unfortunately unable to quantify the abundance of phosphorylated AKT and the INSR proteins due to low protein yield when extracted. From our preliminary results, GRB10 modification inhibits abundance of AKT potentially causing feedback to increase abundance of PI3K.

Insulin activates the PI3K/AKT/mTORC1 pathway promoting many anabolic processes in the cell including protein synthesis, lipogenesis, and nutrient storage (57). GRB10 has been shown to regulate insulin signaling via interactions with the insulin receptor, insulin like growth factor receptor, epidermal growth factor receptor, growth hormone receptor and platelet derived growth factor (11, 58–60). The role of GRB10 and the impact of GRB10 modifications has yet to be firmly established in swine MSC. Insulin stimulated activation of mTORC1 in skeletal muscle of neonatal pigs can be regulated by the abundance of proteins, including GRB10 (22). In swine, insulin induced activation of mTORC1 stimulates the phosphorylation of GRB10 which stabilizes the protein to provide negative feedback on INSR (61)**.** GRB10 has been implicated as both a positive and negative regulator of mitogenesis depending on the experimental conditions (42, 62, 63)**.** We noted reduction in basal glucose uptake but no difference under insulin stimulation while insulin removal from media was also lower over time in the GRB10-KD cells. While the changes in insulin signaling were not expected based on the previous literature (2) it is interesting to note that genetically modified swine MSC may have a different phenotype compared to other models that needs further investigation. In addition to disrupted insulin signaling, we noted reduced proliferation of modified swine MSC. GRB10 functions as a stimulatory mitogenic signaling adapter via its BPS domain (60). As an adaptor protein, GRB10 has been implicated in multiple aspects of cellular metabolism and when stimulated by insulin, via interactions with MAPK (64). These results highlight the role of GRB10 in cellular growth and glucose metabolism. However, due to a lack of reliable protein quantification, we were unable to quantify the change in GRB10 abundance. Further work is necessary to further uncover the role GRB10 plays in MSC glucose metabolism and mitogenesis.

References

1. Wilcox, G. (2005) Insulin and Insulin Resistance. *Clin Biochem Rev* **26**
2. Holt, L. J. and Siddle, K. (2005) Grb10 and Grb14: enigmatic regulators of insulin action-and more? *Biochem. J* **388**
3. Bevan, P. (2001) Insulin signalling. *J Cell Sci* **114**, 1429–1430
4. Manning, B. D. and Toker, A. (2017) AKT/PKB Signaling: Navigating the Network. *Cell* **169**, 381–405
5. Liu, M., Bai, J., He, S., Villarreal, R., Hu, D., Zhang, C., Yang, X., Liang, H., Slaga, T. J., Yu, Y., Zhou, Z., Blenis, J., Scherer, P. E., Dong, L. Q., and Liu, F. (2014) Grb10 promotes lipolysis and thermogenesis by phosphorylation-dependent feedback inhibition of mTORC1. *Cell Metab* **19**, 967–980
6. Hsu, P. P., Kang, S. A., Rameseder, J., Zhang, Y., Ottina, K. A., Lim, D., Peterson, T. R., Choi, Y., Gray, N. S., Yaffe, M. B., Marto, J. A., and Sabatini, D. M. (2011) The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science (1979)* **332**, 1317–1322
7. Wick, K. L. R., Werner, E. D., Langlais, P., Ramos, F. J., Dong, L. Q., Shoelson, S. E., and Liu, F. (2003) Grb10 inhibits insulin-stimulated insulin receptor substrate (IRS)-phosphatidylinositol 3-kinase/Akt signaling pathway by disrupting the association of IRS-1/IRS-2 with the insulin receptor. *Journal of Biological Chemistry* **278**, 8460–8467
8. Liu, F. and Roth, R. A. (1995) Grb-IR: A SH2-domain-containing protein that binds to the insulin receptor and inhibits its function (phosphatidylinositol 3-kinase/yeast two-hybrid system/insulin-receptor substrate 1). **92**

9. O-Sullivan, I. S., Chopra, A., Carr, J., Tae, S. K., and Cohen, E. P. (2008) Immunity to growth factor receptor-bound protein 10, a signal transduction molecule, inhibits the growth of breast cancer in mice. *Cancer Res* **68**, 2463–2470

10. Charalambous, M., Smith, F. M., Bennett, W. R., Crew, T. E., Mackenzie, F., and Ward, A. (2003) Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism.

11. Holt, L. J., Turner, N., Mokbel, N., Trefely, S., Kanzleiter, T., Kaplan, W., Ormandy, C. J., Daly, R. J., and Cooney, G. J. (2012) Grb10 regulates the development of fiber number in skeletal muscle. *The FASEB Journal* **26**, 3658–3669

12. Mokbel, N., Hoffman, N. J., Girgis, C. M., Small, L., Turner, N., Daly, R. J., Cooney, G. J., and Holt, L. J. (2014) Grb10 Deletion Enhances Muscle Cell Proliferation, Differentiation and GLUT4 Plasma Membrane Translocation. *J Cell Physiol* **229**, 1753–1764

13. Holt, L. J. and Siddle, K. (2005) Grb10 and Grb14: enigmatic regulators of insulin action-and more? *Biochem. J* **388**

14. Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., and Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147

15. Owen, M. and Friedenstein, A. J. (1988) Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* **136**, 42–60

16. Friedenstein, A. J., Chailakhjan, R. K., and Lalykina, K. S. (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* **3**, 393–403

17. Chen, Q., Shou, P., Zheng, C., Jiang, M., Cao, G., Yang, Q., Cao, J., Xie, N., Velletri, T., Zhang, X., Xu, C., Zhang, L., Yang, H., Hou, J., Wang, Y., and Shi, Y. (2016) Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? *Cell Death Differ* **23**, 1128–1139

18. James, A. W. (2013) Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. *Scientifica (Cairo)* **2013**, 1–17

19. Ringe, J., Kaps, C., Schmitt, B., Büscher, K., Bartel, J., Smolian, H., Schultz, O., Burmester, G. R., Häupl, T., and Sittinger, M. (2002) Porcine mesenchymal stem cells: Induction of distinct mesenchymal cell lineages. *Cell Tissue Res* **307**, 321–327

20. Miller, E. R. and Ullrey, D. E. (1987) THE PIG AS A MODEL FOR HUMAN NUTRITION. *Ann. Rev. Nutr* **7**

21. Laghouaouta, H., Fraile, L., Suárez-Mesa, R., Ros-Freixedes, R., Estany, J., and Pena, R. N. (2022) A genome-wide screen for resilient responses in growing pigs. *Genetics Selection Evolution* **54**

22. Yang, Y., Liang, G., Niu, G., Zhang, Y., Zhou, R., Wang, Y., Mu, Y., Tang, Z., and Li, K. (2017) Comparative analysis of DNA methylome and transcriptome of skeletal muscle in lean-, obese-, and mini-type pigs. *Sci Rep* **7**

23. Zhang, W., Kroscher, K. A., Murray, R. L., Gagliardi, R., Guiltinan, C., Rhoads, R. P., and Stahl, C. H. (2020) Dietary calcium and phosphorus amounts affect development and tissue-specific stem cell characteristics in neonatal pigs. *Journal of Nutrition* **150**, 1086–1092

24. Mahajan, A. and Stahl, C. H. (2009) Dihydroxy-cholecalciferol stimulates adipocytic differentiation of porcine mesenchymal stem cells. *Journal of Nutritional Biochemistry* **20**, 512–520

25. Alexander, L. S., Seabolt, B. S., Rhoads, R. P., and Stahl, C. H. (2012) Neonatal phosphate nutrition alters in vivo and in vitro satellite cell activity in pigs. *Nutrients* **4**, 436–448

26. Smith, F. M., Holt, L. J., Garfield, A. S., Charalambous, M., Koumanov, F., Perry, M., Bazzani, R., Sheardown, S. A., Hegarty, B. D., Lyons, R. J., Cooney, G. J., Daly, R. J., and Ward, A. (2007) Mice with a Disruption of the Imprinted Grb10 Gene Exhibit Altered Body Composition, Glucose Homeostasis, and Insulin Signaling during Postnatal Life . *Mol Cell Biol* **27**, 5871–5886

27. Hansen, H., Svensson, U., Zhu, J., Laviola, L., Giorgino, F., Wolf, G., Smith, R. J., and Riedel, H. (1996) Interaction between the Grb10 SH2 Domain and the Insulin Receptor Carboxyl Terminus*.

28. Riedel, H. (2004) Grb10 exceeding the boundaries of a common signaling adapter. *Front Biosci* **9**, 603–618

29. Shiura, H., Miyoshi, N., Konishi, A., Wakisaka-Saito, N., Suzuki, R., Muguruma, K., Kohda, T., Wakana, S., Yokoyama, M., Ishino, F., and Kaneko-Ishino, T. (2005) Meg1/Grb10 overexpression causes postnatal growth retardation and insulin resistance via negative modulation of the IGF1R and IR cascades. *Biochem Biophys Res Commun* **329**, 909–916

30. Wang, Y., Ding, X., Tan, Z., Xing, K., Yang, T., Pan, Y., Wang, Y., Mi, S., Sun, D., and Wang, C. (2018) Genome-wide association study for reproductive traits in a Large White pig population. *Anim Genet* **49**, 127–131

31. Filho, D. M., de Carvalho Ribeiro, P., Oliveira, L. F., dos Santos, A. L. R. T., Parreira, R. C., Pinto, M. C. X., and Resende, R. R. (2019) Enhancing the Therapeutic Potential of Mesenchymal Stem Cells with the CRISPR-Cas System. *Stem Cell Rev Rep* **15**, 463–473

32. Hazrati, A., Malekpour, K., Soudi, S., and Hashemi, S. M. (2022) CRISPR/Cas9-engineered mesenchymal stromal/stem cells and their extracellular vesicles: A new approach to overcoming cell therapy limitations. *Biomedicine and Pharmacotherapy* **156**

33. Freitas, G. P., Lopes, H. B., Souza, A. T. P., Gomes, M. P. O., Quiles, G. K., Gordon, J., Tye, C., Stein, J. L., Stein, G. S., Lian, J. B., Beloti, M. M., and Rosa, A. L. (2021) Mesenchymal stem cells overexpressing BMP-9 by CRISPR-Cas9 present high in vitro osteogenic potential and enhance in vivo bone formation. *Gene Ther* **28**, 748–759

34. Gerace, D., Martiniello-Wilks, R., Nassif, N. T., Lal, S., Steptoe, R., and Simpson, A. M. (2017) CRISPR-targeted genome editing of mesenchymal stem cell-derived therapies for type 1 diabetes: A path to clinical success? *Stem Cell Res Ther* **8**

35. Bosch, P., Pratt, S. L., and Stice, S. L. (2006) Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. *Biol Reprod* **74**, 46–57

36. Margolis, B., Silvennoinen, O., Comoglio, F., Roonprapunt, C., Skolnik, E., Ullrich, A., and Schlessinger, J. (1992) High-efficiency expression/cloning of epidermal growth factor-receptor-binding proteins with Src homology 2 domains. *Proc Natl Acad Sci U S A* **89**, 8894–8898

37. Luo, H., Peng, F., Weng, B., Tang, X., Chen, Y., Yang, A., Chen, B., and Ran, M. (2020) miR-222 Suppresses Immature Porcine Sertoli Cell Growth by Targeting the GRB10 Gene Through Inactivating the PI3K/AKT Signaling Pathway. *Front Genet* **11**

38. Illert, A. L., Albers, C., Kreutmair, S., Leischner, H., Peschel, C., Miething, C., and Duyster, J. (2015) Grb10 is involved in BCR-ABL-positive leukemia in mice. *Leukemia* **29**, 858–868

39. Ren, L.-L., Wang, Z.-W., Sen, R., Dai, Z.-T., Liao, X.-H., and Shen, L.-J. (2023) GRB10 is a novel factor associated with gastric cancer proliferation and prognosis. *Aging* **15**, 3394–3409

40. Yan, X., Himburg, H. A., Pohl, K., Quarmyne, M., Tran, E., Zhang, Y., Fang, T., Kan, J., Chao, N. J., Zhao, L., Doan, P. L., and Chute, J. P. (2016) Deletion of the Imprinted Gene Grb10 Promotes Hematopoietic Stem Cell Self-Renewal and Regeneration. *Cell Rep* **17**, 1584–1594

41. Morrione, A., Valentinis, B., Resnicoff, M., Xu, S.-Q., and Baserga, R. (1997) The Role of mGrb10 in Insulin-like Growth Factor I-mediated Growth*.

42. Kazi, J. U. and Rönnstrand, L. (2013) FLT3 signals via the adapter protein Grb10 and overexpression of Grb10 leads to aberrant cell proliferation in acute myeloid leukemia. *Mol Oncol* **7**, 402–418

43. Monami, G., Emiliozzi, V., and Morrione, A. (2008) Grb10/Nedd4-mediated multiubiquitination of the insulin-like growth factor receptor regulates receptor internalization. *J Cell Physiol* **216**, 426–437

44. Vecchione, A., Marchese, A., Henry, P., Rotin, D., and Morrione, A. (2003) The Grb10/Nedd4 Complex Regulates Ligand-Induced Ubiquitination and Stability of the Insulin-Like Growth Factor I Receptor. *Mol Cell Biol* **23**, 3363–3372

45. Sala-Gaston, J., Costa-Sastre, L., Pedrazza, L., Martinez-Martinez, A., Ventura, F., and Rosa, J. L. (2023) Regulation of MAPK Signaling Pathways by the Large HERC Ubiquitin Ligases. *Int J Mol Sci* **24**

46. Cargnello, M. and Roux, P. P. (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* **75**, 50–83

47. Morrione, A., Plant, P., Valentinis, B., Staub, O., Kumar, S., Rotin, D., and Baserga, R. (1999) mGrb10 Interacts with Nedd4*.

48. Edick, A. M., Auclair, O., and Burgos, S. A. (2020) Role of Grb10 in mTORC1-dependent regulation of insulin signaling and action in human skeletal muscle cells. *Am J Physiol Endocrinol Metab* **318**, E173–E183

49. Wang, L., Balas, B., Christ-Roberts, C. Y., Kim, R. Y., Ramos, F. J., Kikani, C. K., Li, C., Deng, C., Reyna, S., Musi, N., Dong, L. Q., DeFronzo, R. A., and Liu, F. (2007) Peripheral Disruption of the Grb10 Gene Enhances Insulin Signaling and Sensitivity In Vivo. *Mol Cell Biol* **27**, 6497–6505

50. Fink, T. and Zachar, V. (2011) Adipogenic differentiation of human mesenchymal stem cells. *Methods Mol Biol* **698**, 243–251

51. Ramos, F. J., Langlais, P. R., Hu, D., Dong, L. Q., and Liu, F. (2006) Grb10 mediates insulin-stimulated degradation of the insulin receptor: A mechanism of negative regulation. *Am J Physiol Endocrinol Metab* **290**

52. Langlais, P., Dong, L. Q., Ramos, F. J., Hu, D., Li, Y., Quon, M. J., and Liu, F. (2004) Negative Regulation of Insulin-Stimulated Mitogen-Activated Protein Kinase Signaling by Grb10. *Molecular Endocrinology* **18**, 350–358

53. Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villén, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P., and Blenis, J. (2011) Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science (1979)* **332**, 1322–1326

54. Deng, Y., Bhattacharya, S., Swamy, O. R., Tandon, R., Wang, Y., Janda, R., and Riedel, H. (2003) Growth factor receptor-binding protein 10 (Grb10) as a partner of phosphatidylinositol 3-kinase in metabolic insulin action. *Journal of Biological Chemistry* **278**, 39311–39322

55. Khan, M. I., Johani, A. Al, Hamid, A., Ateeq, B., Manzar, N., Adhami, V. M., Lall, R. K., Rath, S., Sechi, M., Siddiqui, I. A., Choudhry, H., Zamzami, M. A., Havighurst, T. C., Huang, W., Ntambi, J. M., and Mukhtar, H. (2019) Proproliferative function of adaptor protein GRB10 in prostate carcinoma. *The FASEB Journal* **33**, 3198–3211

56. Saltiel, A. R. (2021) Insulin signaling in health and disease. *Journal of Clinical Investigation* **131**

57. He, W., Rose, D. W., Olefsky, J. M., and Gustafson, T. A. (1998) Grb10 Interacts Differentially with the Insulin Receptor, Insulin-like Growth Factor I Receptor, and Epidermal Growth Factor Receptor via the Grb10 Src Homology 2 (SH2) Domain and a Second Novel Domain Located between the Pleckstrin Homology and SH2 Domains*.

58. Moutoussamy, S., Oise Renaudie, F., Lago, F., Kelly, P. A., and Finidori, J. (1998) Grb10 Identified as a Potential Regulator of Growth Hormone (GH) Signaling by Cloning of GH Receptor Target Proteins*.

59. Wang, J., Dai, H., Yousaf, N., Moussaif, M., Deng, Y., Boufelliga, A., Rama Swamy, O., Leone, M. E., and Riedel, H. (1999) Grb10, a Positive, Stimulatory Signaling Adapter in Platelet-Derived Growth Factor BB-, Insulin-Like Growth Factor I-, and Insulin-Mediated Mitogenesis. *Mol Cell Biol* **19**

60. Suryawan, A. and Davis, T. A. (2018) Amino Acid- and insulin-induced activation of mTORC1 in neonatal piglet skeletal muscle involves Sestin2-GATOR2, Rag A/C-mTOR, and RHEB-mTOR complex formation. *Journal of Nutrition* **148**, 825–833

61. O'Neill, T. J., Rose, D. W., Pillay, T. S., Hotta, K., Olefsky, J. M., and Gustafson, T. A. (1996) Interaction of a GRB-IR splice variant (a human GRB10 homolog) with the insulin and insulin-like growth factor I receptors. Evidence for a role in mitogenic signaling. *J Biol Chem* **271**, 22506–22513

62. Tezuka, N., Brown, A. M. C., and Yanagawa, S. (2007) GRB10 binds to LRP6, the Wnt co-receptor and inhibits canonical Wnt signaling pathway. *Biochem Biophys Res Commun* **356**, 648–654

63. Langlais, P., Wang, C., Dong, L. Q., Carroll, C. A., Weintraub, S. T., and Liu, F. (2005) Phosphorylation of Grb10 by mitogen-activated protein kinase: Identification of Ser150 and Ser476 of human Grb10ζ as major phosphorylation sites. *Biochemistry* **44**, 8890–8897

Chapter 3: Disrupted insulin signaling due to an acute immune response in swine.

Introduction

Swine production and the consumption of pork products provide an important food source around the world (1). The agricultural industry relies on muscle accretion to generate food and revenue for farmers. A key hormone in muscle accretion and appropriate nutrient utilization is insulin. Insulin is an anabolic hormone that drives metabolism of nutrients such as glucose. Insulin is secreted by the pancreas and acts on peripheral tissues to drive glucose from the bloodstream into tissues to drive lipogenesis, protein synthesis, glycolysis, and glycogenesis (2). The insulin signaling pathway is a series of tyrosine kinases that possess intrinsic autophosphorylation capabilities to activate themselves or other downstream proteins. Briefly, when glucose levels are elevated after a meal, insulin is released from the pancreas and binds to the insulin receptor (INSR) prompting autophosphorylation. Once activated INSR process to phosphorylate the insulin receptor substrate-1 (IRS1). IRS1 will move on to activate the next enzyme, phosphoinositide-3, kinase (PI3K). PI3K will then ultimately phosphorylate and activate protein kinase b (AKT) which is the major mediator of insulin action. AKT activations allow for the translocation of the glucose transporter-4 (Glut4) to the plasma membrane to remove glucose from the bloodstream (3).

The disruption of insulin signaling is commonly referred to as insulin resistance where tissues are unable to respond to insulin appropriate to remove glucose from

circulation insulin. Insulin resistance can be caused by obesity (4), autoimmune disorders (5), polycystic ovarian syndrome (6), inflammatory responses (7), and adaptor proteins (8). While there is a large amount literature regarding insulin resistance in human physiology, there are still unanswered questions around disrupted insulin signaling in swine.

Pigs are subjected to a variety of both physical and physiological stressors including over-crowding, heat stress and pathogen exposure that can affect growth rates and muscle accretion (9). Disease in commercial swine production can cause financial loses that varies with disease severity between $11 and $29 USD per pig (10). If exposed to a pathogen, the animals' immune system becomes activated to combat the infection and the animal will exhibit signs of illness including a reduction in appetite, growth rate and poor nutrient metabolism (11). In addition to the release of proinflammatory cytokines, when affected by pathogens, swine have displayed altered insulin signaling (12) and changes in other metabolic parameters (13).

Lipopolysaccharide (LPS) is an endotoxin produced by gram-negative bacteria that causes severe inflammation and illness in post weaning pigs (14). Stressors that reduce gut barrier function will lead to increased LPS in circulation. LPS has been used to assess the inflammatory response in vivo, as it acts through the toll like receptor 4 (TLR4) and nuclear factor kappa beta (NFKB) signaling pathway (15). When the immune system is activated, the energy and nutrient demand increases, partitioning away nutrients from their normal physiologic purposes (16). The impact of LPS on the

immune system has been well characterized and often results in hyperthermia, changes in hemodynamics, production of pro-inflammatory cytokines, clinical signs, and dramatically disrupted metabolism (17–21). During an LPS infection, macrophages are recruited and activated by polarizing signals to produce inflammatory cytokines and induce antimicrobial functions. Polarizing signaling often influences metabolic signaling pathways including aspects of the insulin signaling cascade such as mammalian target of rapamycin (MTOR) and AKT (22). As swine production relies on efficient nutrient metabolism, we must understand how their physiology changes during different metabolic states, including the impact of pathogens.

During an active infection, whole body energy expenditure increases roughly by 50% (23). To meet the energy demand, glucose is freed as typical insulin sensitive tissues (adipose and skeletal muscle) become briefly resistant and hepatic glucose output increases (24, 25). The impact of LPS exposure on overall metabolic function will vary depending on animal model, age, model culture system, and exposure. In cattle exposed to LPS, LPS acts as an insulin secretagogue to induce hyperinsulinemia during LPS exposure (20). While, in pigs administered LPS, the observed increase in circulating insulin was only apparent within the first 24 hours and then resolved with continued exposure as the animals acclimated (26). The full scope of how LPS impacts tissues on a transcriptional and protein level after an acute immune response is still unclear. We aim to characterize the changes in metabolism during an acute LPS induced immune response in post-weaning swine. Establishing parameters on how insulin signaling and glucose

metabolism changes under non-homeostatic conditions is beneficial to further our understanding of swine health to improve production.

Materials and Methods

Animals

All experimental procedures involving animals were approved by the University of Maryland Animal Care and Use committee. 58 commercial crossbred barrows and gilts aged five weeks weighing 12.52 ±0.21 kg was used in this experiment. The 58 animals were randomly divided into two experimental subsets where ten animals were designated for repeated blood sampling and the remaining 48 animals underwent timed euthanasia for tissue collection. The repeated blood sampling group underwent a surgical procedure to place a jugular catheter. The timed euthanasia animals were designated for tissue collection post exposure to LPS. All animals were group housed with a 12:12 light dark cycle with ad libitum access to food and water. All animals were fed a meal diet that met the requirements outlined in the NRC (27).

Repeated Blood Sampling

After a three-day acclimation period, ten animals (12.1±0.5 kg) were randomly selected and placed into single animal housing. All animals were fasted for a minimum of 12 hours before the surgical procedure. Anesthesia was induced with an intramuscular injection of 0.04 ml/kg of body weight (BW) Telazol (100 mg/ml), Ketamine (50 mg/ml), and Xylazine (50 mg/ml) (MWI Animal Health). After induction, anesthesia was maintained via administration of sevoflurane at a rate of 5-10ml/kg/min using a tight-

fitting nose cone. The animal was placed in dorsal recumbency with legs retracted and secured caudally. Hair was trimmed from the surgical area and skin was prepped with alternating scrubs of dilute chlorohexidine and betadine. The jugular furrow was identified and a 1–2-inch incision was made in this plane to provide access. Before isolating the vein, the subcutaneous tissue, fat, and musculature was bluntly dissected to expose the jugular vein to generate a working length of 2-4cm. The vein was partially occluded using 3-0 nylon suture 2 cm above and below the working area. The vein was punctured with a vein pick and a 20g/3fr polyurethane catheter was gently threaded through the lumen of the vessel and extended 10cm towards the animal's chest. The catheter was secured to the musculature of the neck using 3-0 nylon in a simple interrupted pattern. Placement was confirmed via aspirating a small amount of blood and the catheter was well flushed with 0.1% heparinized saline. The skin incision was closed using 4-0 vicryl in a simple interrupted pattern. The catheter was securely wrapped to the body using self-adhesive material and exteriorized to the dorsal aspect of the neck. Of the ten animals utilized, nine survived surgery and seven had patent catheters at the time of experiment outlined below.

Animals were given 48 hours to recover from the procedure before treatment began. Animals were randomly assigned to receive an intraperitoneal injection of LPS (15 μg/kg) or an equivalent volume of 0.9% Saline. Of the seven animals in this phase of the experiment, four received an injection of LPS and three received the saline injection. A basal blood sample (timepoint 0) was collected before each injection. Blood was then collected from the catheter at 2, 4, 8, 12, 24 and 48 hours post injection with saline or

LPS. The blood was stored on ice for no more than two hours and allowed to clot. The serum was then isolated via centrifugation of whole blood for 10 minutes at 1000g. Serum was removed, aliquoted and stored in -20°C until later time. Animals were euthanized via penetrative captive bolt at the 48-hour mark.

Timed euthanasia

The remaining 48 animals were randomly divided evenly into two groups to receive an intraperitoneal injection of LPS (15ug/kg) or an equivalent volume of 0.9% Saline. Within the treatment groups, those animals were then randomly assigned to be euthanized 0, 3, 6, 12, or 24 hours post LPS or Saline injection with 8 animals per timepoint and 4 per treatment. The animals were euthanized via penetrative captive bolt and various tissues were collected for analysis. Briefly, tissues were snap frozen in liquid nitrogen, stored briefly in dry ice, and then transferred to -80 until analysis.

Serum analysis: Insulin and glucose

Insulin concentrations were determined using the Human/Canine/Porcine Insulin Quantikine ELISA kit (R&D Systems) against the provided standard curve. Briefly, the plate was pre-rinsed with the provided wash buffer per the manufacturer's instructions. Samples and standards were diluted in 150uL of provided diluent and incubated for five hours at room temperature with constant orbital shaking. After four washes, the provided Insulin Conjugate was added and allowed to incubate two hours at room temperature with constant orbital shaking. Following rinsing instructions, the provided substrate solution was added to each well followed by the addition of the provided stop solution. The

optical density was determined on the Synergy HTX microplate reader using Gen 5.0 v3.0 software (BioTek Instruments, Winooski, VT). A four-parameter logistic curve was generated against the standards using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, Ca). Glucose concentrations were determined using the glucose colorimetric detection kit (Invitrogen, Carlsbad, California) following manufacturer's instructions. All sera were diluted at 1:15 and concentrations were determined against the provided standard curve. The optical density at 560 nm was determined again using the Synergy HTX microplate reader and Graphpad Prism 9 was used to generate a four-parameter standard curve.

RNA/RT-qPCR

Frozen tissue was stored in -80°C until time for RNA extraction. Approximately 30mg of tissue homogenized in TRIzol (Invitrogen) using FastPrep-24 5G System (MP Biomedicals, Irvine, Ca). Total RNA was isolated from homogenized tissue using RNeasy Mini (Qiagen) and quantified via spectrophotometry using Synergy HTX Gen5.0 v3 (BioteK Instruments, Winooski, VT). A maximum of 5000ng of total RNA was reverse transcribed to cDNA with the SuperScript IV First-Strand Synthesis System (Invitrogen) and treated with RNase H to remove excess RNA. The newly synthesized cDNA was quantified using the Quant-iT OligoGreen assay (Invitrogen). The cDNA was quantified using the Synergy HTX system as described previously. A total of 20ng of cDNA was used for both singleplex and multiplex qRT-PCR using iTaq Universal SYBR Green Supermix and iQ Multiplex Powermix respectively via Bio-Rad's CFX96 Touch Real Time PCR Detection system (Biorad**)**. All primers and probes utilized were ordered

from Integrated DNA Technologies (Coralville, IA). For singleplex qRT-PCR after optimization, a 300nM concentration of forward and reverse primer (Table 2-1) were used for each assay. The assays consisted of amplification for 30s at 60°C for 40 cycles. For multiplex qRT-PCR after optimization, a 2:1 primer-to-probe ratio was utilized for gene expression while a 1:1 ratio was used for RPL4, the reference gene (Table 2-2). For each assay, the samples were amplified for 45s at 60°C for 40 cycles. Genes of interest were normalized to RPL4 using the $2^{-\Delta\Delta CT}$ method for both single and multiplex rt-qPCR. Analysis of genes of interest expression and amplification plots were accomplished using CFX Manager Software (Version 3.1, Biorad).

Table 3-1: Primers used in single reaction RT-qPCR

Symbol	Gene ID		Sequence (5'-3')
ACC	397324	F	CCGTAGAAATCAAATTCCGCAG
		R	CCTTCAGCTTGCTCTCCAG
ACLY	100125957	F	TCACAACACCATCATCTG CG
		R	CTTACTGAACATCTTGGC TGC
CHREBP	100170769	F	ATGTTCGATGACTATGTC CGG
		R	ACACCATCCCATTGAAGG AC
FASN	397561	F	CTCAACTTCCGAGACGTC ATG
		R	ACCATTCCCATCACGCG
GAPDH	396823	F	CCCCAACGTGTCGGTTGT
		R	CTCGGACGCCTGCTTCAC
GCKR	100625192	F	TTCCCATTTCACCTTCTC CC
		R	TTCTCTTTCACCTGCTCC AC
PPARy	397671	F	AGATGATGACCAAGA AGCCC
		R	GCAGAAGACGTCCAG GATG
PNPLA2	100049704	F	ATGGTGCCCTACACGCTG
		R	GCCTGTCTGCTCCTTTATCC
PCK	403165	F	CTGGGAAGGCATTGATCAGC
		R	AGCGAGAGTTAGGATGTACA
GCK	100514142	F	CCGACTTCCTGGACAAGCAT
		R	ATCGTGGCCACAGTGTCATT
G6PC	100134959	F	TGAACGTCTGTCTGTCACGA
		R	ATACTTCTTGAGGCTGGCGT
PC	397630	F	GGACTTCACTGCCACCTTTG
		R	GCTCCACCTCAAACTCCTCT
PK	100158154	F	CCCACTGAAGTCACCGCTAT
		R	GAGGAAGCCACGGAGTTTT
FBP	397038	F	AGCTGGCTATGCGCTCTATG
		R	CGAACTCCTTGGCATAGCCT
Glut4	396754	F	TAAGACAAGATGCCGTCGGG
		R	GAGAAGACGGCGAGGACAAG
HK	100152344	F	TTCAATCCGACAGCCATAG
		R	CTCCGACTCCATAAGAACAT
CPT1	399528	F	ATGGTGGGCGACTAACT
		R	TGCCTGCTGTCTGTGAG
GRB10	100188977	F	TCAGGCTCCTCAAGTACGGA
		R	GCCCAGAAAATCCATCGCC
IGF1	397491	F	CACATCACATCCTCTTCG
		R	CTGGAGCCGTACCCTGTG
RPL4	100038029	F	AGGAGGCTGTTCTGCTTCTG
		R	TCCAGGGATGTTTCTGAAGAAGG

Table 3-2: Primers used in multiplex RT-qPCR reactions.

Symbol	Gene ID		Sequence (5'-3')	Probe Sequence
RPL4*	100038029	F	GTGTGCTCGTCCACTGATA	/5CY5/CACTTTGCC/TAO/TGCTGTGTTCAAGGC/3IAbRQSp/
		R	TCACGATGTCTGGTCGAATG	
IRS1*	100512686	F	GGATGCAGGTGGATGATTCT	/5HEX/ATGAGACAA/ZEN/TCCAGGCAATGC/3IABkFQ/
		R	TTAGAGGAGGACTGGCTCTT	
IGFR*	100512686	F	GCCATCAGGATCGAGAAGAA	/5TEXRD-XN/ACCTCTGCTACCTCTCTA/3IAbRQSp/
		R	CTTGTTGCCCACGATGTAGTTA	
MTOR *	100127359	F	GCGTCAAGAAGGAGAAGGAA	/56-FAM/CTGCTTTCT/ZEN/GTGGCTGTGAGGTCT/3IABkFQ/
		R	CAAGGCTGCTCGGATGAT	
RPL4▪	100038029	F	ACTTCCTTTGGTGGTTGAAGA	/CGY5/ACCCAAGGA/TAO/GGCTGTTCTGCTTCT/3IAbRQSp/
		R	CTCATTCGCTGAGAGGCATAA	
PI3K▪	396979	F	TCTCATGGTGGGAGGTGTAAG	/5HEX/CGGGTCAGT/ZEN/GAACACAGAATCTTACAACT/3IABkFQ/
		R	AGTCAAGCTGTGGTGAATCTAA	
AKT▪	100126861	F	CCGAGAAGAACGTGGTGTATC	/56-FAM/TTGTCCAGC/ZEN/ATGAGGTTCTCCAGC/3IABkFQ/
		R	CCGAAGTCAGTGATCTTGATGT	
INSR▪	396755	F	CAGAAAGTGTGCCCGACCAT	/5TEXRD-XN/CGATGTTTTGGGCAACTGCTCTGA/3IAbRQSp/
		R	ATCTGCCGTCCAGGTAGAAGT	

Symbols * and ▪ indicate different multiplex sets

Western blot analysis of metabolic proteins

Frozen tissues were placed into ice cold RIPA buffer (Invitrogen) with protease and phosphatase inhibitors (Invitrogen) per manufacturer's instructions. While still on ice, a handheld homogenizer the Tissue Tearer (BioSpec Products) was used to homogenize the tissue. Protein extract was then centrifuged at 16,000g for 20 minutes at 4°C to pellet debris. The supernatant was then removed, aliquoted and stored at -20°C until analysis. Protein samples were quantified using the Pierce BCA assay (Invitrogen). A total of 40ug total protein was loaded into each gel and transferred to a PVDF membrane. Membranes were blocked in 5% non-fat milk in TBST for one hour at room temperature. Primary antibodies (Table 3-3) were diluted in blocking buffer overnight at

4°C. Membranes were rinsed three times in TBST. Secondary antibodies HRP conjugated goat-anti rabbit (Cell signaling technology, 1:1000) and goat-anti mouse (Novus Biologicals 1:20,000) in blocking buffer and incubated at room temperature for one hour. After rinsing, blots were visualized using EliteSignal Fire (Cell Signaling Technology) using BioRad gel XR imaging system. Blots designated for stripping (phosho-AKT) were then rinsed three more times in TBST after imaging to remove excess reagent. The blot was incubated with Restore PLUS Western Blot Stripping Buffer (Invitrogen) for 10 minutes at 37°C and then rinsed again. The membrane was then incubated with the appropriate secondary and imaged to confirm full strip of previous antibodies. The membrane was blocked for 1 hour at room temperature in blocking buffer and antibody incubation steps were completed as described above.

Table 3-3: Primary and secondary antibodies used for western blotting analysis.

Symbol	Source	UniProt ID	Dilution	Catalog Number
Beta Actin	Cell Signaling Technology	P60709	1:1000	4970
INSR alpha	Cell Signaling Technology	P06213	1:500	74118
INSR beta	Novus Biologicals	P14616	1:1000	NBP1-19192
PI3K	Proteintech	P42336	1:1000	60225-1-Ig
FBP	Proteintech	P09467	1:2000	12842-1-AP
PCK1	Proteintech	P35558	1:10000	16754-1-AP
AKT	Cell Signaling Technology	P31751 , Q9Y243 , P31748	1:1000	9272
p-AKT	Cell Signaling Technology	P31751 , Q9Y243 , P31749	1:1000	9271

Statistics

The analysis of repeated blood sampling data contained seven animals with n=4 receiving LPS and n=3 receiving saline. Serum glucose and insulin concentrations are reported using GraphPad Prism9 and evaluated using a mixed-effects model with Giesser-greenhouse correction and uncorrected Fisher's LSD. All timed euthanasia points contained eight animals with four receiving LPS and four receiving saline injections. RT-qPCR and western blot data were all analyzed using unpaired two-tailed t-tests. The probability of $p<0.05$ was significant and $p<0.10$ was considered to be a trend. All statistical procedures were analyzed using GraphPad Prism 10 (GraphPad Software, INC, La Jolla, CA). Data are reported as mean ± SEM.

Results

Acute LPS exposure alters glucose and insulin levels

After acute exposure to a mild dose of LPS there was an initial decrease in insulin concentration two hours post injection ($p<0.05$) followed by an increase in insulin concentration at the 12 and 24 post insulin injection ($p<0.05$). There was no discernible difference between the groups at the remaining time points (Figure 3-1A). Following LPS exposure, glucose levels followed a different pattern over the sampling period. Glucose concentrations increased two hours post LPS injection ($p<0.05$) and trended to be increased at the 24-hour mark (Figure 3-1B).

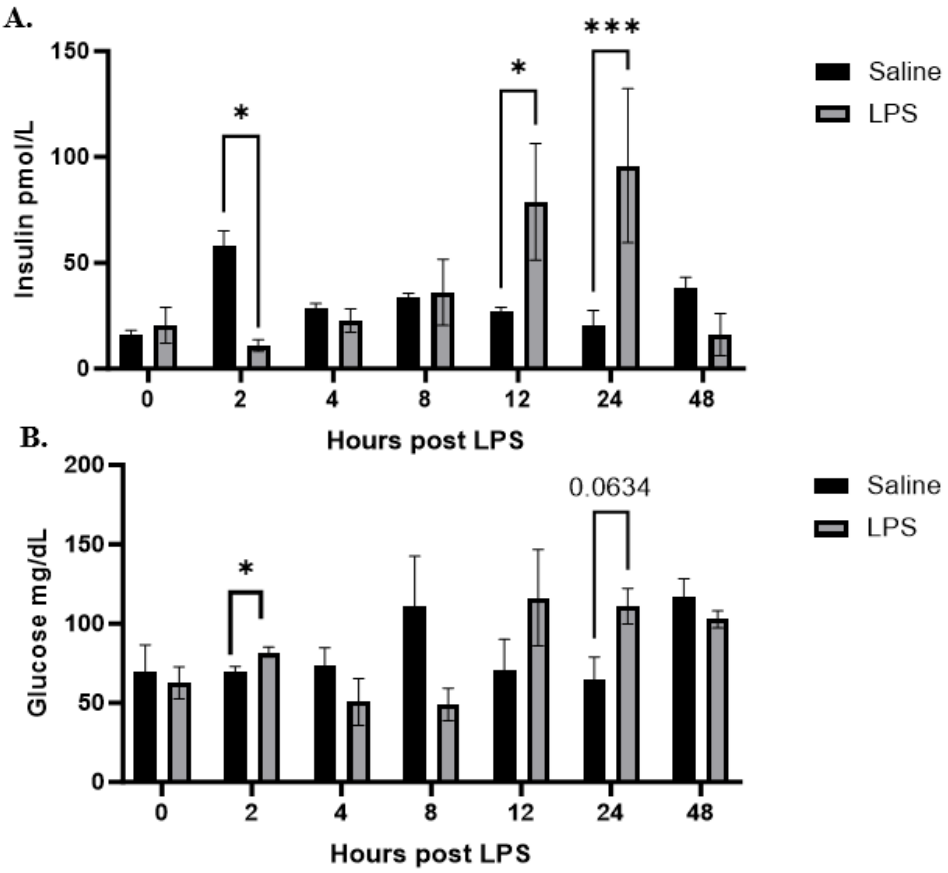


Figure 3-1: Impact of LPS on amount of insulin and glucose in serum. (A) Insulin and glucose (B) concentration over 48 hours after acute exposure to LPS. For both assays n=4 received LPS and n=3 received saline. Bar graphs report data as mean ± SEM. * indicates $p<0.05$ *** indicates $p<0.001$

Acute exposure to LPS reduces insulin signaling genes in the liver.

To assess the impact of LPS on insulin signaling in the liver, gene expression analysis was conducted focusing on genes in the insulin signaling cascade as well as other associated proteins. There was a decrease ($p<0.01$) in insulin receptor (*INSR*) transcript abundance at 6 hours post LPS and a trend for a reduction ($p<0.10$) 12 hours post LPS (Figure 3-2A). The next step in the cascade is insulin receptor substrate 1 (*IRS1*) which exhibited a decrease in expression at 3 ($p<0.01$), 6 ($p<0.05$) and 12 ($p<0.10$) hours post LPS injection (Figure 3-2B). Phosphoinositide 3-kinase (*PI3K*) was

decreased at 3 and 6 hours post LPS injection ($p<0.05$) (Figure 3-2C). The major mediator of insulin action, protein kinase B (*AKT*) was decreased ($p<0.05$) at three hours post LPS injection (Figure 3-2D). The insulin like growth factor receptor (*IGFR*) was decreased at 3 ($p<0.10$) and 12 hours ($p<0.001$) post LPS injection (Figure 3-2E). The inhibitor of insulin signaling, growth factor receptor bound protein 10 (*GRB10*) was decreased ($p<0.05$) at 3, 6, 12 and 24 hours post LPS injection (Figure 3-2F).

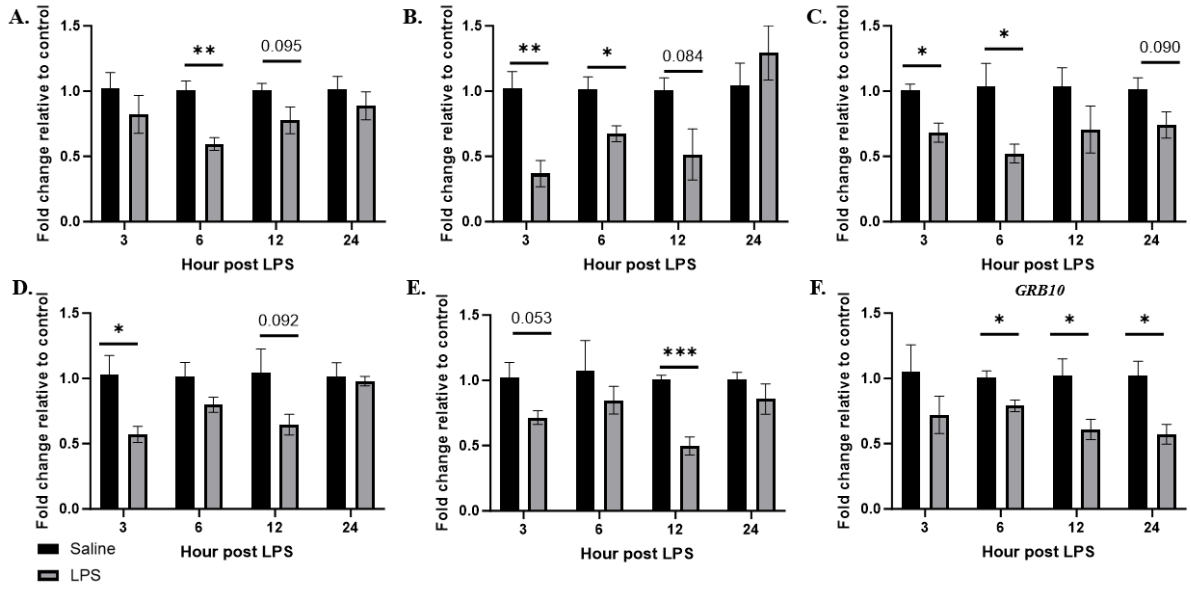


Figure 3-2: LPS induced differential expression of insulin signaling related genes over 24 hours in the liver. Animals were administered LPS via intraperitoneal injection and then euthanized at a designated period of time (A). Gene expression of *INSR* (B) Gene expression of *IRS1* (C) Gene expression of *PI3K* (D) Gene expression of *AKT* (E) Gene expression of *IGFR* (F) Gene expression of *GRB10.* All data are expressed as mean ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

LPS alters gene expression associated with gluconeogenesis, glycolysis and fatty acid metabolism in the liver.

The liver functions to maintain homeostasis by balancing catabolic and anabolic processes. The rate limiting step in gluconeogenesis is fructose 1,6-bisphosphatase (*FBP*) which converts fructose 1,6-bisphosphatase to fructose-6-phosphate to be converted into glucose. *FBP* expression was decreased ($p<0.05$) in the liver at 3, 6, and 12 hours post LPS injection (Figure 3-3A). Another gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (*PCK*) had decreased gene expression at 3 ($p<0.10$), 6 ($p<0.01$) and 12 ($p<0.01$) post LPS injection (Figure 3-3B). The final step in glycolysis is catalyzed by pyruvate kinase (PK) which had decreased expression in the liver 3 and 12 ($p<0.05$) hours post injection and a trend ($p<0.10$) of a reduction at 6 hours (Figure 3-3C). A regulator of glucose metabolism, glucokinase regulatory protein (*GCKR*) was decreased ($p<0.05$) at 6 and 12 hours post LPS injection (Figure 3-3D). There were also alterations in lipogenic genes in the liver, the rate limiting step of lipogenesis acetyl CoA carboxylase (*ACC*) was decreased at 3 ($p<0.05$), 6 ($p<0.01$) and 12 hours ($p<0.05$) post LPS exposure (Figure 3-3E). The final step of lipogenesis is controlled by fatty acid synthase (*FASN*) which was decreased at 3 ($p<0.05$), 6 ($p<0.10$) and 12 ($p<0.05$) hours post LPS administration (Figure 3-3F). Patatin like phospholipase domain containing 2 (*PNPLA2*) encodes the enzyme adipose triglyceride lipase which is the first step of hydrolysis of triglycerides was decreased ($p<0.05$) at 3 and 6 hours post LPS injection (Figure 3-3G).

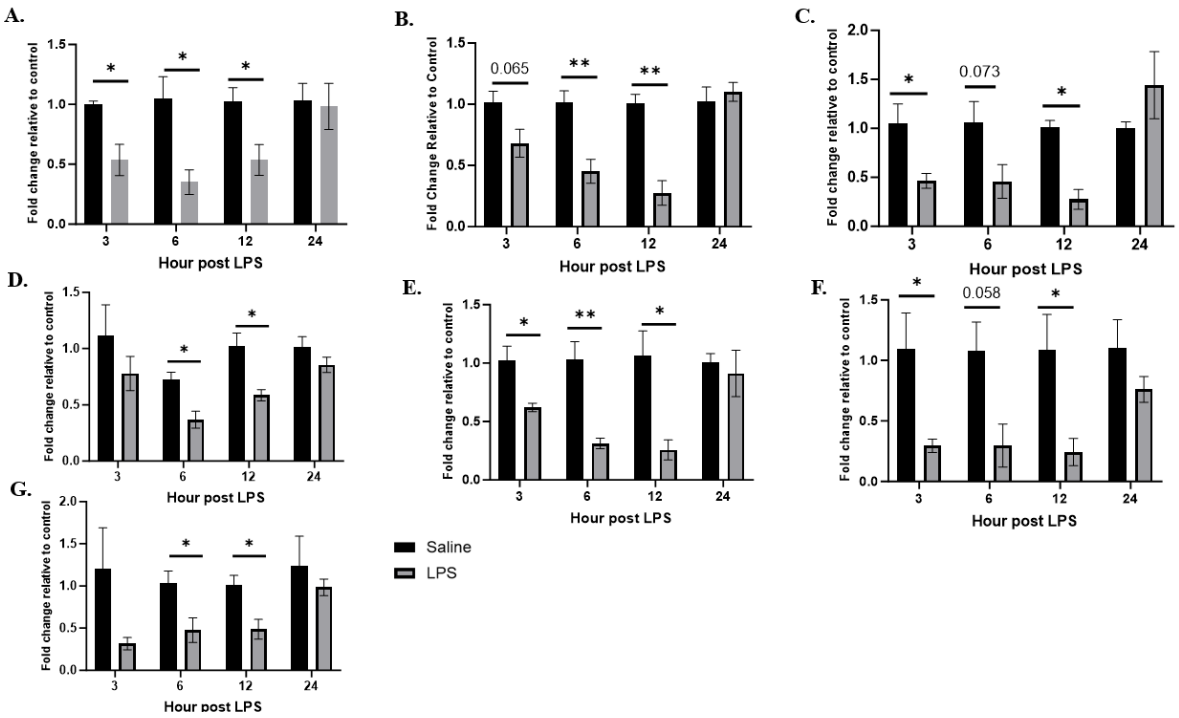


Figure 3- 3: LPS induced changes in genes associated with gluconeogenesis and fatty acid metabolism in the liver (A). Gene expression of *FBP* (B) Gene expression of *PCK* (C) Gene expression of *PK* (D) Gene expression of *GCKR* (E) Gene expression of *ACC* (F) Gene expression of *FASN.* (G) Gene expression of *PNPLA2.* All data are expressed as mean ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

LPS induced changes in metabolic gene expression of *longissimus dorsi* muscle.

The skeletal *longissimus dorsi* (LD) muscle exhibited changes in gene expression post LPS injection. Expression of *IGFR* in skeletal muscle was increased ($p<0.05$) 3 and six hours post LPS injection (Figure 3-4A). The inhibitor of insulin *GRB10*, was increased at 12 hours post LPS administration (Figure 3-4B). Expression of the mammalian target of rapamyacin (*MTOR*) which functions to promote muscle synthesis under insulin action was decreased ($p<0.10$) at three hours post injection and increased ($p<0.05$) 12 hours post LPS (Figure 3-4C). The prevalence of *PI3K* was increased ($p<0.05$) at 12 hours post LPS injection (Figure 3-4D). The expression of *INSR* was increased ($p<0.05$) in skeletal muscle at 12 hours post LPS (Figure 3-4E). The initial

enzyme of glycolysis in muscle, hexokinase (HK), was decreased at both three ($p<0.001$) and six ($p<0.05$) hours post exposure to LPS (Figure 3-4F).

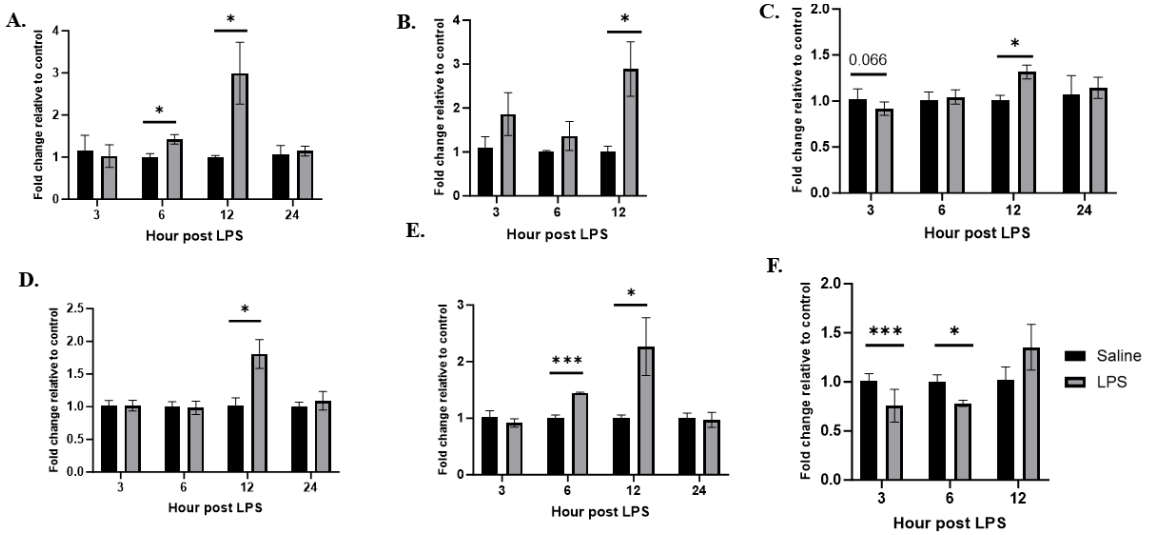


Figure 3-4: LPS induces a variety of changes in gene expression in LD muscle. (A) Gene expression of *IGFR* (B) Gene expression of *GRB10* (C) Gene expression of *MTORC1* (D) Gene expression of *PI3K* (E) Gene expression of *INSR* (F) Gene expression of *HK*. All data are expressed as mean ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

Differential expression of fatty acid metabolism genes in *longissimus dorsi* muscle

Skeletal muscle can synthesize and breakdown lipids for energy and storage in muscle tissue. Expression of genes associated with fatty acid metabolism differ after administration of LPS. The lipogenic transcription actor peroxisome proliferator activated receptor gamma (*PPARy*) was decreased ($p<0.05$) at 6 hours post LPS injection (Figure 3-5A). A key precursor to de novo lipogenesis ATP citrate lyase (*ACLY*) was decreased ($p<0.05$) at 6 hours post LPS (Figure 3-5B). The lipogenic genes *ACC* and *FASN* were altered by LPS exposure in skeletal muscle. *ACC* was decreased ($p<0.05$) 6 hours post LPS (Figure 3-5C) exposure while *FASN* was increased ($p<0.10$) at 6 hours post LPS and a decrease ($p<0.05$) in expression 12 hours after injection (Figure 3-5D). *PNPLA2*

functions in hydrolysis of fatty acids and its expression was increased ($p<0.05$) 12 hours post LPS exposure (Figure 5E).

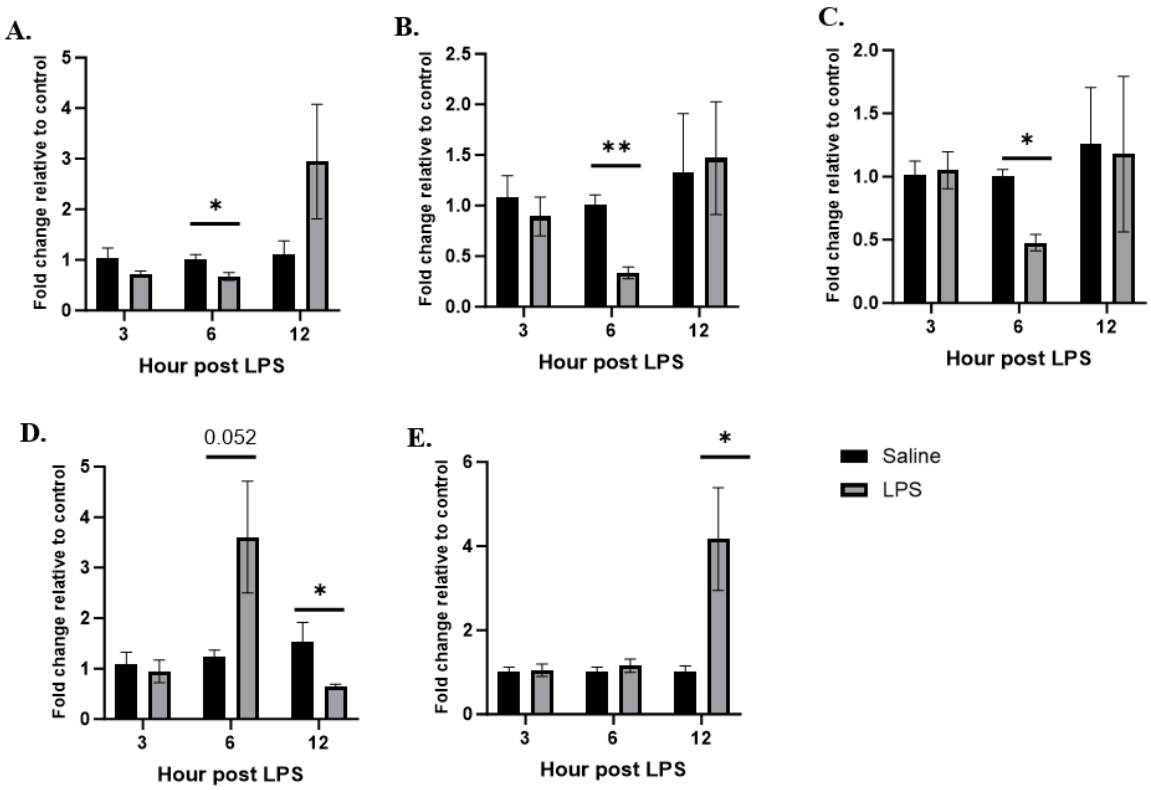


Figure 3-5: LPS transcriptional changes in genes related to fatty acid metabolism LD muscle. (A) Gene expression of *PPARy* (B) Gene expression of *ACLY* (C) Gene expression of *ACC* (D) Gene expression of *FAS* (E) Gene expression of *PNPLA2.* All data are expressed as mean ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

<u>LPS differentially alters gene expression of fat depots.</u>

Fats are required for energy storage and insulation, but different deposits vary in metabolic responses and processes. We examined gene expression in peri-renal and subcutaneous fat samples. Peri-renal saw a twofold decrease ($p<0.05$) in *PI3K* expression 3 hours post LPS injection (Figure 3-6A). Also, at 3 hours post injection *IRS1*

expression decreased ($p<0.05$) compared to saline (Figure 3-6B). The expression of the glucose transporter *GLUT4* was decreased ($p<0.05$) at both 3 and 12 hours post injection (Figure 3-6B). The subcutaneous fat deposit showed decreased expression at 3 ($p<0.05$) and 12 hours ($p<0.05$) of the tyrosine kinase *IRS1*. The final step in lipogenesis, *FASN* was also decreased ($p<0.05$) 6 hours post LPS injection.

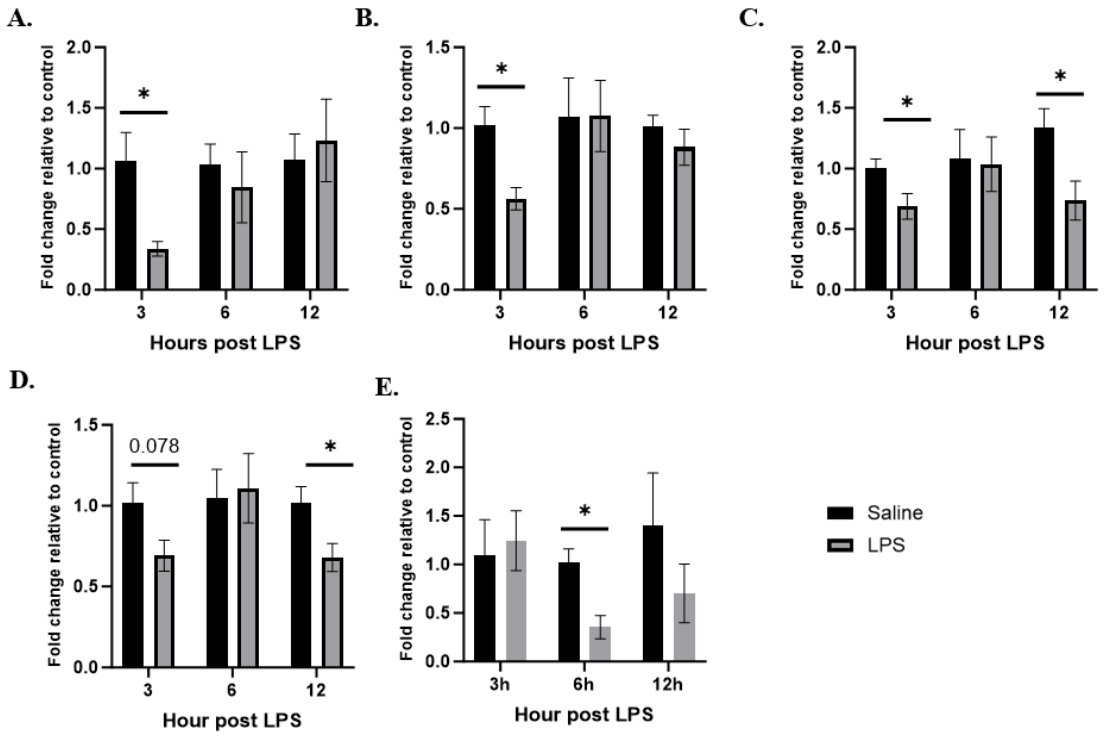


Figure 3-6: Varying gene expression after LPS in both peri-renal (A-C) and subcutaneous fat (D-E). (A) Gene expression of *PI3K* (B) Gene expression of *IRS1* (C) Gene expression of *GLUT4* (D) Gene expression of *IRS1* (E) Gene expression of *FASN*. All data are expressed as mean ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$).

Western blot alters protein abundance in the liver after LPS injection.

Acute exposure to LPS decreased ($p<0.05$) total protein abundance of AKT 3 hours after injection (Figure 3-7A). There was no difference in the level of total AKT 6 hours post LPS (Figure 3-7B). The activated form, phosphorylated AKT (pAKT), was

decreased ($p<0.05$) three hours post exposure to LPS (Figure 3-7C). The ratio of pAKT: AKT was not changed (Figure 3-7D) by exposure to LPS after three hours (Table 3-4). The tyrosine kinase PI3K was decreased ($p<0.05$) 3 hours post LPS (Figure 3-7E) and there was no difference in expression level at 6 and 12 hours post LPS injection (Figure 3-7F, G). The gluconeogenic enzyme PCK was unchanged after LPS injection at 3- and 6-hours post (Figure 3-8A, B) and reduced ($p<0.05$) after 12 hours (Figure 3-8C). Abundance of FBP was not changed after a single exposure to LPS over 12 hours (Figure 3-8D, F). Table 4 details densitometry data and significance for western blots in Figure 7 and Figure 8.

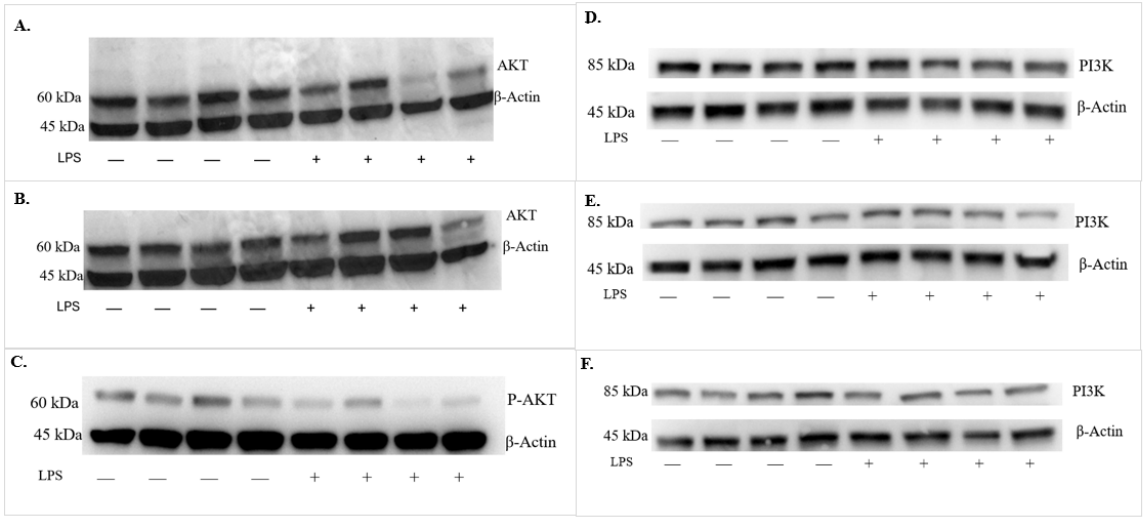


Figure 3-7: Protein abundance of insulin signaling proteins in the liver post LPS injection. (A) Total abundance AKT three hours after LPS. (B) Total AKT abundance six hours after LPS. (C) Phosphorylated-AKT three hours post LPS (D) PI3K abundance in the liver three hours post LPS (E) PI3K abundance six hours post LPS injection (F) PI3K abundance 12 hours post LPS injection.

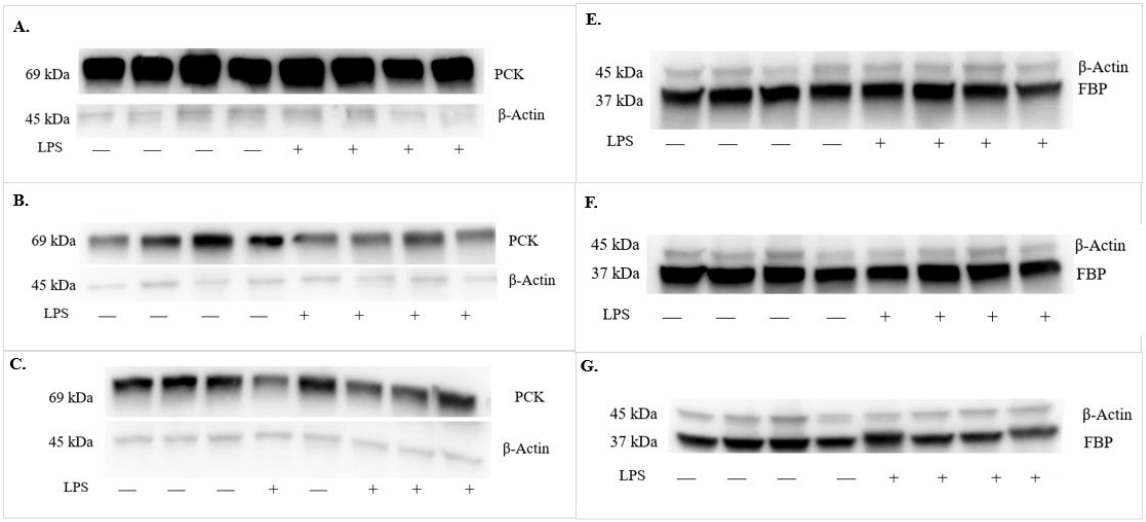


Figure 3-8: Protein abundance of gluconeogenic proteins in the liver post LPS injection. (A) Total abundance PCK three hours after LPS. (B) Total PCK abundance six hours after LPS. (C) Total PCK 12 hours post LPS (D) Total FBP three hours post LPS. (E) FBP abundance in the liver three hours post LPS (F) FBP abundance six hours post LPS injection (G) FBP abundance 12 hours post LPS injection.

Table 3-4: Effect of LPS exposure in relative abundance of proteins involved in insulin signaling and gluconeogenesis in the liver

		Relative fold change ± SEM		
Protein	**Hour post LPS**	**Saline**	**LPS**	**P-Value***
AKT	**3**	**1 ± 0.054**	**0.573 ± 0.111**	**0.012***
AKT	6	1 ± 0.038	0.868 ± 0.101	0.27
pAKT	**3**	**1 ±0.012**	**0.501 ± 0.079**	**0.018***
pAKT: AKT	3	1 ± 0.11	0.868 ± 0.14	0.48
PI3K	**3**	**1 ± 0.043**	**0.832 ± 0.033**	**0.021***
PI3K	6	1 ± 0.081	0.892 ± 0.123	0.496
PI3K	12	1 ± 0.12	0.886 ± 0.066	0.437
PCK	3	1 ± 0.047	1.04 ± 0.053	0.576
PCK	6	1 ± 0.253	0.80 ±0.059	0.253
PCK	**12**	**1 ±0.015**	**0.77 ± 0.085**	**0.038***
FBP	3	1 ± 0.088	1.13 ± 0.07	0.285
FBP	6	1 ± 0.103	0.846 ± 0.079	0.283
FBP	12	1 ± 0.179	0.915 ±0.026	0.65

*Bolded items and * indicate significance of $p<0.05$*

Discussion

The systemic activation of the immune system via LPS action has been well documented (28, 29) and different groups are investigating the impact of LPS on metabolism in different species (19, 30, 31). In pigs, LPS exposure has been shown to increase inflammatory markers, decrease feed intake, reduce growth rate, and induce pyrexia (21, 32, 33). In the present study, we examined the metabolic impact of acute exposure to LPS on a weaned pig model. Our results demonstrate that LPS alters insulin signaling and nutrient processing in the liver and other peripheral tissues. Understanding the metabolic changes during an activated immune response can provide useful information on leaky gut, sepsis, and overall animal health.

This studfy was split into two sections of experiments where a portion of the animals were allocated for repeated blood sampling and the remainder were designated for timed collections after a single dose of LPS. During the progression of LPS induced sepsis, there is a gradual shift from hypoglycemia to hyperglycemia and insulin resistance over a relatively short period of time (12). In our study, two hours post LPS exposure, we noted hyperglycemia and a decrease in insulin concentrations in serum. This contrasts with previous data in adult males where hepatic and peripheral insulin sensitivity was increased after two hours of LPS infusion (34). Early studies in rats also reported elevated glucose concentrations after LPS injection that resolved within two hours (24). Hyperinsulinemia was noted at 12 and 24 hours post injection with hyperglycemia at 24 hours. Other studies in pigs report similar findings within 24 hours, weaned pigs exhibit changes in inflammatory cytokines with a single smaller dose of LPS but no difference in

glucose concentration over 24 hours (29). Finishing gilts under chronic exposure to a 0.1μg/kg dose of LPS showed a marked insulin resistance at 24 hours post LPS that was resolved by 72 hours (26). While in mice, injected with a low dose (0.1uμ/kg) of LPS displayed improved glucose tolerance (35). The body's metabolic response to an endotoxin challenge transiently alters insulin concentrations and glucose utilization over 24 hours.

There is still much to be elucidated about how the pig liver responds to an acute exposure to LPS. Our study aimed to investigate changes in gene expression and protein abundance over the first 24 hours after a single LPS injection. Our results displayed those transcripts associated with the insulin signaling cascade, *INSR, IRS1, PI3K,* and *AKT* were decreased over the LPS exposure and recovery period. Previous studies in pigs report that after chronic exposure to LPS there was no change in gene expression of *INSR*, *IRS1* or *AKT* in pigs after five days. This finding can be attributed to the pigs acclimating to the low dose of LPS over time as an initial insulin resistance was noted after 24 hours (19). When released in response to LPS exposure inflammatory cytokines, such as TNFa and IL-6, can phosphorylate IRS1 on alternate serine residues leading to reduced insulin signaling (36) providing a potential explanation for the reduced transcript abundance in our study. Similar findings are observed in other models as mice undergoing sustained LPS induced endotoxemia showed marked decrease in *INSR* and *IRS1* (37).

The insulin induced activation of PI3K-AKT pathway promotes metabolism, proliferation, and cell survival (38). While there was a decrease in liver *PI3K* at multiple time points, there was only a detectable change in PI3K protein abundance on western blot at 3 hours post LPS. This is inconsistent with previous work in neonatal pigs as LPS did not impact activation of INSR, IRS1 or PI3K after 8 hours (17). At three hours post LPS exposure there was a decrease in total *AKT* mRNA expression, protein abundance and a decrease in phosphorylated AKT, these results indicate suppression of insulin signaling in the liver post LPS. By contrast, mice exposed to a low dose of LPS exhibited an increase in total AKT while p-AKT was unchanged (35). Rats exposed to LPS exhibited decreased p-AKT in cerebral tissue 8 hours post injection (39). However, at 6 hours post LPS exposure there was a numerical but not significant difference of total *AKT* expression at the mRNA or protein level indicating a small period of disrupted insulin signaling in the liver.

The adaptor protein GRB10 has been established as an inhibitor of insulin signaling (8)**.** GRB10 is activated by insulin stimulated activation of MTORC to provide inhibition at the level of the insulin receptor (40). There was an unexpected decrease in mRNA level of *GRB10* after LPS injection in the liver. In skeletal muscle however, we noted that there was an increase in *GRB10* expression after 12 hours. The function of GRB10 in an immune response has yet to be elucidated fully however, GRB10 has been implicated in different immune dysfunctions including osteoarthritis (41) and leukemia (42). Human myotubes exposed to LPS in vitro did not have increased *GB10* expression (43). As GRB10 interacts with a variety of proteins to function in processes such as cell

growth, apoptosis, metabolism, and cell migration (8) it is plausible that suppression of GRB10 has a role in the immune response to LPS.

During severe endotoxemia the liver suffers significant impairment and suppression of hepatic gluconeogenesis (25).We found changes in genes expression and protein abundance of enzymes associated with gluconeogenesis, glycolysis, and fatty acid metabolism in the liver. We noted a decrease in *PCK* mRNA abundance with a decrease in protein abundance after 12 hours. PCK is responsible for the conversion of oxaloacetate to phosphoenolpyruvate (PEP) as the first step in gluconeogenesis. Previous studies in rodent models provide contradicting information as studies in rats showed that there is an increase in *PCK* mRNA after LPS injection (31, 44). While another study of using rat hepatocytes showed LPS inhibits gluconeogenesis via reducing *PCK* after two hours (45). Another key step in gluconeogenesis is catalyzed by FBP which converts fructose-1,6-bisphosphate into fructose-6 phosphate. We noted a decrease in *FBP* gene expression and while there is minimal reporting of *FBP* changes after LPS, there is data showing growing pigs with long-term infection of porcine reproductive and respiratory syndrome (PRRS) exhibited decreased FBP in the liver (46). The substrate for FBP, fructose-1,5-bisphosphate, has been implicated in the treatment of sepsis due to its anti-inflammatory characteristics (47). These results suggest that the abundance of *FBP* could be related to the metabolic response to immune system activation in addition to altering glucose metabolism.

One of three rate limiting steps in glycolysis, PK is the final step to convert PEP into pyruvate processing via the Krebs cycle. In this study we focused on the liver/red blood cell specific isoform which showed reduced expression after LPS. This finding is in line with rats exposed to LPS displaying reduced flux through PK as the precursor, PEP, was depleted (48). Glucokinase is the first step of glycolysis in the liver and is allosterically inhibited by glucokinase regulatory protein (GCKR) (49). We noted a decrease in GCKR in the liver potentially allowing for increase glucokinase activity as there was no change in mRNA expression of glucokinase after LPS. Various isoforms and expression of GCKR have been associated with reduced insulin signaling in men (50). The alterations in the glycolytic pathway can allow for reduced production of pyruvate as a precursor to the Krebs cycle.

Both aspects of fatty acid metabolism, lipolysis, and lipogenesis, were impacted by LPS in the liver. The mRNA *PNPLA2* encodes adipose triglyceride lipase (ATGL), which is the first step in lipolysis of triacylglycerol to diacylglycerol, was decreased post LPS injection. Decreased lipolysis has also been noted 6 hours after intramuscular injection of 15µg/kg LPS (51). We investigated lipogenesis via transcript abundance of *ACC* and *FAS* in the liver and observed a decrease in both post LPS. This finding is consistent with other studies showing reduced de novo lipogenesis after exposure to LPS (51–53). Taken together, we noted drastic suppression of lipogenesis, insulin signaling, gluconeogenesis and glycolysis in the liver after a single LPS injection.

Skeletal muscle is the primary site for insulin mediated glucose disposal (54) and impaired insulin action can result in inefficient glucose utilization. In our study, we observed interesting alterations in gene expression post LPS injection. Hexokinase (HK) enzyme catalyzes the first step on glycolysis in non-hepatic tissues. We observed a decrease in HK after LPS injection which is not consistent with other swine studies where after a single intramuscular injection of 15 μg/kg LPS weaned pigs showed increase in glycolysis related genes (55). After an acute exposure to LPS, skeletal muscle also exhibited an increase in expression of genes associated with insulin signaling such as *INSR*, *PI3K*, and *IGFR* suggesting an increase in glucose uptake. This observation is not consistent with human myotubes stimulated with LPS which had reduced insulin-stimulated activation of IRS1, AKT with reduced glucose transport (56). However, a different study suggests that acute endotoxemia due to LPS exposure does not impair insulin signaling via the PI3K pathway in skeletal muscle, rather from decreased blood flow in tissues (57). The protein complex MTORC is responsible for insulin induced protein synthesis once activated. After exposure to LPS we observed an initial increase in *MTORC* abundance at 3 hours post LPS and then an increase in *MTOR* expression after 12 hours. This variation in mTOR expression is reinforced in the literature that there are tissue specific changes in protein metabolism after an inflammatory challenge (58).

De novo lipogenesis in skeletal muscle is not a major contributor to fatty acid metabolism, yet there is some evidence that muscle de novo lipogenesis provides signaling functionality in addition to the typical lipid storage (59). The altered gene expression of de novo lipogenesis in skeletal muscle after LPS injections could aid in

communication between the immune system and skeletal muscle as in macrophages treated with LPS there is an increase in de novo lipogenesis genes (60). We observed no changes in expression of *ACC* in either fat depot or reduced expression of *FAS* in subcutaneous fat after LPS injection. This aligns partially to previous work indicating decreased lipid metabolism genes in pig back fat (61). Our findings of reduced expression of genes associated with insulin signaling in fat depots correlates with subcutaneous fat remove from human participants after a 3 ng/kg bolus of LPS (62). Changes in insulin signaling expression have been well characterized in skeletal muscle after LPS exposure (18, 56) but there is still missing information regarding fat tissue response.

The hyperglycemic and hyperinsulinemic state after LPS administration has been largely attributed to decreased responsiveness of glucose transporters to insulin in skeletal muscle and adipose tissue leading to lower glucose uptake and higher glucose concentrations in circulation (31). This study provides more information about the liver's role in glucose metabolism during an acute immune response. The insulin resistance and hyperglycemia during an acute immune response is in part, a result of reduced glucose metabolism and uptake in the liver. We also observed some changes in expression in extra hepatic tissues such as skeletal muscle, subcutaneous and peri-renal fat regarding insulin signaling, glucose metabolism and fatty acid metabolism. While these changes in gene expression are promising, more information is required to understand the metabolic response to an acute immune response

References

1. Maes, D. G. D., Dewulf, J., Piñeiro, C., Edwards, S., and Kyriazakis, I. (2020) A critical reflection on intensive pork production with an emphasis on animal health and welfare. *J Anim Sci* **98**, S15–S26

2. Chen, L., Chen, R., Wang, H., and Liang, F. (2015) Mechanisms Linking Inflammation to Insulin Resistance. *Int J Endocrinol* **2015**

3. Bevan, P. (2001) Insulin signalling. *J Cell Sci* **114**, 1429–1430

4. Hotamisligil, G. S. (2006) Inflammation and metabolic disorders. *Nature* **444**, 860–867

5. DiMeglio, L. A., Evans-Molina, C., and Oram, R. A. (2018) Type 1 diabetes. *Lancet* **391**, 2449–2462

6. Purwar, A. and Nagpure, S. (2022) Insulin Resistance in Polycystic Ovarian Syndrome. *Cureus* **14**, e30351

7. Abdul-Ghani, M. A. and DeFronzo, R. A. (2010) Pathogenesis of insulin resistance in skeletal muscle. *J Biomed Biotechnol* **2010**, 476279

8. Holt, L. J. and Siddle, K. (2005) Grb10 and Grb14: enigmatic regulators of insulin action-and more? *Biochem. J* **388**

9. Johnson, R. W. and von Borell, E. (1994) Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin. *J Anim Sci* **72**, 309–314

10. Cornelison, A. S., Karriker, L. A., Williams, N. H., Haberl, B. J., Stalder, K. J., Schulz, L. L., and Patience, J. F. (2018) Impact of health challenges on pig growth performance,

carcass characteristics, and net returns under commercial conditions. *Transl Anim Sci* **2**, 50–61

11. Johnson, R. W. and von Borell, E. (1994) Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin. *J Anim Sci* **72**, 309–314

12. Agwunobi, A. O., Reid, C., Maycock, P., Little, R. A., and Carlson, G. L. (2000) Insulin Resistance and Substrate Utilization in Human Endotoxemia. *J Clin Endocrinol Metab* **85**

13. Curry, S. M., Burrough, E. R., Schwartz, K. J., Yoon, K. J., Lonergan, S. M., and Gabler, N. K. (2018) Porcine epidemic diarrhea virus reduces feed efficiency in nursery pigs. *J Anim Sci* **96**, 85–97

14. Li, Q., Liu, Y., Che, Z., Zhu, H., Meng, G., Hou, Y., Ding, B., Yin, Y., and Chen, F. (2012) Dietary L-arginine supplementation alleviates liver injury caused by Escherichia coli LPS in weaned pigs. *Innate Immun* **18**, 804–814

15. Pålsson-McDermott, E. M. and O'Neill, L. A. J. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* **113**, 153–162

16. Lochmiller, R. L. and Deerenberg, C. (2000) Trade-offs in evolutionary immunology: Just what is the cost of immunity? *Oikos* **88**, 87–98

17. Orellana, R. A., Kimball, S. R., Nguyen, H. V., Bush, J. A., Suryawan, A., Thivierge, M. C., Jefferson, L. S., and Davis, T. A. (2004) Regulation of Muscle Protein Synthesis in Neonatal Pigs during Prolonged Endotoxemia. *Pediatr Res* **55**, 442–449

18. Orellana, R. A., J O, P. M., Nguyen, H. V, Bush, J. A., Suryawan, A., Carole Thivierge, M., Fiorotto, M. L., Davis, T. A., and Carole Thivi-erge, M. (2002) Endotoxemia reduces

skeletal muscle protein synthesis in neonates. *Am J Physiol Endocrinol Metab* **283**, 909–916

19. Bidne, K. L., Kvidera, S. S., Ross, J. W., Baumgard, L. H., and Keating, A. F. (2018) Impact of repeated lipopolysaccharide administration on ovarian signaling during the follicular phase of the estrous cycle in post-pubertal pigs. *J Anim Sci* **96**, 3622–3634

20. Kvidera, S. K., Horst, E. A., Mayorga, E. J., Sanz-Fernandez, M. V., Abuajamieh, M., and Baumgard, L. H. (2017) Estimating glucose requirements of an activated immune system in growing pigs. *J Anim Sci* **95**, 5020–5029

21. Nordgreen, J., Munsterhjelm, C., Aae, F., Popova, A., Boysen, P., Ranheim, B., Heinonen, M., Raszplewicz, J., Piepponen, P., Lervik, A., Valros, A., and Janczak, A. M. (2018) The effect of lipopolysaccharide (LPS) on inflammatory markers in blood and brain and on behavior in individually-housed pigs. *Physiol Behav* **195**, 98–111

22. Langston, P. K., Shibata, M., and Horng, T. (2017) Metabolism Supports Macrophage Activation. *Front Immunol* **8**, 61

23. Lang, C. H. and Spitzer, J. A. (1987) Glucose kinetics and development of endotoxin tolerance during long-term continuous endotoxin infusion. *Metabolism* **36**, 469–474

24. Lang, C. H., Spolarics, Z., Ottlakan, A., and Spitzer, J. J. (1993) Effect of high-dose endotoxin on glucose production and utilization. *Metabolism* **42**, 1351–1358

25. Filkins, J. P. and Cornell, R. P. (1974) Depression of hepatic gluconeogenesis and the hYP gY o 1 cemia of endotoxin shock. *AMERICAN JOURNAL OF PHYSIOLOGY* **227**

26. Bidne, K. L., Kvidera, S. S., Ross, J. W., Baumgard, L. H., and Keating, A. F. (2018) Impact of repeated lipopolysaccharide administration on ovarian signaling during the follicular phase of the estrous cycle in post-pubertal pigs. *J Anim Sci* **96**, 3622–3634

27. National Research Council. (2012) Nutrient Requirements of Swine. National Academies Press, Washington, D.C.

28. Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Chabo, C., Waget, A., Delmée, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrières, J., Tanti, J. F., Gibson, G. R., Casteilla, L., Delzenne, N. M., Alessi, M. C., and Burcelin, R. (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56**, 1761–1772

29. Webel, D. M., Finck, B. N., Baker, D. H., and Johnson, R. W. (1997) Time Course of Increased Plasma Cytokines, Cortisol, and Urea Nitrogen in Pigs Following Intraperitoneal Injection of Lipopolysaccharide 1.

30. Campos, P. H. R. F., Merlot, E., Renaudeau, D., Noblet, J., and Le Floc'h, N. (2019) Postprandial insulin and nutrient concentrations in lipopolysaccharide-challenged growing pigs reared in thermoneutral and high ambient temperatures. *J Anim Sci* **97**, 3354–3368

31. Sugita, H., Kaneki, M., Tokunaga, E., Sugita, M., Koike, C., Yasuhara, S., Tompkins, R. G., Jeevendra Martyn, J. A., and Tomp-kins, R. G. (2002) Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance.

32. Duan, G., Huang, P., Zheng, C., Zheng, J., Yu, J., Zhang, P., Wan, M., Li, F., Guo, Q., Yin, Y., and Duan, Y. (2023) Development and Recovery of Liver Injury in Piglets by Incremental Injection of LPS. *Antioxidants* **12**

33. Terenina, E., Sautron, V., Ydier, C., Bazovkina, D., Sevin-Pujol, A., Gress, L., Lippi, Y., Naylies, C., Billon, Y., Liaubet, L., Mormede, P., and Villa-Vialaneix, N. (2017) Time course study of the response to LPS targeting the pig immune gene networks. *BMC Genomics* **18**

34. Van Der Crabben, S. N., Blümer, R. M. E., Stegenga, M. E., Ackermans, M. T., Endert, E., Tanck, M. W. T., Serlie, M. J., Van Der Poll, T., and Sauerwein, H. P. (2009) Early endotoxemia increases peripheral and hepatic insulin sensitivity in healthy humans. *Journal of Clinical Endocrinology and Metabolism* **94**, 463–468

35. Stevens, J. R., McMillan, R. P., Resendes, J. T., Lloyd, S. K., Ali, M. M., Frisard, M. I., Hargett, S., Keller, S. R., and Hulver, M. W. (2017) Acute low-dose endotoxin treatment results in improved whole-body glucose homeostasis in mice. *Metabolism* **68**, 150–162

36. Kanety, H., Feinstein, R., Papa, M. Z., Hemi, R., and Karasik, A. (1995) Tumor Necrosis Factor Phosphorylation of Insulin Receptor Substrate-1 (IRS-1) POSSIBLE MECHANISM FOR SUPPRESSION OF INSULIN-STIMULATED TYROSINE PHOSPHORYLATION OF IRS-1*.

37. McCowen, K. C., Malhotra, A., and Bistrian, B. R. (2001) Stress-induced hyperglycemia. *Crit Care Clin* **17**, 107–124

38. Hemmings, B. A. and Restuccia, D. F. (2012) PI3K-PKB/Akt pathway. *Cold Spring Harb Perspect Biol* **4**

39. Eklind, S., Mallard, C., Arvidsson, P., and Hagberg, H. (2005) Lipopolysaccharide induces both a primary and a secondary phase of sensitization in the developing rat brain. *Pediatr Res* **58**, 112–116

40. Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villén, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P., and Blenis, J. (2011) Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science (1979)* **332**, 1322–1326

41. Deng, Y. J., Ren, E. H., Yuan, W. H., Zhang, G. Z., Wu, Z. L., and Xie, Q. Q. (2020) GRB10 and E2F3 as diagnostic markers of osteoarthritis and their correlation with immune infiltration. *Diagnostics* **10**

42. Kazi, J. U. and Rönnstrand, L. (2013) FLT3 signals via the adapter protein Grb10 and overexpression of Grb10 leads to aberrant cell proliferation in acute myeloid leukemia. *Mol Oncol* **7**, 402–418

43. Dollet, L., Kuefner, M., Caria, E., Rizo-Roca, D., Pendergrast, L., Abdelmoez, A. M., Karlsson, H. K. R., Björnholm, M., Dalbram, E., Treebak, J. T., Harada, J., Naslund, E., Rydén, M., Zierath, J. R., Pillon, N. J., and Krook, A. (2022) Glutamine Regulates Skeletal Muscle Immunometabolism in Type 2 Diabetes. *Diabetes* **71**, 624–636

44. Beier, J. I., Guo, L., Von Montfort, C., Kaiser, J. P., Joshi-Barve, S., and Arteel, G. E. (2008) New role of resistin in lipopolysaccharide-induced liver damage in mice. *Journal of Pharmacology and Experimental Therapeutics* **325**, 801–808

45. Tanaka, H., Nishikawa, Y., Fukushima, T., Taniguchi, A., Fujita, Y., Tsuda, K., Inagaki, N., and Hosokawa, M. (2018) Lipopolysaccharide inhibits hepatic gluconeogenesis in rats: The role of immune cells. *J Diabetes Investig* **9**, 494–504

46. Helm, E. T., Curry, S. M., De Mille, C. M., Schweer, W. P., Burrough, E. R., Zuber, E. A., Lonergan, S. M., and Gabler, N. K. (2019) Impact of porcine reproductive and respiratory syndrome virus on muscle metabolism of growing pigs1. *J Anim Sci* **97**, 3213–3227

47. Nunes, F. B., Graziottin, C. M., Carlos, J., Alves Filho, F., Lunardelli, A., Pires, M. G. S., Wächter, P. H., and De Oliveira, J. R. (2003) An assessment of fructose-1,6-bisphosphate as an antimicrobial and anti-inflammatory agent in sepsis. *Pharmacol Res* **47**

48. Jones, C. G. and Titheradge, M. A. (1993) The effect of treatment of the rat with bacterial endotoxin on gluconeogenesis and pyruvate metabolism in subsequently isolated hepatocytes. *Biochem. J* **289**

49. Mohás, M., Kisfali, P., Járomi, L., Maász, A., Fehér, E., Csöngei, V., Polgár, N., Sáfrány, E., Cseh, J., Sümegi, K., Hetyésy, K., Wittmann, I., and Melegh, B. (2010) GCKR gene functional variants in type 2 diabetes and metabolic syndrome: Do the rare variants associate with increased carotid intima-media thickness? *Cardiovasc Diabetol* **9**

50. Onuma, H., Tabara, Y., Kawamoto, R., Shimizu, I., Kawamura, R., Takata, Y., Nishida, W., Ohashi, J., Miki, T., Kohara, K., Makino, H., and Osawa, H. (2010) The GCKR rs780094 polymorphism is associated with susceptibility of type 2 diabetes, reduced fasting plasma glucose levels, increased triglycerides levels and lower HOMA-IR in Japanese population. *J Hum Genet* **55**, 600–604

51. Liu, Z., Liu, W., Huang, Y., Guo, J., Zhao, R., and Yang, X. (2015) Lipopolysaccharide significantly influences the hepatic triglyceride metabolism in growing pigs. *Lipids Health Dis* **14**

52. Pekala, P. H., Kawakamit, M., Angus', C. W., Laneo, M. D., and Ceramit, A. (1983) Selective inhibition of synthesis of enzymes for de novo fatty acid biosynthesis by an endotoxin-induced mediator from exudate cells (acetyl-CoA carboxylase/fatty acid synthetase/3T3-L1 preadipocytes/shock/macrophage). *Proc. NatL Acad. Sci. USA* **80**

53. Feingold, K. R., Wang, Y., Moser, A., Shigenaga, J. K., and Grunfeld, C. (2008) LPS decreases fatty acid oxidation and nuclear hormone receptors in the kidney. *J Lipid Res* **49**, 2179–2187

54. DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., and Felber, J. P. (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* **30**, 1000–1007

55. Sun, H., Huang, Y., Yin, C., Guo, J., Zhao, R., and Yang, X. (2016) Lipopolysaccharide markedly changes glucose metabolism and mitochondrial function in the longissimus muscle of pigs. *Animal* **10**, 1204–1212

56. Liang, H., Hussey, S. E., Sanchez-Avila, A., Tantiwong, P., and Musi, N. (2013) Effect of Lipopolysaccharide on Inflammation and Insulin Action in Human Muscle. *PLoS One* **8**

57. Mulligan, K. X., Morris, R. T., Otero, Y. F., Wasserman, D. H., and McGuinness, O. P. (2012) Disassociation of muscle insulin signaling and insulin-stimulated glucose uptake during endotoxemia. *PLoS One* **7**

58. Klasing, K. C. and Austic, R. E. (1984) Changes in protein synthesis due to an inflammatory challenge. *Proc Soc Exp Biol Med* **176**, 285–291

59. Funai, K., Song, H., Yin, L., Lodhi, I. J., Wei, X., Yoshino, J., Coleman, T., and Semenkovich, C. F. (2013) Muscle lipogenesis balances insulin sensitivity and strength through calcium signaling. *J Clin Invest* **123**

60. Li, H. and Sartorelli, V. (2018) ATP Citrate Lyase: A New Player Linking Skeletal Muscle Metabolism and Epigenetics. *Trends in Endocrinology and Metabolism* **29**, 202–204

61. Guo, J., Liu, Z., Sun, H., Huang, Y., Albrecht, E., Zhao, R., and Yang, X. (2015) Lipopolysaccharide challenge significantly influences lipid metabolism and proteome of white adipose tissue in growing pigs. *Lipids Health Dis* **14**

62. Mehta, N. N., McGillicuddy, F. C., Anderson, P. D., Hinkle, C. C., Shah, R., Pruscino, L., Tabita-Martinez, J., Sellers, K. F., Rickels, M. R., and Reilly, M. P. (2010) Experimental endotoxemia induces adipose inflammation and insulin resistance in humans. *Diabetes* **59**, 172–181

Chapter 4: General Conclusions

Swine are considered a dual purposes species and can be used for agriculture and biomedical purposes It is well established that insulin signaling is tightly controlled to maintain euglycemia as drastic changes in glucose concentrations can be detrimental in both the short and long term. To obtain the best product yield in swine production, there must be efficient nutrient utilization. Our goal was to investigate insulin signaling in atypical circumstances using pigs as our dual-purpose model system. Study one highlights the role of growth factor receptor bound protein 10 (GRB10) in porcine mesenchymal stem cells (MSC). While GRB10 is not well studied in pigs, studying GRB10 and MSC can have an impact on other areas of research. Study two focuses on insulin signaling after an acute immune response induced by an endotoxin, lipopolysaccharide (LPS). Disease prevalence and subsequent inflammation in a commercial swine system can be detrimental to the financial health of the operation. LPS has been used for decades in literature to model systemic immune response and recently, the focus has shifted from other model species into pigs.

Our first study proved that we could successfully genetically edit swine MSC, something that has not been documented frequently in the literature. With the generation of the GRB10-KD cells, we did see a decrease in the proliferation rate of these cells. While having a phenotype of modified cells is ideal, attempting to perform culture experiments with slowly proliferating cells is suboptimal. Given this reduction in proliferation rate, there is potential for GRB10 as a regulator of cell division in swine MSC via the mitogen activated protein kinase (MAPK) family, in addition to its role as an insulin inhibitor. While we did see changes in insulin signaling in our modified MSC, the results mostly did not align with the previous literature. From previous

studies, we expected improved glucose metabolism and insulin sensitivity with a reduction in GRB10. For our in vitro studies, we observed a worsening glucose metabolism and insulin signaling. However, insulin signaling when the MSC undergoes adipogenesis appears to be approved as evidenced by increased mRNA abundance of insulin and lipogenic related genes. Unfortunately, we could not verify the degree of GRB10 depression in our modified cells, we were only able to see reduction in *GRB10* based on transcript abundance. Luckily however, we did see a phenotype for modified cells that is indicative of changes in protein abundance. This study has highlighted GRB10 as a regulator of multiple aspects of metabolism. However, the role of GRB10 can vary depending on the physiologic status, animal model, tissue, or cell type.

The second study transitioned to evaluate altered insulin signaling in a different swine system. Overwhelming bacterial infection, often due to the presence of LPS, has been shown to cause hypoglycemia and hyperinsulinemia as the immune system requires glucose to fight the infection. Previous literature has shown that LPS in swine has caused insulin resistance and tissue level metabolic alterations when under chronically exposed. The repeated blood sampling portion of the study reinforced that LPS induces insulin resistance, as indicated by hyperglycemia and hyperinsulinemia, at 24 hours post exposure. A new finding this study uncovered a marked reduction in insulin concentration in the LPS administered pigs after 2 hours. The difference in insulin concentration between treatment groups may be impacted by the animals having free access to food. This finding has not been previously published and warrants further investigation, potentially with fasted animals. When examining the timed collection of animals and their tissues, we noted a marked reduction in transcripts associated with insulin signaling, glucose metabolism and lipogenesis in the liver. To further investigate the impact of

LPS, we looked at transcript abundance of metabolic genes in both the skeletal muscle and different fat deposits. The results were not as comprehensive as those seen in the liver but were still relevant when considering whole body metabolism. These results displayed the importance of the liver in the metabolic response to an LPS exposure

As study one was focused on GRB10, we opted to investigate the transcript abundance of *GRB10* in the liver and skeletal muscle of LPS exposed pigs. We found a novel reduction in the mRNA level of *GRB10* in the liver after LPS administration. While GRB10 has been implicated in cancer development, this appears to be the first indication that GRB10 can be modulated by an activated immune system. The hormone insulin is required to maintain homeostasis and can be disrupted through a variety of mechanisms. Both altering GRB10 and LPS exposure induced changes in insulin signaling and glucose metabolism that deviated from their normal physiological state. Given that we disrupted insulin signaling using a single adaptor protein and an acute endotoxin exposure, more work is required to investigate how GRB10 and LPS can influence metabolism.

Moving forward, more focus should be placed on the mechanism of action for both our in vitro and in vivo model of disrupted insulin signaling. Our in vitro system using GRB10 and MSC established that GRB10 modification does disrupt insulin signaling and greatly reduce proliferation rate. Establishing which tyrosine kinase GRB10 prefers, whether INSR or EGFR, will be beneficial to determine how GRB10 functions in MSC. While this study focuses on the impact of GRB10 on insulin signaling in an undetermined stem cell, there is potential for GRB10 ablation studies in MSC that have been differentiated into adipocytes. As there could be differing

mechanisms of how an undifferentiated MSC and an adipocyte control insulin signaling via GRB10. With the in vivo study, we determined that a single LPS injection does significantly reduce liver metabolism at a variety of levels while also causing systemic insulin resistance. Going forward, emphasis should be placed on feeding schedules in future studies in order to have animals be on a comparable plane of nutrition during the challenge. Given the significant changes in the liver, it is definitely worth considering the transcriptomic and proteomic changes after an LPS challenge. In closing, we highlighted those atypical causes of altered insulin signaling, modulation via adaptor proteins and acute immune response, can be studied via a commercial pig model.

Bibliography

Abdul-Ghani, M. A., & DeFronzo, R. A. (2010). Pathogenesis of insulin resistance in skeletal muscle. *Journal of Biomedicine & Biotechnology*, *2010*, 476279. https://doi.org/10.1155/2010/476279

Agwunobi, A. O., Reid, C., Maycock, P., Little, R. A., & Carlson, G. L. (2000). Insulin Resistance and Substrate Utilization in Human Endotoxemia. In *J Clin Endocrinol Metab* (Vol. 85). https://academic.oup.com/jcem/article/85/10/3770/2853946

Alexander, L. S., Seabolt, B. S., Rhoads, R. P., & Stahl, C. H. (2012). Neonatal phosphate nutrition alters in vivo and in vitro satellite cell activity in pigs. *Nutrients*, *4*(6), 436–448. https://doi.org/10.3390/nu4060436

Baker, D. H. (2008). Animal Models in Nutrition Research 1,2. In *The Journal of Nutrition Symposium: Animal Models in Nutrition Research J. Nutr* (Vol. 138).

Barkholt, L., Flory, E., Jekerle, V., Lucas-Samuel, S., Ahnert, P., Bisset, L., Büscher, D., Fibbe, W., Foussat, A., Kwa, M., Lantz, O., Mačiulaitis, R., Palomäki, T., Schneider, C. K., Sensebé, L., Tachdjian, G., Tarte, K., Tosca, L., & Salmikangas, P. (2013). Risk of tumorigenicity in mesenchymal stromal cell-based therapies--bridging scientific observations and regulatory viewpoints. *Cytotherapy*, *15*(7), 753–759. https://doi.org/10.1016/j.jcyt.2013.03.005

Beier, J. I., Guo, L., Von Montfort, C., Kaiser, J. P., Joshi-Barve, S., & Arteel, G. E. (2008). New role of resistin in lipopolysaccharide-induced liver damage in mice. *Journal of*

Pharmacology and Experimental Therapeutics, *325*(3), 801–808. https://doi.org/10.1124/jpet.108.136721

Berg, J. S., Lin, K. K., Sonnet, C., Boles, N. C., Weksberg, D. C., Nguyen, H., Holt, L. J., Rickwood, D., Daly, R. J., & Goodell, M. A. (2011). Imprinted genes that regulate early mammalian growth are coexpressed in somatic stem cells. *PLoS ONE*, *6*(10). https://doi.org/10.1371/journal.pone.0026410

Bevan, P. (2001). Insulin signalling. *Journal of Cell Science*, *114*(8), 1429–1430. www.biologists.com/jcs

Bidne, K. L., Kvidera, S. S., Ross, J. W., Baumgard, L. H., & Keating, A. F. (2018). Impact of repeated lipopolysaccharide administration on ovarian signaling during the follicular phase of the estrous cycle in post-pubertal pigs. *Journal of Animal Science*, *96*(9), 3622–3634. https://doi.org/10.1093/jas/sky226

Bosch, P., Pratt, S. L., & Stice, S. L. (2006). Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. *Biology of Reproduction*, *74*(1), 46–57. https://doi.org/10.1095/biolreprod.105.045138

Boucher, J., Kleinridders, A., & Ronald Kahn, C. (2014). Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harbor Perspectives in Biology*, *6*(1). https://doi.org/10.1101/cshperspect.a009191

Bugianesi, E., McCullough, A. J., & Marchesini, G. (2005). Insulin resistance: A metabolic pathway to chronic liver disease. In *Hepatology* (Vol. 42, Issue 5, pp. 987–1000). https://doi.org/10.1002/hep.20920

Calle, A., Barrajón-Masa, C., Gómez-Fidalgo, E., Martín-Lluch, M., Cruz-Vigo, P., Sánchez-Sánchez, R., & Ramírez, M. Á. (2018). Iberian pig mesenchymal stem/stromal cells from dermal skin, abdominal and subcutaneous adipose tissues, and peripheral blood: In vitro characterization and migratory properties in inflammation. *Stem Cell Research and Therapy*, *9*(1). https://doi.org/10.1186/s13287-018-0933-y

Campos, P. H. R. F., Merlot, E., Renaudeau, D., Noblet, J., & Le Floc'h, N. (2019). Postprandial insulin and nutrient concentrations in lipopolysaccharide-challenged growing pigs reared in thermoneutral and high ambient temperatures. *Journal of Animal Science*, *97*(8), 3354–3368. https://doi.org/10.1093/jas/skz204

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Chabo, C., Waget, A., Delmée, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrières, J., Tanti, J. F., Gibson, G. R., Casteilla, L., … Burcelin, R. (2007). Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes*, *56*(7), 1761–1772. https://doi.org/10.2337/db06-1491

Cargnello, M., & Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiology and Molecular Biology Reviews : MMBR*, *75*(1), 50–83. https://doi.org/10.1128/MMBR.00031-10

Caton, P. W., Nayuni, N. K., Murch, O., & Corder, R. (2009). Endotoxin induced hyperlactatemia and hypoglycemia is linked to decreased mitochondrial phosphoenolpyruvate carboxykinase. *Life Sciences*, *84*(21–22), 738–744. https://doi.org/10.1016/j.lfs.2009.02.024

Cavalcante-Silva, L. H. A., Galvão, J. G. F. M., da Silva, J. S. de F., de Sales-Neto, J. M., & Rodrigues-Mascarenhas, S. (2015). Obesity-Driven Gut Microbiota Inflammatory Pathways to Metabolic Syndrome. *Frontiers in Physiology*, *6*, 341. https://doi.org/10.3389/fphys.2015.00341

Charalambous, M., Smith, F. M., Bennett, W. R., Crew, T. E., Mackenzie, F., & Ward, A. (2003). *Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism*. www.ensembl.org

Chawla, A., Nguyen, K. D., & Goh, Y. P. S. (2011). Macrophage-mediated inflammation in metabolic disease. In *Nature Reviews Immunology* (Vol. 11, Issue 11, pp. 738–749). https://doi.org/10.1038/nri3071

Chen, L., Chen, R., Wang, H., & Liang, F. (2015). Mechanisms Linking Inflammation to Insulin Resistance. In *International Journal of Endocrinology* (Vol. 2015). Hindawi Limited. https://doi.org/10.1155/2015/508409

Chen, Q., Shou, P., Zheng, C., Jiang, M., Cao, G., Yang, Q., Cao, J., Xie, N., Velletri, T., Zhang, X., Xu, C., Zhang, L., Yang, H., Hou, J., Wang, Y., & Shi, Y. (2016). Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? *Cell Death and Differentiation*, *23*(7), 1128–1139. https://doi.org/10.1038/cdd.2015.168

Cornelison, A. S., Karriker, L. A., Williams, N. H., Haberl, B. J., Stalder, K. J., Schulz, L. L., & Patience, J. F. (2018). Impact of health challenges on pig growth performance, carcass characteristics, and net returns under commercial conditions. *Translational Animal Science*, *2*(1), 50–61. https://doi.org/10.1093/tas/txx005

Curry, S. M., Burrough, E. R., Schwartz, K. J., Yoon, K. J., Lonergan, S. M., & Gabler, N. K. (2018). Porcine epidemic diarrhea virus reduces feed efficiency in nursery pigs. *Journal of Animal Science*, *96*(1), 85–97. https://doi.org/10.1093/jas/skx005

Dali-Youcef, N., Mecili, M., Ricci, R., & Andrès, E. (2013). Metabolic inflammation: Connecting obesity and insulin resistance. *Annals of Medicine*, *45*(3), 242–253. https://doi.org/10.3109/07853890.2012.705015

Davis, C., & Lin, B. H. (2005). Factors Affecting U.S. Pork Consumption. *Livestock, Dairy, Poultry Outlook*.

DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., & Felber, J. P. (1981). The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes*, *30*(12), 1000–1007. https://doi.org/10.2337/diab.30.12.1000

Deng, Y., Bhattacharya, S., Swamy, O. R., Tandon, R., Wang, Y., Janda, R., & Riedel, H. (2003). Growth factor receptor-binding protein 10 (Grb10) as a partner of phosphatidylinositol 3-kinase in metabolic insulin action. *Journal of Biological Chemistry*, *278*(41), 39311–39322. https://doi.org/10.1074/jbc.M304599200

Deng, Y. J., Ren, E. H., Yuan, W. H., Zhang, G. Z., Wu, Z. L., & Xie, Q. Q. (2020). GRB10 and E2F3 as diagnostic markers of osteoarthritis and their correlation with immune infiltration. *Diagnostics*, *10*(3). https://doi.org/10.3390/diagnostics10030171

DiMeglio, L. A., Evans-Molina, C., & Oram, R. A. (2018). Type 1 diabetes. *Lancet (London, England)*, *391*(10138), 2449–2462. https://doi.org/10.1016/S0140-6736(18)31320-5

Dollet, L., Kuefner, M., Caria, E., Rizo-Roca, D., Pendergrast, L., Abdelmoez, A. M., Karlsson, H. K. R., Björnholm, M., Dalbram, E., Treebak, J. T., Harada, J., Naslund, E., Rydén, M., Zierath, J. R., Pillon, N. J., & Krook, A. (2022). Glutamine Regulates Skeletal Muscle Immunometabolism in Type 2 Diabetes. *Diabetes*, *71*(4), 624–636. https://doi.org/10.2337/db20-0814

Dong, L. Q., Porter, S., Hu, D., & Liu, F. (1998). *Inhibition of hGrb10 Binding to the Insulin Receptor by Functional Domain-mediated Oligomerization**. http://www.jbc.org

Duan, G., Huang, P., Zheng, C., Zheng, J., Yu, J., Zhang, P., Wan, M., Li, F., Guo, Q., Yin, Y., & Duan, Y. (2023). Development and Recovery of Liver Injury in Piglets by Incremental Injection of LPS. *Antioxidants*, *12*(6). https://doi.org/10.3390/antiox12061143

Edick, A. M., Auclair, O., & Burgos, S. A. (2020). Role of Grb10 in mTORC1-dependent regulation of insulin signaling and action in human skeletal muscle cells. *American Journal of Physiology - Endocrinology and Metabolism*, *318*(2), E173–E183. https://doi.org/10.1152/ajpendo.00025.2019

Eklind, S., Mallard, C., Arvidsson, P., & Hagberg, H. (2005). Lipopolysaccharide induces both a primary and a secondary phase of sensitization in the developing rat brain. *Pediatric Research*, *58*(1), 112–116. https://doi.org/10.1203/01.PDR.0000163513.03619.8D

Elahi, S., Brownlie, R., Korzeniowski, J., Buchanan, R., O'Connor, B., Peppler, M. S., Halperin, S. A., Lee, S. F., Babiuk, L. A., & Gerdts, V. (2005). Infection of newborn piglets with Bordetella pertussis: A new model for pertussis. *Infection and Immunity*, *73*(6), 3636–3645. https://doi.org/10.1128/IAI.73.6.3636-3645.2005

Feingold, K. R., Wang, Y., Moser, A., Shigenaga, J. K., & Grunfeld, C. (2008). LPS decreases fatty acid oxidation and nuclear hormone receptors in the kidney. *Journal of Lipid Research*, *49*(10), 2179–2187. https://doi.org/10.1194/jlr.M800233-JLR200

Fernández-Real, J. M., & Ricart, W. (2003). Insulin resistance and chronic cardiovascular inflammatory syndrome. In *Endocrine Reviews* (Vol. 24, Issue 3, pp. 278–301). Endocrine Society. https://doi.org/10.1210/er.2002-0010

Filho, D. M., de Carvalho Ribeiro, P., Oliveira, L. F., dos Santos, A. L. R. T., Parreira, R. C., Pinto, M. C. X., & Resende, R. R. (2019). Enhancing the Therapeutic Potential of Mesenchymal Stem Cells with the CRISPR-Cas System. In *Stem Cell Reviews and Reports* (Vol. 15, Issue 4, pp. 463–473). Humana Press Inc. https://doi.org/10.1007/s12015-019-09897-0

Filkins, J. P., & Cornell, R. P. (1974). Depression of hepatic gluconeogenesis and the hYP gY o 1 cemia of endotoxin shock. In *AMERICAN JOURNAL OF PHYSIOLOGY* (Vol. 227, Issue 4).

Fink, T., & Zachar, V. (2011). Adipogenic differentiation of human mesenchymal stem cells. *Methods in Molecular Biology (Clifton, N.J.)*, *698*, 243–251. https://doi.org/10.1007/978-1-60761-999-4_19

Freitas, G. P., Lopes, H. B., Souza, A. T. P., Gomes, M. P. O., Quiles, G. K., Gordon, J., Tye, C., Stein, J. L., Stein, G. S., Lian, J. B., Beloti, M. M., & Rosa, A. L. (2021). Mesenchymal stem cells overexpressing BMP-9 by CRISPR-Cas9 present high in vitro osteogenic potential and enhance in vivo bone formation. *Gene Therapy*, *28*(12), 748–759. https://doi.org/10.1038/s41434-021-00248-8

Fried, S. K., Bunkin, D. A., & Greenberg, A. S. (1998). *Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6: Depot Difference and Regulation by Glucocorticoid**. https://academic.oup.com/jcem/article/83/3/847/2865248

Friedenstein, A. J., Chailakhjan, R. K., & Lalykina, K. S. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell and Tissue Kinetics*, *3*(4), 393–403. https://doi.org/10.1111/j.1365-2184.1970.tb00347.x

Funai, K., Song, H., Yin, L., Lodhi, I. J., Wei, X., Yoshino, J., Coleman, T., & Semenkovich, C. F. (2013). Muscle lipogenesis balances insulin sensitivity and strength through calcium signaling. *The Journal of Clinical Investigation*, *123*. https://doi.org/10.1172/JCI65726DS1

Gerace, D., Martiniello-Wilks, R., Nassif, N. T., Lal, S., Steptoe, R., & Simpson, A. M. (2017). CRISPR-targeted genome editing of mesenchymal stem cell-derived therapies for type 1 diabetes: A path to clinical success? In *Stem Cell Research and Therapy* (Vol. 8, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13287-017-0511-8

Golchin, A., Shams, F., & Karami, F. (2020). Advancing Mesenchymal Stem Cell Therapy with CRISPR/Cas9 for Clinical Trial Studies. *Advances in Experimental Medicine and Biology*, *1247*, 89–100. https://doi.org/10.1007/5584_2019_459

Gonzalez, L. M., Moeser, A. J., & Blikslager, A. T. (2015). Porcine models of digestive disease: The future of large animal translational research. In *Translational Research* (Vol. 166, Issue 1, pp. 12–27). Mosby Inc. https://doi.org/10.1016/j.trsl.2015.01.004

Guilbault, C., Saeed, Z., Downey, G. P., & Radzioch, D. (2007). Cystic fibrosis mouse models. In *American Journal of Respiratory Cell and Molecular Biology* (Vol. 36, Issue 1, pp. 1–7). https://doi.org/10.1165/rcmb.2006-0184TR

Guo, J., Liu, Z., Sun, H., Huang, Y., Albrecht, E., Zhao, R., & Yang, X. (2015). Lipopolysaccharide challenge significantly influences lipid metabolism and proteome of white adipose tissue in growing pigs. *Lipids in Health and Disease*, *14*(1). https://doi.org/10.1186/s12944-015-0067-5

Haleem-Smith, H., Derfoul, A., Okafor, C., Tuli, R., Olsen, D., Hall, D. J., & Tuan, R. S. (2005). *High-Efficiency Transfection of Human MSCs 9 MOLECULAR BIOTECHNOLOGY Abstract Optimization of High-Efficiency Transfection of Adult Human Mesenchymal Stem Cells In Vitro*. http://www.amaxa.com

Hamann, A., Nguyen, A., & Pannier, A. K. (2019). Nucleic acid delivery to mesenchymal stem cells: A review of nonviral methods and applications. In *Journal of Biological Engineering* (Vol. 13, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13036-019-0140-0

Han, D. C., Shen, T. L., & Guan, J. L. (2001). The Grb7 family proteins: structure, interactions with other signaling molecules and potential cellular functions. *Oncogene*, *20*(44), 6315–6321. https://doi.org/10.1038/sj.onc.1204775

Han, M. S., Jung, D. Y., Morel, C., Lakhani, S. A., Kim, J. K., Flavell, R. A., & Davis, R. J. (2013). JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation. *Science*, *339*(6116), 218–222. https://doi.org/10.1126/science.1227568

Hansen, H., Svensson, U., Zhu, J., Laviola, L., Giorgino, F., Wolf, G., Smith, R. J., & Riedel, H. (1996). *Interaction between the Grb10 SH2 Domain and the Insulin Receptor Carboxyl Terminus**.

Hazrati, A., Malekpour, K., Soudi, S., & Hashemi, S. M. (2022). CRISPR/Cas9-engineered mesenchymal stromal/stem cells and their extracellular vesicles: A new approach to overcoming cell therapy limitations. In *Biomedicine and Pharmacotherapy* (Vol. 156). Elsevier Masson s.r.l. https://doi.org/10.1016/j.biopha.2022.113943

He, W., Rose, D. W., Olefsky, J. M., & Gustafson, T. A. (1998). *Grb10 Interacts Differentially with the Insulin Receptor, Insulin-like Growth Factor I Receptor, and Epidermal Growth Factor Receptor via the Grb10 Src Homology 2 (SH2) Domain and a Second Novel Domain Located between the Pleckstrin Homology and SH2 Domains**. http://www.jbc.org

Helm, E. T., Curry, S. M., De Mille, C. M., Schweer, W. P., Burrough, E. R., Zuber, E. A., Lonergan, S. M., & Gabler, N. K. (2019). Impact of porcine reproductive and respiratory syndrome virus on muscle metabolism of growing pigs1. *Journal of Animal Science*, *97*(8), 3213–3227. https://doi.org/10.1093/jas/skz168

Hemmings, B. A., & Restuccia, D. F. (2012). PI3K-PKB/Akt pathway. *Cold Spring Harbor Perspectives in Biology*, *4*(9). https://doi.org/10.1101/cshperspect.a011189

Hitchins, M. P., Monk, D., Bell, G. M., Ali, Z., Preece, M. A., Stanier, P., & Moore, G. E. (2001). Maternal repression of the human GRB10 gene in the developing central nervous system; evaluation of the role for GRB10 in Silver-Russell syndrome. *European Journal of Human Genetics : EJHG*, *9*(2), 82–90. https://doi.org/10.1038/sj.ejhg.5200583

Holt, L. J., & Siddle, K. (2005). Grb10 and Grb14: enigmatic regulators of insulin action-and more? In *Biochem. J* (Vol. 388).

Holt, L. J., Turner, N., Mokbel, N., Trefely, S., Kanzleiter, T., Kaplan, W., Ormandy, C. J., Daly, R. J., & Cooney, G. J. (2012). Grb10 regulates the development of fiber number in skeletal muscle. *The FASEB Journal*, *26*(9), 3658–3669. https://doi.org/10.1096/fj.11-199349

Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature*, *444*(7121), 860–867. https://doi.org/10.1038/nature05485

Houpt, K. A., Houpt, T. R., & Pondb, W. G. (1979). The Pig as a Model for the Study of Obesity and of Control of Food Intake: A Review. In *BIOLOGY AND MEDICINE* (Vol. 52).

Hsu, P. P., Kang, S. A., Rameseder, J., Zhang, Y., Ottina, K. A., Lim, D., Peterson, T. R., Choi, Y., Gray, N. S., Yaffe, M. B., Marto, J. A., & Sabatini, D. M. (2011). The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science*, *332*(6035), 1317–1322. https://doi.org/10.1126/science.1199498

Illert, A. L., Albers, C., Kreutmair, S., Leischner, H., Peschel, C., Miething, C., & Duyster, J. (2015). Grb10 is involved in BCR-ABL-positive leukemia in mice. *Leukemia*, *29*(4), 858–868. https://doi.org/10.1038/leu.2014.283

Jahn, T., Seipel, P., Urschel, S., Peschel, C., & Duyster, J. (2002). Role for the Adaptor Protein Grb10 in the Activation of Akt. *Molecular and Cellular Biology*, *22*(4), 979–991. https://doi.org/10.1128/mcb.22.4.979-991.2002

James, A. W. (2013). Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. *Scientifica*, *2013*, 1–17. https://doi.org/10.1155/2013/684736

Janderová, L., McNeil, M., Murrell, A. N., Mynatt, R. L., & Smith, S. R. (2003). Human mesenchymal stem cells as an in vitro model for human adipogenesis. *Obesity Research*, *11*(1), 65–74. https://doi.org/10.1038/oby.2003.11

Jang, J. H., Shin, H. W., Lee, J. M., Lee, H. W., Kim, E. C., & Park, S. H. (2015). An Overview of Pathogen Recognition Receptors for Innate Immunity in Dental Pulp. In *Mediators of Inflammation* (Vol. 2015). Hindawi Publishing Corporation. https://doi.org/10.1155/2015/794143

Johnson, R. W., & von Borell, E. (1994). Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin. *Journal of Animal Science*, *72*(2), 309–314. https://doi.org/10.2527/1994.722309x

Jones, C. G., & Titheradge, M. A. (1993). The effect of treatment of the rat with bacterial endotoxin on gluconeogenesis and pyruvate metabolism in subsequently isolated hepatocytes. In *Biochem. J* (Vol. 289).

Kanety, H., Feinstein, R., Papa, M. Z., Hemi, R., & Karasik, A. (1995). *Tumor Necrosis Factor Phosphorylation of Insulin Receptor Substrate-1 (IRS-1) POSSIBLE MECHANISM FOR SUPPRESSION OF INSULIN-STIMULATED TYROSINE PHOSPHORYLATION OF IRS-1*.*

Kawasaki, T., & Kawai, T. (2014). Toll-like receptor signaling pathways. In *Frontiers in Immunology* (Vol. 5, Issue SEP). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2014.00461

Kazi, J. U., & Rönnstrand, L. (2013). FLT3 signals via the adapter protein Grb10 and overexpression of Grb10 leads to aberrant cell proliferation in acute myeloid leukemia. *Molecular Oncology*, *7*(3), 402–418. https://doi.org/10.1016/j.molonc.2012.11.003

Khan, M. I., Johani, A. Al, Hamid, A., Ateeq, B., Manzar, N., Adhami, V. M., Lall, R. K., Rath, S., Sechi, M., Siddiqui, I. A., Choudhry, H., Zamzami, M. A., Havighurst, T. C., Huang, W., Ntambi, J. M., & Mukhtar, H. (2019). Proproliferative function of adaptor protein GRB10 in prostate carcinoma. *The FASEB Journal*, *33*(3), 3198–3211. https://doi.org/10.1096/fj.201800265RR

Kido, Y., Nakae, J., & Accili, D. (2001). Clinical review 125: The insulin receptor and its cellular targets. *The Journal of Clinical Endocrinology and Metabolism*, *86*(3), 972–979. https://doi.org/10.1210/jcem.86.3.7306

Klasing, K. C., & Austic, R. E. (1984). Changes in protein synthesis due to an inflammatory challenge. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, *176*(3), 285–291. https://doi.org/10.3181/00379727-176-41872

Kvidera, S. K., Horst, E. A., Mayorga, E. J., Sanz-Fernandez, M. V., Abuajamieh, M., & Baumgard, L. H. (2017). Estimating glucose requirements of an activated immune system in growing pigs. *Journal of Animal Science*, *95*(11), 5020–5029. https://doi.org/10.2527/jas2017.1830

Laghouaouta, H., Fraile, L., Suárez-Mesa, R., Ros-Freixedes, R., Estany, J., & Pena, R. N. (2022). A genome-wide screen for resilient responses in growing pigs. *Genetics Selection Evolution*, *54*(1). https://doi.org/10.1186/s12711-022-00739-1

Lang, C. H., & Spitzer, J. A. (1987). Glucose kinetics and development of endotoxin tolerance during long-term continuous endotoxin infusion. *Metabolism: Clinical and Experimental*, *36*(5), 469–474. https://doi.org/10.1016/0026-0495(87)90045-x

Lang, C. H., Spolarics, Z., Ottlakan, A., & Spitzer, J. J. (1993). Effect of high-dose endotoxin on glucose production and utilization. *Metabolism: Clinical and Experimental*, *42*(10), 1351–1358. https://doi.org/10.1016/0026-0495(93)90137-d

Langlais, P., Dong, L. Q., Ramos, F. J., Hu, D., Li, Y., Quon, M. J., & Liu, F. (2004). Negative Regulation of Insulin-Stimulated Mitogen-Activated Protein Kinase Signaling by Grb10. *Molecular Endocrinology*, *18*(2), 350–358. https://doi.org/10.1210/me.2003-0117

Langlais, P., Wang, C., Dong, L. Q., Carroll, C. A., Weintraub, S. T., & Liu, F. (2005). Phosphorylation of Grb10 by mitogen-activated protein kinase: Identification of Ser150 and Ser476 of human Grb10ζ as major phosphorylation sites. *Biochemistry*, *44*(24), 8890–8897. https://doi.org/10.1021/bi050413i

Langston, P. K., Shibata, M., & Horng, T. (2017). Metabolism Supports Macrophage Activation. *Frontiers in Immunology*, *8*, 61. https://doi.org/10.3389/fimmu.2017.00061

Lanuza-Masdeu, J., Isabel Arévalo, M., Vila, C., Barberà, A., Gomis, R., & Caelles, C. (2013). In vivo jnk activation in pancreatic β-cells leads to glucose intolerance caused by insulin resistance in pancreas. *Diabetes*, *62*(7), 2308–2317. https://doi.org/10.2337/db12-1097

Lee, B. C., & Lee, J. (2014). Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. In *Biochimica et Biophysica Acta - Molecular Basis of Disease* (Vol. 1842, Issue 3, pp. 446–462). https://doi.org/10.1016/j.bbadis.2013.05.017

Li, H., & Sartorelli, V. (2018). ATP Citrate Lyase: A New Player Linking Skeletal Muscle Metabolism and Epigenetics. In *Trends in Endocrinology and Metabolism* (Vol. 29, Issue 4, pp. 202–204). Elsevier Inc. https://doi.org/10.1016/j.tem.2018.01.006

Li, Q., Liu, Y., Che, Z., Zhu, H., Meng, G., Hou, Y., Ding, B., Yin, Y., & Chen, F. (2012). Dietary L-arginine supplementation alleviates liver injury caused by Escherichia coli LPS in weaned pigs. *Innate Immunity*, *18*(6), 804–814. https://doi.org/10.1177/1753425912441955

Liang, H., Hussey, S. E., Sanchez-Avila, A., Tantiwong, P., & Musi, N. (2013). Effect of Lipopolysaccharide on Inflammation and Insulin Action in Human Muscle. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0063983

Litten-Brown, J. C., Corson, A. M., & Clarke, L. (2010). Porcine models for the metabolic syndrome, digestive and bone disorders: A general overview. *Animal*, *4*(6), 899–920. https://doi.org/10.1017/S1751731110000200

Liu, F., & Roth, R. A. (1995). *Grb-IR: A SH2-domain-containing protein that binds to the insulin receptor and inhibits its function (phosphatidylinositol 3-kinase/yeast two-hybrid system/insulin-receptor substrate 1)* (Vol. 92). https://www.pnas.org

Liu, M., Bai, J., He, S., Villarreal, R., Hu, D., Zhang, C., Yang, X., Liang, H., Slaga, T. J., Yu, Y., Zhou, Z., Blenis, J., Scherer, P. E., Dong, L. Q., & Liu, F. (2014). Grb10 promotes lipolysis and thermogenesis by phosphorylation-dependent feedback inhibition of mTORC1. *Cell Metabolism*, *19*(6), 967–980. https://doi.org/10.1016/j.cmet.2014.03.018

Liu, Z., Liu, W., Huang, Y., Guo, J., Zhao, R., & Yang, X. (2015). Lipopolysaccharide significantly influences the hepatic triglyceride metabolism in growing pigs. *Lipids in Health and Disease*, *14*(1). https://doi.org/10.1186/s12944-015-0064-8

Lochmiller, R. L., & Deerenberg, C. (2000). Trade-offs in evolutionary immunology: Just what is the cost of immunity? *Oikos*, *88*(1), 87–98. https://doi.org/10.1034/j.1600-0706.2000.880110.x

Low, M. J. (2012). Mouse models in gastroenterology research. In *Gastroenterology* (Vol. 143, Issue 6, pp. 1410–1412). W.B. Saunders. https://doi.org/10.1053/j.gastro.2012.10.011

Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. In *Cytokine* (Vol. 42, Issue 2, pp. 145–151). https://doi.org/10.1016/j.cyto.2008.01.006

Luo, H., Peng, F., Weng, B., Tang, X., Chen, Y., Yang, A., Chen, B., & Ran, M. (2020). miR-222 Suppresses Immature Porcine Sertoli Cell Growth by Targeting the GRB10 Gene Through Inactivating the PI3K/AKT Signaling Pathway. *Frontiers in Genetics*, *11*. https://doi.org/10.3389/fgene.2020.581593

Maes, D. G. D., Dewulf, J., Piñeiro, C., Edwards, S., & Kyriazakis, I. (2020). A critical reflection on intensive pork production with an emphasis on animal health and welfare. *Journal of Animal Science*, *98*, S15–S26. https://doi.org/10.1093/jas/skz362

Mahajan, A., & Stahl, C. H. (2009). Dihydroxy-cholecalciferol stimulates adipocytic differentiation of porcine mesenchymal stem cells. *Journal of Nutritional Biochemistry*, *20*(7), 512–520. https://doi.org/10.1016/j.jnutbio.2008.05.010

Mair, K. H., Sedlak, C., Käser, T., Pasternak, A., Levast, B., Gerner, W., Saalmüller, A., Summerfield, A., Gerdts, V., Wilson, H. L., & Meurens, F. (2014). The porcine innate immune system: An update. In *Developmental and Comparative Immunology* (Vol. 45, Issue 2, pp. 321–343). Elsevier Ltd. https://doi.org/10.1016/j.dci.2014.03.022

Maitra, S. R., Gestring, M. L., El-Maghrabi, M. R., Lang, C. H., & Henry, M. C. (1999). Endotoxin-induced alterations in hepatic glucose-6-phosphatase activity and gene expression. *Molecular and Cellular Biochemistry*, *196*(1–2), 79–83.

Manning, B. D., & Toker, A. (2017). AKT/PKB Signaling: Navigating the Network. In *Cell* (Vol. 169, Issue 3, pp. 381–405). Cell Press. https://doi.org/10.1016/j.cell.2017.04.001

Margolis, B., Silvennoinen, O., Comoglio, F., Roonprapunt, C., Skolnik, E., Ullrich, A., & Schlessinger, J. (1992). High-efficiency expression/cloning of epidermal growth factor-receptor-binding proteins with Src homology 2 domains. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(19), 8894–8898. https://doi.org/10.1073/pnas.89.19.8894

McCowen, K. C., Malhotra, A., & Bistrian, B. R. (2001). Stress-induced hyperglycemia. *Critical Care Clinics*, *17*(1), 107–124. https://doi.org/10.1016/s0749-0704(05)70154-8

Mehta, N. N., McGillicuddy, F. C., Anderson, P. D., Hinkle, C. C., Shah, R., Pruscino, L., Tabita-Martinez, J., Sellers, K. F., Rickels, M. R., & Reilly, M. P. (2010). Experimental endotoxemia induces adipose inflammation and insulin resistance in humans. *Diabetes*, *59*(1), 172–181. https://doi.org/10.2337/db09-0367

Meng, Y., Eirin, A., Zhu, X. Y., Tang, H., Chanana, P., Lerman, A., van Wijnen, A. J., & Lerman, L. O. (2018). Obesity-induced mitochondrial dysfunction in porcine adipose

tissue-derived mesenchymal stem cells. *Journal of Cellular Physiology*, *233*(8), 5926–5936. https://doi.org/10.1002/jcp.26402

Miller, E. R., & Ullrey, D. E. (1987). THE PIG AS A MODEL FOR HUMAN NUTRITION. In *Ann. Rev. Nutr* (Vol. 7). www.annualreviews.org

Mohammad, S., & Thiemermann, C. (2020). Role of Metabolic Endotoxemia in Systemic Inflammation and Potential Interventions. *Frontiers in Immunology*, *11*, 594150. https://doi.org/10.3389/fimmu.2020.594150

Mohás, M., Kisfali, P., Járomi, L., Maász, A., Fehér, E., Csöngei, V., Polgár, N., Sáfrány, E., Cseh, J., Sümegi, K., Hetyésy, K., Wittmann, I., & Melegh, B. (2010). GCKR gene functional variants in type 2 diabetes and metabolic syndrome: Do the rare variants associate with increased carotid intima-media thickness? *Cardiovascular Diabetology*, *9*. https://doi.org/10.1186/1475-2840-9-79

Mokbel, N., Hoffman, N. J., Girgis, C. M., Small, L., Turner, N., Daly, R. J., Cooney, G. J., & Holt, L. J. (2014). Grb10 Deletion Enhances Muscle Cell Proliferation, Differentiation and GLUT4 Plasma Membrane Translocation. *Journal of Cellular Physiology*, *229*(11), 1753–1764. https://doi.org/10.1002/jcp.24628

Monami, G., Emiliozzi, V., & Morrione, A. (2008). Grb10/Nedd4-mediated multiubiquitination of the insulin-like growth factor receptor regulates receptor internalization. *Journal of Cellular Physiology*, *216*(2), 426–437. https://doi.org/10.1002/jcp.21405

Morrione, A., Plant, P., Valentinis, B., Staub, O., Kumar, S., Rotin, D., & Baserga, R. (1999). *mGrb10 Interacts with Nedd4**. http://www.jbc.org

Morrione, A., Valentinis, B., Resnicoff, M., Xu, S.-Q., & Baserga, R. (1997). *The Role of mGrb10 in Insulin-like Growth Factor I-mediated Growth**. http://www.jbc.org

Moutoussamy, S., Oise Renaudie, F., Lago, F., Kelly, P. A., & Finidori, J. (1998). *Grb10 Identified as a Potential Regulator of Growth Hormone (GH) Signaling by Cloning of GH Receptor Target Proteins**. http://www.jbc.org

Mulligan, K. X., Morris, R. T., Otero, Y. F., Wasserman, D. H., & McGuinness, O. P. (2012). Disassociation of muscle insulin signaling and insulin-stimulated glucose uptake during endotoxemia. *PLoS ONE*, *7*(1). https://doi.org/10.1371/journal.pone.0030160

National Research Council. (2012). *Nutrient Requirements of Swine* (11th ed.). National Academies Press. https://doi.org/10.17226/13298

Nordgreen, J., Munsterhjelm, C., Aae, F., Popova, A., Boysen, P., Ranheim, B., Heinonen, M., Raszplewicz, J., Piepponen, P., Lervik, A., Valros, A., & Janczak, A. M. (2018). The effect of lipopolysaccharide (LPS) on inflammatory markers in blood and brain and on behavior in individually-housed pigs. *Physiology and Behavior*, *195*, 98–111. https://doi.org/10.1016/j.physbeh.2018.07.013

Nunes, F. B., Graziottin, C. M., Carlos, J., Alves Filho, F., Lunardelli, A., Pires, M. G. S., Wächter, P. H., & De Oliveira, J. R. (2003). An assessment of fructose-1,6-bisphosphate as an antimicrobial and anti-inflammatory agent in sepsis. In *Pharmacological Research* (Vol. 47).

Odegaard, J. I., & Chawla, A. (2013). Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis. In *Science* (Vol. 339, Issue 6116, pp. 172–177).

American Association for the Advancement of Science. https://doi.org/10.1126/science.1230721

Oggu, G. S., Sasikumar, S., Reddy, N., Ella, K. K. R., Rao, C. M., & Bokara, K. K. (2017). Gene Delivery Approaches for Mesenchymal Stem Cell Therapy: Strategies to Increase Efficiency and Specificity. *Stem Cell Reviews and Reports*, *13*(6), 725–740. https://doi.org/10.1007/s12015-017-9760-2

O'Neill, T. J., Rose, D. W., Pillay, T. S., Hotta, K., Olefsky, J. M., & Gustafson, T. A. (1996). Interaction of a GRB-IR splice variant (a human GRB10 homolog) with the insulin and insulin-like growth factor I receptors. Evidence for a role in mitogenic signaling. *The Journal of Biological Chemistry*, *271*(37), 22506–22513. https://doi.org/10.1074/jbc.271.37.22506

Onuma, H., Tabara, Y., Kawamoto, R., Shimizu, I., Kawamura, R., Takata, Y., Nishida, W., Ohashi, J., Miki, T., Kohara, K., Makino, H., & Osawa, H. (2010). The GCKR rs780094 polymorphism is associated with susceptibility of type 2 diabetes, reduced fasting plasma glucose levels, increased triglycerides levels and lower HOMA-IR in Japanese population. *Journal of Human Genetics*, *55*(9), 600–604. https://doi.org/10.1038/jhg.2010.75

Ooi, J., Yajnik, V., Immanuel, D., Gordon, M., Moskow, J. J., Buchberg, A. M., & Margolis, B. (1995). The cloning of Grb10 reveals a new family of SH2 domain proteins. *Oncogene*, *10*(8), 1621–1630.

Orellana, R. A., J O, P. M., Nguyen, H. V, Bush, J. A., Suryawan, A., Carole Thivierge, M., Fiorotto, M. L., Davis, T. A., & Carole Thivi-erge, M. (2002). Endotoxemia reduces

skeletal muscle protein synthesis in neonates. *Am J Physiol Endocrinol Metab*, *283*, 909–916. https://doi.org/10.1152/ajpendo.00220.2002.-Pro

Orellana, R. A., Kimball, S. R., Nguyen, H. V., Bush, J. A., Suryawan, A., Thivierge, M. C., Jefferson, L. S., & Davis, T. A. (2004). Regulation of Muscle Protein Synthesis in Neonatal Pigs during Prolonged Endotoxemia. *Pediatric Research*, *55*(3), 442–449. https://doi.org/10.1203/01.PDR.0000110526.02282.F3

O-Sullivan, I. S., Chopra, A., Carr, J., Tae, S. K., & Cohen, E. P. (2008). Immunity to growth factor receptor-bound protein 10, a signal transduction molecule, inhibits the growth of breast cancer in mice. *Cancer Research*, *68*(7), 2463–2470. https://doi.org/10.1158/0008-5472.CAN-07-5685

Owen, M., & Friedenstein, A. J. (1988). Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Foundation Symposium*, *136*, 42–60. https://doi.org/10.1002/9780470513637.ch4

Pålsson-McDermott, E. M., & O'Neill, L. A. J. (2004). Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. In *Immunology* (Vol. 113, Issue 2, pp. 153–162). https://doi.org/10.1111/j.1365-2567.2004.01976.x

Pandey, A., Duan, H., Fiore, P. Di, Dixit, V. M., Santoro, M., Carlomagno, F., Romano, A., Bottaro, D. P., Dathan, N. A., Grieco, M., Fusco, A., Vecchio, G., Matˇoškova, M. M., Kraus, B., & Difiore, P. (1995). *The Ret Receptor Protein Tyrosine Kinase Associates with the SH2-containing Adapter Protein Grb10* Ret is a receptor protein tyrosine kinase that has been implicated in the development of the enteric nervous, endocrine, and renal systems. Mutations associated with multiple endocrine neoplasia types 2A and 2B (MEN*

2A and 2B) have been shown to activate the in-trinsic kinase and transforming ability of ret (.

Park, B. S., & Lee, J. O. (2013). Recognition of lipopolysaccharide pattern by TLR4 complexes. In *Experimental and Molecular Medicine* (Vol. 45, Issue 12). Nature Publishing Group. https://doi.org/10.1038/emm.2013.97

Pederson, T. M., Kramer, D. L., & Rondinone, C. M. (2001). Original Articles Serine/Threonine Phosphorylation of IRS-1 Triggers Its Degradation Possible Regulation by Tyrosine Phosphorylation. In *24 DIABETES* (Vol. 50). http://diabetesjournals.org/diabetes/article-pdf/50/1/24/366872/0500024.pdf

Pekala, P. H., Kawakamit, M., Angus', C. W., Laneo, M. D., & Ceramit, A. (1983). Selective inhibition of synthesis of enzymes for de novo fatty acid biosynthesis by an endotoxin-induced mediator from exudate cells (acetyl-CoA carboxylase/fatty acid synthetase/3T3-L1 preadipocytes/shock/macrophage). In *Proc. NatL Acad. Sci. USA* (Vol. 80).

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S., & Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science (New York, N.Y.)*, *284*(5411), 143–147. https://doi.org/10.1126/science.284.5411.143

Purwar, A., & Nagpure, S. (2022). Insulin Resistance in Polycystic Ovarian Syndrome. *Cureus*, *14*(10), e30351. https://doi.org/10.7759/cureus.30351

Ragheb, R., & M. Medhat, A. (2011). Mechanisms of Fatty Acid-Induced Insulin Resistance in Muscle and Liver. *Journal of Diabetes & Metabolism*, *02*(04). https://doi.org/10.4172/2155-6156.1000127

Ramos, F. J., Langlais, P. R., Hu, D., Dong, L. Q., & Liu, F. (2006). Grb10 mediates insulin-stimulated degradation of the insulin receptor: A mechanism of negative regulation. *American Journal of Physiology - Endocrinology and Metabolism*, *290*(6). https://doi.org/10.1152/ajpendo.00609.2005

Ren, L.-L., Wang, Z.-W., Sen, R., Dai, Z.-T., Liao, X.-H., & Shen, L.-J. (2023). GRB10 is a novel factor associated with gastric cancer proliferation and prognosis. *Aging*, *15*(9), 3394–3409. https://doi.org/10.18632/aging.204603

Rhee, S. H. (2014). Lipopolysaccharide: Basic Biochemistry, Intracellular Signaling, and Physiological Impacts in the Gut. *Intestinal Research*, *12*(2), 90. https://doi.org/10.5217/ir.2014.12.2.90

Riedel, H. (2004). Grb10 exceeding the boundaries of a common signaling adapter. *Frontiers in Bioscience : A Journal and Virtual Library*, *9*, 603–618. https://doi.org/10.2741/1227

Ringe, J., Kaps, C., Schmitt, B., Büscher, K., Bartel, J., Smolian, H., Schultz, O., Burmester, G. R., Häupl, T., & Sittinger, M. (2002). Porcine mesenchymal stem cells: Induction of distinct mesenchymal cell lineages. *Cell and Tissue Research*, *307*(3), 321–327. https://doi.org/10.1007/s00441-002-0525-z

Sakurai, H., Suzuki, S., Kawasaki, N., Nakano, H., Okazaki, T., Chino, A., Doi, T., & Saiki, I. (2003). Tumor necrosis factor-α-induced IKK phosphorylation of NF-κB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *Journal of Biological Chemistry*, *278*(38), 36916–36923. https://doi.org/10.1074/jbc.M301598200

Sala-Gaston, J., Costa-Sastre, L., Pedrazza, L., Martinez-Martinez, A., Ventura, F., & Rosa, J. L. (2023). Regulation of MAPK Signaling Pathways by the Large HERC Ubiquitin

Ligases. *International Journal of Molecular Sciences*, *24*(5). https://doi.org/10.3390/ijms24054906

Saltiel, A. R. (2021). Insulin signaling in health and disease. In *Journal of Clinical Investigation* (Vol. 131, Issue 1). American Society for Clinical Investigation. https://doi.org/10.1172/JCI142241

Secunda, R., Vennila, R., Mohanashankar, A. M., Rajasundari, M., Jeswanth, S., & Surendran, R. (2015). Isolation, expansion and characterisation of mesenchymal stem cells from human bone marrow, adipose tissue, umbilical cord blood and matrix: a comparative study. *Cytotechnology*, *67*(5), 793–807. https://doi.org/10.1007/s10616-014-9718-z

Senn, J. J., Klover, P. J., Nowak, I. A., & Mooney, R. A. (2002). Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes*, *51*(12), 3391–3399. https://doi.org/10.2337/diabetes.51.12.3391

Sharma, M., Vikram, N. K., Misra, A., Bhatt, S. P., Tarique, M., Parray, H. A., Pandey, R. M., & Luthra, K. (2013). Assessment of 11-b hydroxysteroid dehydrogenase (11-bHSD1) 4478T>G and tumor necrosis factor-a (TNF-a)-308 G>A polymorphisms with obesity and insulin resistance in Asian Indians in North India. *Molecular Biology Reports*, *40*(11), 6261–6270. https://doi.org/10.1007/s11033-013-2738-5

Shimobayashi, M., & Hall, M. N. (2014). Making new contacts: The mTOR network in metabolism and signalling crosstalk. *Nature Reviews Molecular Cell Biology*, *15*(3), 155–162. https://doi.org/10.1038/nrm3757

Shiura, H., Miyoshi, N., Konishi, A., Wakisaka-Saito, N., Suzuki, R., Muguruma, K., Kohda, T., Wakana, S., Yokoyama, M., Ishino, F., & Kaneko-Ishino, T. (2005). Meg1/Grb10

overexpression causes postnatal growth retardation and insulin resistance via negative modulation of the IGF1R and IR cascades. *Biochemical and Biophysical Research Communications*, *329*(3), 909–916. https://doi.org/10.1016/j.bbrc.2005.02.047

Smith, F. M., Holt, L. J., Garfield, A. S., Charalambous, M., Koumanov, F., Perry, M., Bazzani, R., Sheardown, S. A., Hegarty, B. D., Lyons, R. J., Cooney, G. J., Daly, R. J., & Ward, A. (2007). Mice with a Disruption of the Imprinted Grb10 Gene Exhibit Altered Body Composition, Glucose Homeostasis, and Insulin Signaling during Postnatal Life . *Molecular and Cellular Biology*, *27*(16), 5871–5886. https://doi.org/10.1128/mcb.02087-06

Sohani, Z. N., Anand, S. S., Robiou-Du-pont, S., Morrison, K. M., McDonald, S. D., Atkinson, S. A., Teo, K. K., & Meyre, D. (2016). Risk alleles in/near ADCY5, ADRA2A, CDKAL1, CDKN2A/B, GRB10, and TCF7L2 elevate Plasma glucose levels at birth and in early childhood: Results from the FAMILY study. *PLoS ONE*, *11*(4). https://doi.org/10.1371/journal.pone.0152107

Song, C., Jiang, J., Han, X., Yu, G., & Pang, Y. (2014). Effect of immunological stress to neuroendocrine and gene expression in different swine breeds. *Molecular Biology Reports*, *41*(6), 3569–3576. https://doi.org/10.1007/s11033-014-3219-1

Soria, B., Quesada, I., Ropero, A. B., Pertusa, J. A., Martín, F., & Nadal, A. (2004). Novel players in pancreatic islet signaling: from membrane receptors to nuclear channels. *Diabetes*, *53 Suppl 1*, S86-91. https://doi.org/10.2337/diabetes.53.2007.s86

Spurlock, M. E., & Gabler, N. K. (2008). The Development of Porcine Models of Obesity and the Metabolic Syndrome 1,2. In *The Journal of Nutrition Symposium: Animal Models in Nutrition Research J. Nutr* (Vol. 138).

Stevens, J. R., McMillan, R. P., Resendes, J. T., Lloyd, S. K., Ali, M. M., Frisard, M. I., Hargett, S., Keller, S. R., & Hulver, M. W. (2017). Acute low-dose endotoxin treatment results in improved whole-body glucose homeostasis in mice. *Metabolism: Clinical and Experimental*, *68*, 150–162. https://doi.org/10.1016/j.metabol.2016.12.008

Sugita, H., Kaneki, M., Tokunaga, E., Sugita, M., Koike, C., Yasuhara, S., Tompkins, R. G., Jeevendra Martyn, J. A., & Tomp-kins, R. G. (2002). *Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance*. https://doi.org/10.1152/ajpendo.00087.2001.-The

Sun, H., Huang, Y., Yin, C., Guo, J., Zhao, R., & Yang, X. (2016). Lipopolysaccharide markedly changes glucose metabolism and mitochondrial function in the longissimus muscle of pigs. *Animal*, *10*(7), 1204–1212. https://doi.org/10.1017/S1751731116000100

Suryawan, A., & Davis, T. A. (2018). Amino Acid- and insulin-induced activation of mTORC1 in neonatal piglet skeletal muscle involves Sestin2-GATOR2, Rag A/C-mTOR, and RHEB-mTOR complex formation. *Journal of Nutrition*, *148*(6), 825–833. https://doi.org/10.1093/jn/nxy044

Swindle, M. M., Makin, A., Herron, A. J., Clubb, F. J., & Frazier, K. S. (2012). Swine as Models in Biomedical Research and Toxicology Testing. *Veterinary Pathology*, *49*(2), 344–356. https://doi.org/10.1177/0300985811402846

Tanaka, H., Nishikawa, Y., Fukushima, T., Taniguchi, A., Fujita, Y., Tsuda, K., Inagaki, N., & Hosokawa, M. (2018). Lipopolysaccharide inhibits hepatic gluconeogenesis in rats: The role of immune cells. *Journal of Diabetes Investigation*, *9*(3), 494–504. https://doi.org/10.1111/jdi.12729

Terenina, E., Sautron, V., Ydier, C., Bazovkina, D., Sevin-Pujol, A., Gress, L., Lippi, Y., Naylies, C., Billon, Y., Liaubet, L., Mormede, P., & Villa-Vialaneix, N. (2017). Time course study of the response to LPS targeting the pig immune gene networks. *BMC Genomics*, *18*(1). https://doi.org/10.1186/s12864-017-4363-5

Tezuka, N., Brown, A. M. C., & Yanagawa, S. (2007). GRB10 binds to LRP6, the Wnt co-receptor and inhibits canonical Wnt signaling pathway. *Biochemical and Biophysical Research Communications*, *356*(3), 648–654. https://doi.org/10.1016/j.bbrc.2007.03.019

Thomas, J. W., Touchman, J. W., Blakesley, R. W., Bouffard, G. G., Beckstrom-Sternberg, S. M., Margulies, E. H., Blanchette, M., Siepel, A. C., Thomas, P. J., McDowell, J. C., Maskeri, B., Hansen, N. F., Schwartz, M. S., Weber, R. J., Kent, W. J., Karolchik, D., Bruen, T. C., Bevan, R., Cutler, D. J., … Green, E. D. (2003). Comparative analyses of multi-species sequences from targeted genomic regions. *Nature*, *424*(6950), 788–793. https://doi.org/10.1038/nature01858

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science (New York, N.Y.)*, *282*(5391), 1145–1147. https://doi.org/10.1126/science.282.5391.1145

Van Der Crabben, S. N., Blümer, R. M. E., Stegenga, M. E., Ackermans, M. T., Endert, E., Tanck, M. W. T., Serlie, M. J., Van Der Poll, T., & Sauerwein, H. P. (2009). Early endotoxemia increases peripheral and hepatic insulin sensitivity in healthy humans. *Journal of Clinical Endocrinology and Metabolism*, *94*(2), 463–468. https://doi.org/10.1210/jc.2008-0761

Vecchione, A., Marchese, A., Henry, P., Rotin, D., & Morrione, A. (2003). The Grb10/Nedd4 Complex Regulates Ligand-Induced Ubiquitination and Stability of the Insulin-Like Growth Factor I Receptor. *Molecular and Cellular Biology*, *23*(9), 3363–3372. https://doi.org/10.1128/mcb.23.9.3363-3372.2003

Waldron, M. R., Kulick, A. E., Bell, A. W., & Overton, T. R. (2006). Acute experimental mastitis is not causal toward the development of energy-related metabolic disorders in early postpartum dairy cows. *Journal of Dairy Science*, *89*(2), 596–610. https://doi.org/10.3168/jds.S0022-0302(06)72123-3

Waldron, M. R., Nishida, T., Nonnecke, B. J., & Overton, T. R. (2003). Effect of lipopolysaccharide on indices of peripheral and hepatic metabolism in lactating cows. *Journal of Dairy Science*, *86*(11), 3447–3459. https://doi.org/10.3168/jds.S0022-0302(03)73949-6

Wang, J., Dai, H., Yousaf, N., Moussaif, M., Deng, Y., Boufelliga, A., Rama Swamy, O., Leone, M. E., & Riedel, H. (1999). Grb10, a Positive, Stimulatory Signaling Adapter in Platelet-Derived Growth Factor BB-, Insulin-Like Growth Factor I-, and Insulin-Mediated Mitogenesis. In *MOLECULAR AND CELLULAR BIOLOGY* (Vol. 19, Issue 9).

Wang, L., Balas, B., Christ-Roberts, C. Y., Kim, R. Y., Ramos, F. J., Kikani, C. K., Li, C., Deng, C., Reyna, S., Musi, N., Dong, L. Q., DeFronzo, R. A., & Liu, F. (2007). Peripheral Disruption of the Grb10 Gene Enhances Insulin Signaling and Sensitivity In Vivo. *Molecular and Cellular Biology*, *27*(18), 6497–6505. https://doi.org/10.1128/mcb.00679-07

Wang, Y., Ding, X., Tan, Z., Xing, K., Yang, T., Pan, Y., Wang, Y., Mi, S., Sun, D., & Wang, C. (2018). Genome-wide association study for reproductive traits in a Large White pig population. *Animal Genetics*, *49*(2), 127–131. https://doi.org/10.1111/age.12638

Webel, D. M., Finck, B. N., Baker, D. H., & Johnson, R. W. (1997). *Time Course of Increased Plasma Cytokines, Cortisol, and Urea Nitrogen in Pigs Following Intraperitoneal Injection of Lipopolysaccharide 1*. https://academic.oup.com/jas/article/75/6/1514/4625013

Weekers, F., Giulietti, A. P., Michalaki, M., Coopmans, W., Van Herck, E., Mathieu, C., & Van Den Berghe, G. (2003). Metabolic, Endocrine, and Immune Effects of Stress Hyperglycemia in a Rabbit Model of Prolonged Critical Illness. *Endocrinology*, *144*(12), 5329–5338. https://doi.org/10.1210/en.2003-0697

Weiss, M., Steiner, D. F., & Philipson, L. H. (2014). *Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships*. MDText.com, INC. https://www.ncbi.nlm.nih.gov/books/NBK279029/

Wick, K. L. R., Werner, E. D., Langlais, P., Ramos, F. J., Dong, L. Q., Shoelson, S. E., & Liu, F. (2003). Grb10 inhibits insulin-stimulated insulin receptor substrate (IRS)-phosphatidylinositol 3-kinase/Akt signaling pathway by disrupting the association of

IRS-1/IRS-2 with the insulin receptor. *Journal of Biological Chemistry*, *278*(10), 8460–8467. https://doi.org/10.1074/jbc.M208518200

Wilcox, G. (2005). Insulin and Insulin Resistance. In *Clin Biochem Rev* (Vol. 26).

Wyns, H., Plessers, E., De Backer, P., Meyer, E., & Croubels, S. (2015). In vivo porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. In *Veterinary Immunology and Immunopathology* (Vol. 166, Issues 3–4, pp. 58–69). Elsevier. https://doi.org/10.1016/j.vetimm.2015.06.001

Yan, X., Himburg, H. A., Pohl, K., Quarmyne, M., Tran, E., Zhang, Y., Fang, T., Kan, J., Chao, N. J., Zhao, L., Doan, P. L., & Chute, J. P. (2016). Deletion of the Imprinted Gene Grb10 Promotes Hematopoietic Stem Cell Self-Renewal and Regeneration. *Cell Reports*, *17*(6), 1584–1594. https://doi.org/10.1016/j.celrep.2016.10.025

Yang, Y., Liang, G., Niu, G., Zhang, Y., Zhou, R., Wang, Y., Mu, Y., Tang, Z., & Li, K. (2017). Comparative analysis of DNA methylome and transcriptome of skeletal muscle in lean-, obese-, and mini-type pigs. *Scientific Reports*, *7*. https://doi.org/10.1038/srep39883

Yu, C., Chen, Y., Cline, G. W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J. K., Cushman, S. W., Cooney, G. J., Atcheson, B., White, M. F., Kraegen, E. W., & Shulman, G. I. (2002). Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *Journal of Biological Chemistry*, *277*(52), 50230–50236. https://doi.org/10.1074/jbc.M200958200

Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villén, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P., & Blenis, J. (2011). Phosphoproteomic analysis identifies

Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science*, *332*(6035), 1322–1326. https://doi.org/10.1126/science.1199484

Zhang, W., Kroscher, K. A., Murray, R. L., Gagliardi, R., Guiltinan, C., Rhoads, R. P., & Stahl, C. H. (2020). Dietary calcium and phosphorus amounts affect development and tissue-specific stem cell characteristics in neonatal pigs. *Journal of Nutrition*, *150*(5), 1086–1092. https://doi.org/10.1093/jn/nxaa011

Zhu, X.-Y., Ma, S., Eirin, A., Woollard, J. R., Hickson, L. J., Sun, D., Lerman, A., & Lerman, L. O. (2016). Functional Plasticity of Adipose-Derived Stromal Cells During Development of Obesity. *Stem Cells Translational Medicine*, *5*(7), 893–900. https://doi.org/10.5966/sctm.2015-0240