Summer 8-15-2013

# Type 1 Diabetes and the Male Reproductive Axis

Erica Schoeller
*Washington University in St. Louis*

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Molecular Genetics and Genomics

Dissertation Examination Committee:
Kelle H. Moley, Chair
Sanjay Jain
Liang Ma
Joan Riley
Tim Schedl
David Wilson

# Type 1 Diabetes and the Male Reproductive Axis

by

Erica Lee Schoeller

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2013

St. Louis, Missouri

**Table of Contents:**

**List of Figures:**

**Acknowledgements:**

I must first thank my advisor, Kelle Moley, for all her guidance over the years. I couldn't have asked for a more wonderful mentor, and I appreciate all her advice, patience, and encouragement. I kindly request that she keep the photo of me, Toni, and the monster truck displayed prominently in her office forever. I'd also like to thank all the members of my thesis committee for keeping me on track in graduate school and always being available, especially Joan Riley, whose unfortunate proximity to my bench led to innumerable opportunities for both experimental and life advice.

I'd also like to thank all the past and present member of the Moley lab, especially Maggie Chi, who has been in the lab since I started as a graduate student. Her kindness and helpfulness make the Moley lab a wonderful environment. I'd also like to thank the former graduate students, Lauren Flessner, Antonina Frolova, and Katie Adastra for welcoming me into the lab and helping with countless experiments, and well as all the other Moley lab members who truly are the best coworkers and friends. I'd also like to thank the program coordinators and directors for helping to develop such a great research community.

Thanks so much to all my St. Louis friends. You have all made graduate school an unforgettable time in my life and I'm grateful for the countless activities we've done over the past six years. Any group of friends that will outfit themselves in prom gear three years running to help celebrate a birthday is the best group of friends a girl could have. I've certainly been fortunate to have made some lifelong friends during graduate school.

Lastly, I need to thank my family for their support throughout graduate school. I am grateful to have a mother who will send cookies for no reason, and a father who will always answer his work phone to tell me why a dog's nose is cold (it was important at the time). I couldn't ask for better parents and I appreciate everything you do for me. My two older brothers have turned out to be pretty excellent human beings despite some rather "rough" childhood experiences (though I was at fault for more than a few of those). Thanks for turning out to be great brothers! Thanks to my Aunt Joy and Uncle Art who are wonderful people and I appreciate their love and support.

**ABSTRACT OF THE DISSERTATION**

Type 1 Diabetes and the Male Reproductive Axis

by

Erica Lee Schoeller

Doctor of Philosophy in Molecular Genetics and Genomics

Washington University in St. Louis, 2013

Kelle H. Moley, M.D., Chairperson

Glucose regulation is important in both spermatogenesis as well as mature sperm function. The impact of diabetes on sperm function is becoming increasingly clear, though much work is still needed to elucidate the mechanism by which diabetes impairs spermatogenesis. Diabetes causes disruptions in the hypothalamic pituitary gonadal (HPG) axis, the regulatory system that controls reproduction. The HPG axis functions by secreting GnRH pulses from the hypothalamus, which bind to receptors on the pituitary and cause secretion of follicle stimulating hormone and luteinizing hormone, which then bind to receptors in the testes and facilitate spermatogenesis. Type 1 diabetes can severely impact function of the HPG axis, leading to fertility defects. Using the Akita mouse model, a genetic model of type 1 diabetes, we show that in the absence of sufficient insulin, spermatogenesis is arrested and the mice are infertile. These mice have lower levels of luteinizing hormone, follicle stimulating hormone, and testosterone. Upon treatment with either insulin or leptin, spermatogenesis is restored, despite persistent levels of high blood glucose. We conclude that the lack of insulin and leptin during the pathogenesis of type 1 diabetes causes an arrest in spermatogenesis by abrogating function of the HPG axis. It is possible that insulin and leptin have redundant roles regulating reproductive function within the

hypothalamus, and a mechanism for the precise molecular targets of both insulin and leptin that restore HPG axis function remains to be determined.

We additionally found that insulin secretion occurs within the testes in addition to that secreted by the pancreas. Insulin protein was localized to the Sertoli, or "nurse", cell of the testes, which is the cell responsible for providing hormones and nutrients for sperm cells as they develop from spermatogonia to spermatozoa. Additionally, we determined the presence of glucose transporter 4 (GLUT4) on the mature sperm cell. As this is an insulin responsive transporter, this provides the potential for insulin regulation of sperm cells. We show the critical nature of both GLUT4 and the basal glucose transporter, GLUT1, in glucose uptake and fertilization capacity of sperm cells. These findings demonstrate the importance of hexose utilization to sperm cell competence, and further identification and characterization of glucose transporters in sperm cells will clarify the role of hexose metabolism during sperm cell maturation and fertilization reactions.

# Chapter 1: Introduction

A portion of this introduction was published as a review in the journal *Cell and Tissue Research.*

**The effects of type 1 diabetes on the hypothalamic, pituitary and testes axis.** Schoeller EL, Schon S, Moley KH. Cell Tissue Res. 2012 Sep;349(3):839-47. doi: 10.1007/s00441-012-1387-7. Epub 2012 Apr 15. Review.

# PART I: Glucose Transporters in Spermatozoa

## Spermatogenesis

Spermatogenesis is the process by which germ cells in the testes (spermatogonial stem cells) divide and mature into spermatids, which can then be released from the testes to the epididymis, where they acquire motility, and then to the vas deferens where they can be released from the male tract. Upon entry into the female tract, sperm cells undergo rapid metabolic changes, collectively termed "capacitation", which prepare the sperm cells to reach the oocyte and penetrate the cumulus cells surrounding the oocyte and the zona pellucida to allow entry and fertilization. Spermatogenesis and fertilization are complex processes, dependent on hormonal regulation as well as proper energy metabolism. Mature haploid sperm cells differentiate from a population of spermatogonial stem cells. These cells can divide into either Type A spermatogonia, which can renew the stem cell population, or Type B spermatogonia, which are destined to differentiate into mature sperm cells. Spermatogonia mitotically divide into primary spermatocytes, which then undergo meiosis I to become secondary spermatocytes and then meiosis II to become round spermatids. These cells then elongate to form mature spermatozoa, which are then released into the lumen of the seminiferous tubule and exit the testis **(Fig 1-1)**. This process is dependent on nutrient support from Sertoli cells, the "nurse cells" of the testis, and on the hypothalamic pituitary gonadal axis, which integrates hormonal signals from the central nervous system to promote gonadal function.

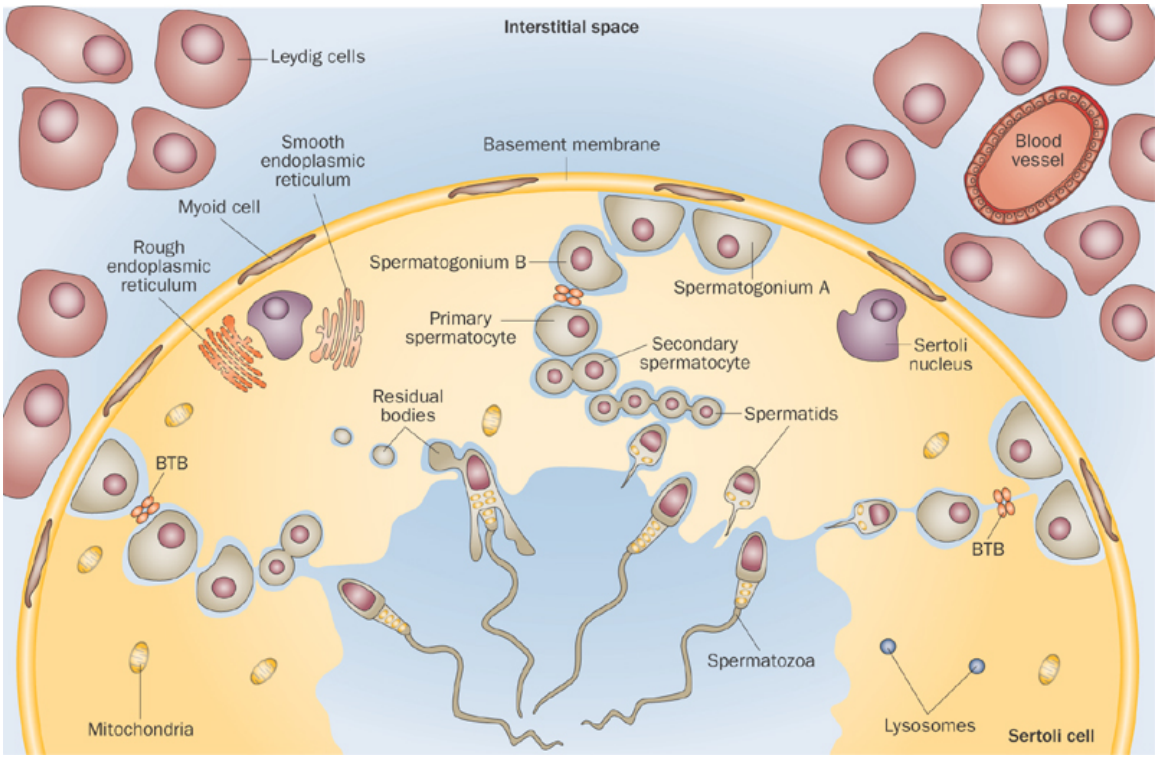



Figure 1-1: Schematic representation of spermatogenesis.

Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Urology] (Rato et al., 2012), copyright (2012).

The metabolism of sperm cells dramatically changes upon exit from the male tract and entry into the female tract. During spermatogenesis, developing sperm cells utilize primary lactate provided to them by the Sertoli cells of the testis (Rato et al., 2012). As sperm cells are released from the testis, they enter the cauda epididymis, where they further mature and acquire motility. Here they use glucose and lactate as energy substrates (Voglmayr & White, 1979). As sperm cells mix with seminal fluid, they encounter a high percentage of glucose and fructose (Martikainen, Sannikka, Suominen, & Santti, 1980). Finally, upon entry into the female tract, sperm cells utilize glucose,

fructose, and lactate, and these cells encounter environmental changes that trigger changes in metabolism and motility (Hamner, 1973). These changes in substrate availability are coupled with changes in energy metabolism as sperm cells adapt to various microenvironments.

## Capacitation

Sperm cells are not competent for fertilization immediately following ejaculation. In order for sperm cells to successfully penetrate the zona pellucida, sperm cells must acquire progressive (forward) motility, achieve hyperactivation, and undergo acrosomal exocytosis. As sperm cells enter the female tract, they undergo a set of reactions that allow the sperm to develop fertilization competence, collectively termed "capacitation". Capacitation is induced by the presence of serum albumin, calcium, and sodium bicarbonate that are present in the female uterine fluid, as well as the energy substrates pyruvate, lactate, and glucose (Visconti et al., 2002). Capacitation is characterized by increased membrane fluidity, increased tyrosine phosphorylation, increased pH levels, increased intracellular cAMP, and calcium influx.

## Hyperactivation

During capacitation, sperm cells also undergo a process known as hyperactivation, which is a specific motility pattern characterized by wild flagellar beating and an asymmetrical beating pattern **(Fig 1-2)**. This distinct motility pattern is hypothesized to assist the sperm cell in traversing the viscous uterine fluid and penetrating the cumulus cells layer and zona pellucida of the oocyte. Hyperactivation, and consequently, fertilization, are not achieved in the absence of

glucose, highlighting the importance of glycolytic pathways in mature spermatozoa (Travis et al., 2001). Hyperactivation is induced by the activation of sperm-specific ion channels, Catsper and Ksper (Slo3) (Lishko et al., 2012) that result in a calcium influx into the cell. More recently, progesterone was identified as one of the important signals from the cumulus cells to trigger sperm activation (Bronson, Peresleni, & Golightly, 1999; Jensen & Publicover, 2012) The concentration of progesterone can be in the micromolar range near the site of fertilization. Progesterone is thought to induce hyperactivation by activating the Catsper ion channels and triggering a calcium influx that precedes hyperactivation. Indeed, mice with a genetic knockout of any of the four known Catsper channels lack the ability to undergo hyperactivation (Carlson et al., 2005; Qi et al., 2007; Quill et al., 2003; Ren et al., 2001) These mice are infertile, highlighting the importance of hyperactivation in sperm function. The cumulative processes of hyperactivation and capacitation are thought to prepare the sperm cells for the exocytosis of the acrosomal cap, which releases hydrolytic enzymes to help penetrate the zona pellucida (Yamagata et al., 1998).

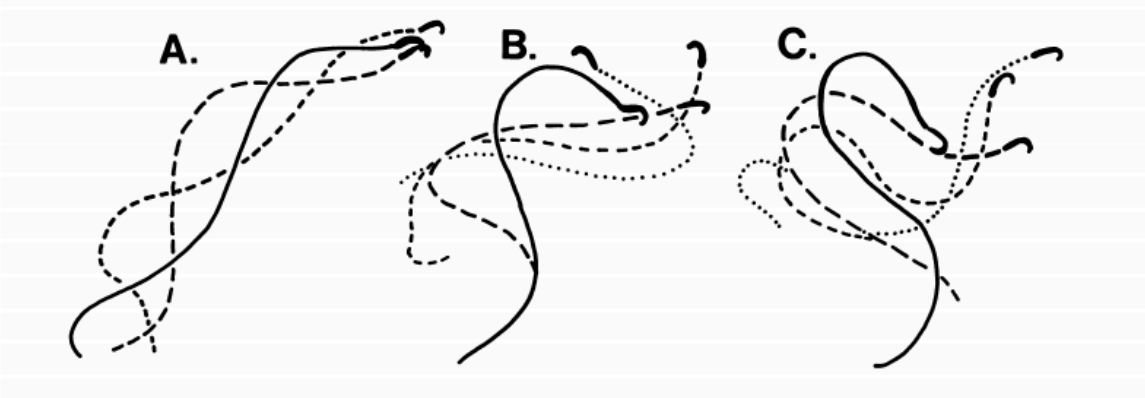



Figure 1-2: Hyperactivation. A) Capacitated sperm cell prior to hyperactivated motility. B) Transitional sperm cell. C) Hyperactivated sperm cell. (Suarez, Katz, Owen, Andrew, & Powell, 1991)

## Acrosome Reaction

Capacitation is a prerequisite for the induction of the acrosome reaction, a process where the cap of the mature sperm cell is exocytosed, releasing acrosin and other enzymes that help to digest the cumulus cells and break through the zona pellucida (Vigil, Orellana, & Cortes, 2011). The acrosome reaction is induced by stimuli including sperm binding to the zona pellucida, where compounds on the surface such as zona pellucida glycoprotein 3 (ZP3) can induce the acrosome reaction, or by progesterone, which is a hormone secreted by the cumulus cells (Florman, Jungnickel, & Sutton, 2008). Both ZP3 and progesterone function by increasing the calcium entry into the sperm cell. Progesterone activates the Catsper channels, causing a membrane depolarization and subsequent calcium influx, and ZP3, causes a similar calcium influx, mediated by G-protein activation and a pH increase (Florman et al., 2008; O'Toole, Arnoult, Darszon, Steinhardt, & Florman, 2000). The presence of glucose, but not pyruvate, promotes induction of the acrosome reaction, highlighting the importance of glucose metabolism to generate energy for this process (Fraser & Quinn, 1981).

## Hexose Utilization in Sperm

Sperm cells are notable in that they utilize different sources of energy throughout their development. During spermatogenesis in the testes, germ cells use primarily lactate, which is provided to them by the Sertoli, or “nurse cells” of the testes (Rato et al., 2012). Sertoli cells metabolize glucose obtained from the bloodstream in the interstitium into lactate using lactate dehydrogenase. Spermatogonia are also in contact with the interstitium of the testis, and have access to the blood supply and utilize glucose as an energy substrate. Spermatocytes and

spermatids are beyond the blood testes barrier, and acquire energy through the lactate provided to them by the Sertoli cells. Spermatocytes and spermatids are not viable when cultured with glucose alone, and must be supplemented with either lactate or pyruvate to generate ATP (Jutte, Grootegoed, Rommerts, & van der Molen, 1981; Nakamura, Okinaga, & Arai, 1984). Spermatocytes have a greater enzyme activity for glycolytic and pentose phosphate pathway enzymes than spermatids, which rely mainly on the TCA cycle. Once mature, spermatozoa have a large glycolytic potential and thus switch to metabolizing glucose and fructose (Bajpai, Gupta, & Setty, 1998). The reason for this switch in metabolism is unclear, but the ability to switch metabolism may be critical to the adaptability of sperm cells to their various environments.

**Glucose Transporter Expression in Spermatozoa**

Sperm activation is associated with metabolic changes in the sperm cell, and glucose transporter expression may be critical during these processes. Sperm cells need to adapt their metabolic needs during capacitation and the acrosome reaction and require energy from ATP to support this increase in motility and acrosomal exocytosis. Spermatozoa preferentially use glucose as an energy substrate (Leese, Astley, & Lambert, 1981) and this entry of glucose into cells is regulated by a family of facilitative glucose transporters (GLUTs). GLUTs are a family of 14 proteins that have 12 membrane-spanning domains and are able to move hexoses and other substrates across a cell membrane. Each GLUT has a unique substrate affinity and specificity and each cell type has a unique set of glucose transporters. Glucose transporter characterization in sperm cells is incomplete and further investigation in these cells is warranted, as they are critical in understanding sperm cell metabolism. (Augustin, 2010; Thorens & Mueckler, 2010)

**Glucose Transporter Function**

Glucose transporters are divided into three classes. Class 1 includes GLUTs 1-5 and these are known as the classical glucose transporters. They are named so because they were the first GLUTs to be discovered and thus are the most well studied. GLUT1 is expressed ubiquitously and is highly expressed in erythrocytes and in the endothelial and epithelial barriers of the brain and placenta. GLUT1 transports mainly glucose but also galactose, mannose and glucosamine (Carruthers, DeZutter, Ganguly, & Devaskar, 2009). GLUT2 is expressed in the pancreas, liver, kidney, and intestine. This glucose transporter is known for transporting glucose but it is also a glucose sensor important in regulating insulin secretion by the pancreas. It transports glucose as well as mannose and glucosamine (Im et al., 2005; Thorens, Wu, Leahy, & Weir, 1992). GLUT3 is expressed highly in the brain and testes and also in the placenta and preimplantation embryo. It has a high affinity and capacity for glucose but also transports galactose, mannose, maltose, xylose, and dehydroascorbic acid (Simpson et al., 2008). GLUT4 is expressed in muscle, heart, and adipose tissue and is primary known for its ability to translocate from intracellular compartments to the plasma membrane upon insulin-stimulation. GLUT4 transports glucose as well as dehydroascorbic acid and glucosamine. (Huang & Czech, 2007)GLUT14 is a newly discovered GLUT that is specifically expressed in testes. It is the result of a gene duplication of GLUT3 and is 95% homologous to GLUT3 at the nucleotide level (Wu & Freeze, 2002).

Class II glucose transporters, known as the “odd” glucose transporters include GLUTs 5, 7, 9, and 11. These GLUTs are known for their ability to transport fructose. GLUT5 is expressed

mainly in the intestine with lower expression in the kidney, brain, skeletal muscle, and adipose tissue. GLUT5 is primarily a fructose transporter with little affinity for glucose (Douard & Ferraris, 2008). GLUT7 is expressed in the small intestine and colon, and mRNA has been detected in the testes and prostate. GLUT7 has a high affinity for glucose and fructose, but does not transport galactose, 2-DG, or xylose (Cheeseman, 2008). GLUT9 is expressed primarily in the kidney and liver and also to the beta cells of the pancreas. GLUT9 transports glucose and fructose with a high affinity, and also transports uric acid (Augustin et al., 2004; Doblado & Moley, 2009). GLUT11 has three splice variants. GLUT11A is expressed in heart, skeletal muscle and kidney, GLUT11B is expressed in placenta, adipose tissue and kidney, and GLUT11C is expressed in adipose tissue, heart, muscle, and pancreas. GLUT11 transports glucose and fructose but not galactose (Doege et al., 2001; Scheepers et al., 2005).

Class III glucose transporters include GLUT6, GLUT8, GLUT10, GLUT12 and HMIT. Class III GLUTs are notable for their intracellular localization motif. GLUT6 is expressed in the brain, spleen and leukocytes and transports glucose, though the kinetic data is limited (Lisinski, Schurmann, Joost, Cushman, & Al-Hasani, 2001). GLUT8 is expressed primarily in the testes and brain, with lower levels in the adrenal, spleen, brown adipose tissue, and lung. GLUT8 has a high affinity for glucose, but also transports fructose and galactose. Insulin-stimulated translocation has been reported in blastocysts and sperm cells, but not adipocytes, CHO-cells or neuronal cell types (Carayannopoulos et al., 2000; Schmidt, Joost, & Schurmann, 2009). GLUT10 mRNA has been detected in heart, liver, brain, lung, muscle, pancreas, kidney, and placenta. This GLUT has a high affinity for 2-DG and can also transport glucose and galactose. GLUT12 is primarily expressed in the muscle, heart, small intestine and prostate (Dawson et al.,

2001). GLUT12 can transport glucose, fructose, and galactose, and like GLUT4, GLUT12 is also capable of insulin-responsive translocation to the plasma membrane. (Stuart, Howell, Zhang, & Yin, 2009). HMIT is expressed primarily in the brain, with lower expression detected in the adipose tissue and kidneys. Though HMIT contains all the important sequence motifs responsible for glucose transport activity, this GLUT does not appear to transport any sugars, but instead transports inositol-3-phosphate (Uldry et al., 2001).

**Glucose Transporters in Sperm Cells**

Though the data on glucose transporters in sperm is far from complete, because of the importance of glycolysis to sperm cell function, much work has been done to characterize which glucose transporters are present in sperm cells. This work has mainly focused on the classical glucose transporters (I-IV), though more recent work shows important roles for GLUT8 and GLUT9. Expression of GLUTs 1, 2, 3, 5, 8, and 9 have been reported in sperm cells.

A subset of glucose transporters have been examined in a variety of species of sperm cells. In 1998, Angulo et al. (Angulo et al., 1998) examined the presence of GLUTs 1-5 in human, rat and bull sperm cells. Glut1 was detected in the acrosome of the head in human sperm, and in the head and principal piece of the tail in the rat and bull. GLUT2 was detected in the acrosome of human and rat sperm, and displayed weak expression in the head and midpiece of bull sperm. GLUT3 was expressed in the post equatorial region of the head, the midpiece, and the principal piece of human sperm, the midpeice and faintly in the principal piece in rat sperm, and the head and midpiece of bull sperm. GLUT5 was detected in the subequatorial region and the tail in

human, and in the head and midpiece in both rat and bull sperm. GLUT4 was not detected in human, bull, or rat sperm.

In 2002, Rigau et al studied GLUTs 3 and 5 in dog sperm and examined the effects of various hexoses on motility and APT content (Rigau et al., 2002). GLUT3 was detected in the tail of the sperm while GLUT5 was detected in the acrosome. Incubation of sperm cells with glucose increased cellular APT content, but not as fast or as high as incubation with fructose. A separate group also identified GLUT3 in boar sperm, where it was detected on the membranes of the acrosome and midpiece (Medrano et al., 2006). They also observed an increase in hexokinase activity and intracellular ATP and glucose-6-phosphate levels upon incubation with glucose, indicating an intense activation of glycolysis, possibly mediated by membrane-bound GLUT3. Sancho et al. showed GLUT3 and GLUT5 expression in boar sperm, with GLUT3 localizing strongly to the acrosome and GLUT 5 localizing to the acrosome, midpiece and principal piece (Sancho et al., 2007).

In 2010, Bucci et al. reexamined the localization of GLUTs 1-5 in dog, stallion, donkey, and boar spermatozoa. GLUT1 again localized to the acrosome and the tail. GLUT2 localized to the acrosome. GLUT3 localized to the acrosome and tail in human and boar and just to the tail of dog spermatozoa. GLUT5 localized to the acrosome and the tail of all species examined. This time, the authors examined redistribution of glucose transporters in fresh, capacitated, and acrosome-reacted sperm. Only in dog spermatozoa did the authors observe a change in localization after capacitation, with GLUT1 localizing more intensely to the acrosome, GLUT2 decreasing in the midpiece and increasing in the acrosome, GLUT3 localizing more intensely to

the tail and the acrosome, and GLUT5 localizing more intensely to the tail(Bucci et al., 2010). Differences between the studies of glucose transporter localization in sperm could be due to various fixation techniques and possibly to incubation conditions that may alter localization patterns.

GLUT8 has also been extensively studied in sperm cells. It has been localized to the acrosomal region of type 1 spermatocytes. In mature sperm cells, GLUT8 localizes to the acrosome (Schurmann, Axer, Scheepers, Doege, & Joost, 2002). There is debate over whether GLUT8 is an insulin-responsive transporter. GLUT8 translocation to the acrosome in the presence of insulin has been demonstrated by confocal microscopy (Lampiao, 2010). Within the testis, GLUT8 is localized to intracellular compartments within the forming acrosome (Gomez, Romero, Terrado, & Mesonero, 2006). Despite high expression of GLUT8 within the testis, GLUT8 knockout mice are viable and fertile, though GLUT8 knockout mice have lower testicular ATP levels, lower sperm motility, and lower mitochondrial membrane potential, indicating that GLUT8 is important for energy metabolism in sperm cells. (Gawlik et al., 2008). Interestingly, a recent proteome of mature spermatozoa did not detect GLUT8, despite detecting significant levels of GLUT3 and GLUT5 (Chauvin et al., 2012). It is possible that GLUT 8 is expressed more highly during spermatogenesis than in mature spermatozoa.

GLUT 9 is also expressed in spermatozoa. GLUT9a and GLUT9b are both expressed in the principal piece of caudal sperm cells. Interestingly, GLUT9a is absent from the sperm cells of type 1 diabetic Akita mice, a mouse model resulting from a mutation in the ins2 gene that encodes for insulin. Akita mice also have poor sperm quality and fertilize fewer oocytes than

wild type, potentially due to this abnormal glucose transporter expression (Kim & Moley, 2008). This link between glucose transporter expression and fertility is important, and will be addressed further in chapter 4. It appears that diabetes may affect sperm quality in two separate ways, a direct effect though energy metabolism in the testes and sperm, or through altering hormones important in spermatogenesis through perturbations in the hypothalamic pituitary gonadal testis axis.

## Introduction Part II: Type 1 Diabetes and the Hypothalamic Pituitary Gonadal Axis

### Type 1 diabetes and Fertility

Type 1 diabetes is an autoimmune disorder characterized by a lack of insulin production by the beta cells of the pancreas (Yoon & Jun, 2005). This lack of insulin causes a variety of systemic effects on whole body metabolism. Poorly managed type 1 diabetes can lead to cardiovascular disease, diabetic neuropathy, and diabetic retinopathy (Barber, 2003; Said, 2007; Sowers, Epstein, & Frohlich, 2001; Vinik et al., 1992). Increasingly, even well managed type 1 diabetic patients show damage to peripheral organs related to complications from the disease. Insulin's central role in energy homeostasis renders it an important signaling factor in the reproductive tract as well. Type 1 diabetes has recently been shown to cause defects in sperm and testes, and the aim of this review is to present the known role and effect of insulin on the male reproductive tract. Although fertility complications also occur in type 2 diabetic males, this review will focus on the defects specifically linked with the lack of insulin associated with type 1 diabetes.

The link between type 1 diabetes and fertility has long been established. Accounts dating as far back as the 11$^{th}$ century have described the disease as, "a collapse of sexual functions", highlighting the importance of insulin in the reproductive system. While current understanding of the mechanism of this subfertility has greatly progressed since the 11$^{th}$ century, it is yet

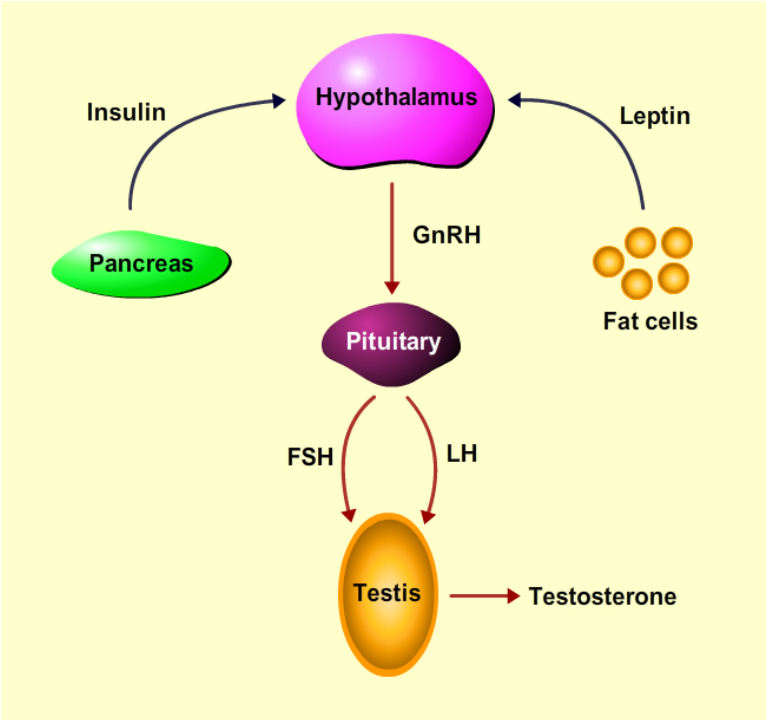
unclear whether the damage to sperm is due to local effects from hyperglycemia or by alterations in hormone levels which disrupt the hypothalamic pituitary gonadal axis.

Diabetes is associated with reduced sperm parameters in affected males. The recent discovery that both the testes and sperm produce insulin brings a new perspective on how diabetes may contribute to subfertility. Indeed, insulin expression in the testes also seems to be affected by diabetes, with streptozotocin-induced diabetic rats expressing less than half of the insulin protein compared to nondiabetic controls (Gomez et al., 2009). This suggests that insulin may have an important role in spermatogenesis. In addition to the testes, sperm cells have also been shown to contain both insulin mRNA and protein (Aquila, Gentile, Middea, Catalano, & Ando, 2005). These cells are activated by insulin to induce pAKT phosphorylation, suggesting a functional role in insulin signaling. Additionally, these cells have been shown to secrete insulin in response to glucose administration. These contributions open a new avenue of research into the functions of insulin in the reproductive tract as the specific role of insulin in the process of spermatogenesis and sperm motility and/or capacitation has not been determined.

.

In a normally functioning hypothalamic pituitary gonadal axis, the hypothalamus releases GnRH pulses that stimulate the pituitary to secrete both luteinizing hormone (LH) and follicle stimulating hormone (FSH) (**Fig. 1-3).** LH and FSH act on the Sertoli cells and the Leydig cells, respectively, to stimulate the process of spermatogenesis. The onset of type 1 diabetes is known to disrupt the HPG axis, resulting in impaired spermatogenesis and subsequent subfertility.

Figure 1-3: The hypothalamic pituitary gonadal axis




Disruptions in any part of the HPG axis impair fertility, and this review will focus on how these disruptions lead to infertility. Specifically, we will focus on the effects of type 1 Diabetes on: 1) insulin and leptin levels, 2) GnRH pulses from the hypothalamus, 3) LH and FSH secretion from the pituitary, 4) testosterone secretion from the Leydig cells, and 5) sperm quality. Additionally, we will address the possibility of local insulin signaling within the testes and how type 1 diabetes may locally affect the gonads.

## Insulin/leptin Effects on the Hypothalamic Pituitary Gonadal Axis

### Insulin levels mediate the function of the HPG axis

Serum insulin has long been known to affect the central nervous system, and these effects could mediate whole body energy homeostasis, including the reproductive axis through further signaling to the pituitary and ultimately, the gonads. A study in 1977 by (Porte, Baskin, & Schwartz, 2005) showed that peripheral insulin injection caused an increase in insulin levels in

cerebral spinal fluids, suggesting that insulin could potentially be a signal to the brain regarding energy stores and promoting whole-body energy homeostasis. Thus, a lack of insulin would signal to the brain a lack of energy supply and the central nervous system could potentially shut down extraneous energy-consuming processes such as reproductive function.

Indeed, insulin levels have dramatic effects on the regulation of the HPG axis. The effects of diabetes on the reproductive axis are mediated at least in part, by signaling in the brain. Insulin mediates its effects through binding with the insulin receptor, resulting in a signaling cascade. Through interactions with the insulin receptor substrate proteins, notably IRS-2, insulin potentiates signaling through PI 3-kinase, which then activates AKT, an important mediator of energy signaling (Boura-Halfon & Zick, 2009).

Insulin signaling in the brain can happen at multiple sites. Insulin receptor expression has been detected in the hypothalamus, the olfactory bulb, and the pituitary (Havrankova, Brownstein, & Roth, 1981). Additionally, insulin concentrations in the brain are markedly higher than plasma insulin levels, suggesting that insulin in the brain is not simply a reflection of serum levels but that there is a critical function of this signaling hormone in the central nervous system(Havrankova, Schmechel, Roth, & Brownstein, 1978).

Exactly how insulin in the brain impacts changes in the reproductive axis is unknown. An experiment using a brain-specific insulin receptor knockout revealed the connection between insulin signaling in the brain and fertility. The development of a neuron-specific insulin receptor knockout mouse (NIRKO) resulted in subfertile male mice. NIRKO mice display a significant

reduction in fertility, with 46% of male mating resulting in offspring versus 76% for controls. Male NIRKO mice display impaired spermatogenesis, which may account for this decline in fecundity. Histological examination revealed that although many of the seminiferous tubules appeared normal, about 20% did not have a lumen and had little or no mature sperm cells. Additionally, the Leydig cells appeared shrunken, suggesting that the lack of proper insulin signaling in the brain reduced the hormonal output necessary to retain the Leydig cell population to successfully promote spermatogenesis in all tubules. Also, there was a 60% reduction in circulating LH, indicating that the lack of insulin response in the brain reduces hypothalamic-pituitary axis function (Bruning et al., 2000). Neither LH nor FSH was measured in these animals, though the shrunken Leydig cells and low LH levels suggest a testosterone deficiency.

Other studies examining the mechanism of insulin signaling in the brain have found that insulin signaling in the brain is required for inhibition of glucose production. Injection of insulin directly into the brain resulted in decreased production of glucose independently of serum insulin levels. Injection of insulin signaling inhibitors resulted in an increased glucose production, despite circulating serum insulin levels. This study further demonstrates that neuronal insulin action is regulated separately from plasma insulin and may be involved in overall energy homeostasis mediated by the central nervous system. (Obici, Zhang, Karkanias, & Rossetti, 2002)

Defects in the IRS signaling proteins can also have detrimental effects on fertility, since the insulin receptor substrate proteins are activated by the binding of insulin to its receptor. IRS-2 knockout males develop severe glucose intolerance and inadequate carbohydrate metabolism. Despite being initially fertile, IRS-2 knockout mice become infertile after the onset of diabetes (Burks et al., 2000). This suggests that it is the diabetic state and not the lack of IRS-2 that is

responsible for the impairment in male reproductive capacity. Therefore, any number of hormones or glucose response pathways could be responsible for the subfertility in IRS-2 knockout males. IRS-2 protein may also mediate its effects through signaling to the brain. IRS-2 protein has been localized to the AR hypothalamus along with insulin receptor. Activation with insulin results in tyrosine phosphorylation of IRS-2 corresponding to activation of PI3-kinase, indicating that insulin signaling via the (Porte et al., 2005)

**Insulin levels are correlated to leptin levels**

Insulin's effects on the reproductive axis are not solely mediated by insulin interactions with receptors in the brain. Insulin levels are also known to directly correlate to circulating levels of leptin, an important molecule involved in maintaining energy homeostasis. Leptin is an important hormone secreted by the fat cells that signals to the hypothalamus and also regulates the reproductive system. Leptin serves as a metabolic signal that informs the brain of nutritional status and provides information regarding an animal's ability to meet the energy demands of reproduction (Barash et al., 1996).

**Type 1 diabetic males have decreased leptin levels, correlating to HPG axis dysfunction**

In type 1 diabetes, the decreased insulin levels can also affect leptin levels. Insulin has been shown *in vitro* to have direct effects on leptin synthesis. (Barr, Malide, Zarnowski, Taylor, & Cushman, 1997; Cammisotto & Bukowiecki, 2002; Wabitsch et al., 1996). In fat cells, administration of insulin promotes an increase in leptin production *in vitro*. *In vivo*, long-term

exposure to hyperinsulinemia promoted an increase in circulating leptin levels (Kolaczynski et al., 1996). However, this effect was not seen instantaneously, but only in the final 24 hours of the study, suggesting that the hypothalamic leptin response to insulin is an indirect mechanism acting through adipose cells. In humans, leptin levels also are affected by type 1 diabetes. Leptin levels were decreased in newly diagnosed type 1 diabetic patients before the administration of insulin treatment, but these levels normalized after the onset of insulin treatment (Azar, Zalloua, Zantout, Shahine, & Salti, 2002). In another study of newly diagnosed children with type 1 diabetes, leptin levels were low prior to insulin treatment, and became elevated after only one day of insulin therapy. This leads to the conclusion that serum leptin is not simply a readout of body fat stores, but that it is regulated by insulin levels as well. (Hanaki, Becker, & Arslanian, 1999)

Further studies have established that both insulin and leptin potentiate insulin signaling in the brain. Injections of either insulin or leptin into the intracerebroventricular region of the brain elicited overlapping but distinct signaling mechanisms. Both hormones were able to affect the IRS and PI3-kinase signaling pathways, while insulin alone resulted in the phosphorylation of AKT serine. (Carvalheira et al., 2005)

**Uncontrolled type 1 diabetes is associated with decreased leptin levels**

It now seems clear that leptin plays an important role in the development of type 1 diabetes. Decreased levels of leptin are associated with insulin resistance and other markers of metabolic syndrome, suggesting an interplay between the two molecules. (German et al., 2010)

Additionally, decreased leptin levels in streptozotocin-induced diabetic Wistar rats contributes to insulin resistance and metabolic syndrome prior to the development of hyperglycemia.(German et al., 2010) Another study showed that leptin therapy alone in a nonobese diabetic mouse restored normal blood glucose levels as well as reversed the catabolic state of the mice.(Wang et al., 2010). These data suggest that leptin effects may be downstream of insulin effects on metabolism, and thus restoring leptin levels in type 1 diabetic males may be able to reverse many of the effects insulin deficiency.

**Disturbances in leptin signaling cause subfertility**

Through interactions with the hypothalamus, leptin signaling regulates fertility. Both the ob/ob and the db/db mouse models, which lack the leptin protein and the leptin receptor protein, respectively, exemplify the central role of leptin in the HPG axis. Both the ob/ob mouse and the db/db mouse have impaired fertility, and this defect is rescued by the administration of exogenous leptin. However, the defect in fertility is not restored with food restriction, suggesting that it is the lack of leptin, and not obesity, that is responsible for this infertility (Mounzih, Lu, & Chehab, 1997). Ob/ob males without leptin treatment have smaller testes, reduced numbers of sperm within the seminiferous tubules, and also have shrunken Leydig cells, indicative of impaired steroidogenesis. (Fujikawa, Chuang, Sakata, Ramadori, & Coppari, 2010). Another study found that free testosterone levels were not altered in the leptin deficient ob/ob mice. (Bhat et al., 2006) The authors speculated that this may be because the leptin deficient mice produce a binding protein, which lowers free biologically active testosterone.

Leptin may mediate its effects by direct interactions with the hypothalamus. Leptin receptors are found in the rat and mouse brain, notably in the hypothalamus as well as in the pituitary, indicating a potential for leptin regulation at both sites. Injections of leptin directly into the rat hypothalamus stimulate the release of GnRH, suggesting that leptin directly acts within the hypothalamus to stimulate gonadotropin-releasing hormone secretion *in vivo* (Watanobe, 2002). Another study found that leptin indirectly regulates the GnRH neurons (Quennell et al., 2009). The authors deleted the leptin receptor either in the forebrain or in the GnRH neurons. Male infertility resulted only from deletion in the forebrain, suggesting that there is an indirect mechanistic relationship between leptin and GnRH pulses. These experiments highlight the importance of leptin signaling to the brain for normal reproductive function.

Leptin receptors are also expressed in the testes, suggesting that leptin secreted by fat cells can directly signal to the gonads (Zamorano et al., 1997). Leptin receptor expression in the testes seems to be stage-specific, indicating a role in spermatogenesis. Expression is restricted to spermatocytes in stage IX and X of the testes, corresponding to the time just after sperm release (El-Hefnawy, Ioffe, & Dym, 2000). The authors postulate that mature sperm may suppress leptin receptor expression through a negative feedback loop that prevents expression of the receptor in the other spermatogenic stages. Thus, disruptions in leptin production during the pathogenesis of diabetes may impact the gonadal axis at multiple levels, through signaling to the hypothalamus affecting GnRH pulses, through signaling to the pituitary affecting LH and FSH secretion, and through direct interactions with developing sperm cells in the testes.

## Fertility Effects of Deficiency in the Hypothalamic Pituitary Gonadal Axis

**GnRH signaling from the hypothalamus is impaired in type 1 diabetic males**

Both leptin and insulin are known to interact with the hypothalamus to ultimately regulate the output of GnRH from the hypothalamus. These GnRH pulses subsequently affect hormones important in spermatogenesis, notably luteinizing hormone and follicle stimulating hormone. Follicle stimulating hormone acts on the Sertoli cells of the testes to stimulate germ cell progression through mitosis and entry into meiosis (O'Shaughnessy, Monteiro, Verhoeven, De Gendt, & Abel, 2010; Selice, Ferlin, Garolla, Caretta, & Foresta, 2011). Luteinizing hormone acts on the Leydig cells of the testes to stimulate testosterone synthesis (Amory & Bremner, 2003). Disruption of the hypothalamic pituitary gonadal axis at any point in this hormonal loop results in a disruption of spermatogenesis.

A human study by Baccetti et al. attempted to examine the function of the HPG axis in diabetic men by measuring LH and FSH secretion in response to GnRH as well as TEM microscopy of sperm cells to look for abnormalities. The authors found that LH secretion in response to GnRH pulses was lower in diabetic men than in healthy controls ($48 \pm 8$ versus $59 \pm 10$ mIU/ml; $P < 0.05$). Upon examination of sperm ultrastructure, sperm from diabetic patients had a greater percentage of abnormally shaped acrosomes (Baccetti et al., 2002), Another group found that impotent diabetic males had a lower LH response to GnRH pulses (Zeidler et al., 1982). LH secretion in response to GnRH administration was also shown to be impaired in type 1 diabetic men in a study conducted by (Lopez-Alvarenga et al., 2002). This study examined young diabetic men without systemic complications of diabetes for an average of 3.7 years. The authors

found that patients with poorly controlled diabetes had lower endogenous LH pulses as well as a decreased LH response to pulsatile GnRH administration. The authors concluded that the lack of LH response stemmed from the acute effects of diabetes including hypoinsulinemia and hyperglycemia and not from long-term systemic complications of the disease. These data together suggest that the pathogenesis of type 1 diabetes affects the hypothalamic pituitary axis resulting in decreased LH secretion. The low LH response may have negative implications on steroidogenesis in the Leydig cells, resulting in impaired spermatogenesis.

**Disruptions in follicle hormone signaling impair spermatogenesis**

Disturbances in the HPG axis impact both LH and FSH levels, thereby impairing spermatogenesis. Scientists have attempted to isolate the roles of these hormones by developing mouse models lacking either FSH or its receptor. Mice lacking the FSH receptor (FORKO mice) have underdeveloped testes and a 50% reduction in the number of Sertoli cells. (Sairam & Krishnamurthy, 2001). FORKO mice also show a decreased seminiferous tubule diameter and reduced sperm count corresponding to low testosterone levels (Krishnamurthy, Danilovich, Morales, & Sairam, 2000). This mouse model also displays a delay in puberty, suggesting that FSH signaling is important in the onset of spermatogenesis (Krishnamurthy, Babu, Morales, & Sairam, 2001). These mice remain fertile, however, suggesting that FSH is required for normal spermatogenesis but not critical for fertility. In humans, mutations in the FSH or its receptor are associated with fertility abnormalities. Three males with FSH beta mutations have been described, all exhibiting subfertility including symptoms of small testes and azoospermia. Mutations in the FSH receptor resulted in low sperm counts or low sperm volume, but none of these patients were infertile. (Meduri et al., 2008)

**Disturbances in LH signaling impair late stages of spermatogenesis**

Luteinizing hormone is necessary for spermatogenesis to properly occur. A study in men who were gonadotropin deficient found that LH replacement restored spermatogenesis (Matsumoto, Paulsen, & Bremner, 1984) In this same study, after azoospermia was achieved via exogenous testosterone administration, treatment with LH resulted in a significant increase in sperm concentration, although not to normal levels.

Mouse models of gonadotropin deficiency have provided more insight into the importance and function of LH in spermatogenesis. Two different groups have made an LH receptor knockout mouse model. (Lei et al., 2001; Zhang, Poutanen, Wilbertz, & Huhtaniemi, 2001). Both groups found that LH-receptor null mice had reduced numbers of Leydig cells and spermatogenesis was arrested at the round spermatid stage. Testosterone replacement therapy in these mice resulted in the completion of spermatogenesis, though the Leydig cell population remained low in number and size. Mice treated with testosterone beginning at 30 days of age remained infertile(Lei et al., 2001), however, when mice were treated with high doses of testosterone beginning at 21 days of age, partial fertility was restored (7 out of 78 matings)(Pakarainen, Zhang, Makela, Poutanen, & Huhtaniemi, 2005). This suggests that FSH alone is able to support the Sertoli cell population and the early stages of spermatogenesis, but that LH is required for the progression and/or maintenance of a mature sperm cell population.

Similar findings have been demonstrated in mice lacking the luteinizing hormone beta-subunit. These mice are completely infertile, with reduced testes size and reduced or absent amounts of the hormones involved in the biosynthesis of testosterone. Additionally, the seminiferous tubules

of these mice contain spermatogonia, spermatocytes, and round spermatids, but they lack any elongated and late-stage spermatids (Ma, Dong, Matzuk, & Kumar, 2004). Both human and mouse data suggests that LH is critical to the completion of normal spermatogenesis through stimulation of Leydig cell steroidogenesis.

**Testosterone levels are decreased in uncontrolled type 1 diabetic males**

Several studies have evaluated the production of testosterone in diabetic men. A study by (M. Maneesh, 2006)found that men with diabetes had reduced testosterone levels compared to age matched control males, while LH and FSH levels remained similar. Another study found that free testosterone levels were decreased while total testosterone levels were similar in type 1 diabetic men versus control males. The authors also demonstrated that LH levels were not different while FSH levels were slightly increased (van Dam et al., 2003). A study of newly diagnosed type 1 diabetic patients revealed that plasma testosterone concentrations were decreased in diabetic patients but returned to normal levels after four days of insulin treatment (Gluud, Madsbad, Krarup, & Bennett, 1982). Similarly, in type 1 diabetic patients whose androgen levels were measured before and after insulin withdrawal. While testosterone levels were similar before insulin withdrawal, testosterone levels decreased below control significantly after 4 hours of insulin withdrawal and remained lower throughout the 12-hour study (Madsbad, Gluud, Bennett, & Krarup, 1986). Together, this data suggests that testosterone levels are impaired by type 1 diabetes in poorly controlled subjects, which could inhibit the process of spermatogenesis.

**Diabetes is associated with poor sperm quality**

Diabetes mellitus is associated with spermatogenic defects in afflicted males as a result, at least in part, of the known perturbations in the HPG axis. The characterization of these spermatogenic defects is an area of active research as new technology for assaying the quality of sperm cells continues to develop. Rather recently, researchers have found that men with diabetes have sperm with significantly higher amounts of DNA damage (Agbaje et al., 2007). Additionally, sperm from diabetic men were shown to have higher DNA fragmentation as well as an increase in RAGE, a receptor protein important in the oxidative stress response (Karimi, Goodarzi, Tavilani, Khodadadi, & Amiri, 2011; Mallidis et al., 2007). Additionally, sperm from type 1 diabetic men have an increased number of mitochondrial DNA deletions as well as an increase in sperm nuclear DNA fragmentation (Agbaje et al., 2007). Sperm from diabetic men also correlates to decreased embryo quality. *In vitro* fertilization using sperm from diabetic men produced far fewer pregnancies compared to controls. However, two-cell embryo fertilization rates from using sperm from diabetic men are not impaired(Mulholland, Mallidis, Agbaje, & McClure, 2011). This indicates that the sperm cells are able to fertilize embryo but there may be DNA damage that prevents competent embryo development. Additionally, work in both streptozotocin-induced and genetically diabetic Akita mice showed that sperm from these mice produced fewer 2-cell embryos, and of those 2-cell embryos, fewer developed successfully to blastocyst stage. This suggests that the severe diabetes in these type I models affects sperm competence, thereby impairing proper embryo development (Kim & Moley, 2008).

A study by (Ballester et al., 2004) sought to establish the linkage between diabetes and fertility

by examining a type 1 diabetic rat model for defects in testicular signaling. The authors treated male Wistar rats with a single dose of streptozotocin (70mg/kg), rendering them diabetic. After 3 months, many of the rats were infertile, had smaller testes and decreased numbers of Leydig cells. Additionally, the serum levels of LH, FSH and testosterone were all decreased. The authors suggested an indirect mechanism between insulin and LH, as they did not find a significant correlation between serum insulin and LH levels. This study shows an important relationship between type 1 diabetes and pituitary hormone signaling to the testes leading to infertility.

**Insulin also may act directly in the testes to regulate spermatogenesis**

In addition to disturbances in the HPG axis, recent data has demonstrated the potential for local hormonal signaling within the testes. Identification of insulin, leptin, adiponectin, and resistin, in particular, provide a framework for

Aside from its effect on the HPG axis, insulin may also have a direct role in the testes and spermatogenesis. Recent research has identified insulin expression in the both the testes and sperm (Gomez et al., 2009). Insulin mRNA and protein expression were detected in human sperm cells and these cells respond to increasing concentrations of glucose with increasing secretion of insulin. (Aquila et al., 2005)Additionally, sperm cells released more insulin upon capacitation, the process by which sperm acquire the ability to fertilize. This provides a potential mechanism by which insulin might have an autocrine role in sperm cells, which could serve to mediate sperm maturation and/or fertilization events.

Additionally, insulin transcripts and protein have been detected in the testes, raising the possibility that insulin may interact directly with receptors in the testes to promote the process of spermatogenesis. Detection of insulin in control and diabetic rat testes had also been established, with lower levels of insulin protein in diabetic testes(Gomez et al., 2009). Histological examination localized insulin to the spermatids and Leydig cells. Additionally, the insulin receptor was localized to the cytoplasmic droplets of elongating spermatids just prior to release into the lumen (Gomez et al., 2009). Together, these studies demonstrate the importance of local insulin signaling within the testes in addition to systemic effects, although the specific role has not yet been determined. A study of cultured cells derived from chicken testes showed that incubation of testicular cells with insulin increased proliferation, as demonstrated by the incorporation of 3H-thymidine into the cells. This indicates that insulin may have a mitogenic effect in the testes (Bobes, Castro, Miranda, & Romano, 2001). The presence of insulin and its receptor has implications on the pathogenesis of diabetes, as the hypoinsulinemia associated with type 1 diabetes may directly interfere with insulin signaling at the level of the testes.

It is also important to note that other signaling molecules in addition to insulin have recently been identified within the testes, including resistin and adiponectin. This leads to the possibility of a local signaling network that could potentially regulate testis function. Adiponectin, an adipocyte hormone important in glucose homeostasis, has been detected in rat testes and localized to the interstitial Leydig cells (Caminos et al., 2008). In this same study, adiponectin was shown to decrease testosterone secretion on testicular tissue, displaying an important role for adiponectin in local regulation of spermatogenesis.

Resistin is another adipocyte hormone recently identified in testis. Resistin is a hormone involved in insulin sensitivity whose levels increase during in type 1 diabetic patients(Shalev, Patterson, Hirshberg, Rother, & Harlan, 2004). This hormone has recently been identified in the Leydig cells, and, to a lesser degree, the Sertoli cells of the testis (Nogueiras et al., 2004). Within the testis, resistin has been shown to decrease in response to fasting as well as central leptin administration. Additionally, testicular sections incubated with resistin increased production of testosterone, demonstrating the potential role of resistin as a regulator of spermatogenesis.

Hormone levels likely affect spermatogenesis on multiple levels, both through the HPG axis as well as through local signaling within the testis, However, the direct involvement of these hormones on spermatogenesis within the testes is still not thoroughly defined. Although type 1 diabetes is known to affect serum hormone levels of adiponectin, leptin, resistin, and insulin, a clear connection between diabetes and these testicular hormone levels remains to be established.

**Concluding Remarks:** Disruptions in the hypothalamic pituitary gonadal axis have severe reproductive consequences. Type 1 diabetes can impact many aspects of the functional axis, resulting in subfertility. Low insulin levels attributable to type 1 diabetes lead to decreased leptin levels, leading to decreased GnRH secretion, and subsequent decreased in LH and FSH signaling to the testes. However, it is probable that not all of the diabetic outcomes on fertility are mediated through the HPG axis, but also by the detrimental effects of hyperglycemia and oxidative DNA damage to the testes and sperm cells. Additionally, the presence of insulin transcripts in testes and sperm brings up the possibility that insulin signaling may be important within the testes and may play a part in the diabetic pathogenesis of infertility. Additional work

in the field is necessary to establish the precise role of direct insulin signaling in the testes and sperm cells, and to determine whether diabetes has an impact on this local signaling. Identification of the cell types in the testis responsible for insulin signaling will help to clarify the potential role of insulin in the gonads. Additionally, an insulin or insulin receptor knockout specifically in the testis will allow the distinction between systemic insulin deficiency and testis-specific insulin deficiency.

**References:**


**Agbaje, I. M., Rogers, D. A., McVicar, C. M., McClure, N., Atkinson, A. B., Mallidis, C., & Lewis, S. E. (2007). Insulin dependant diabetes mellitus: implications for male reproductive function. *Hum Reprod, 22*(7), 1871-1877. doi: 10.1093/humrep/dem077**

**Amory, J. K., & Bremner, W. J. (2003). Regulation of testicular function in men: implications for male hormonal contraceptive development. *J Steroid Biochem Mol Biol, 85*(2-5), 357-361. doi: S096007600300205X [pii]**

**Angulo, C., Rauch, M. C., Droppelmann, A., Reyes, A. M., Slebe, J. C., Delgado-Lopez, F., . . . Concha, II. (1998). Hexose transporter expression and function in mammalian spermatozoa: cellular localization and transport of hexoses and vitamin C. *J Cell Biochem, 71*(2), 189-203.**

**Aquila, S., Gentile, M., Middea, E., Catalano, S., & Ando, S. (2005). Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology, 146*(2), 552-557. doi: 10.1210/en.2004-1252**

**Augustin, R. (2010). The protein family of glucose transport facilitators: It's not only about glucose after all. *IUBMB Life, 62*(5), 315-333. doi: 10.1002/iub.315**

**Augustin, R., Carayannopoulos, M. O., Dowd, L. O., Phay, J. E., Moley, J. F., & Moley, K. H. (2004). Identification and characterization of human glucose transporter-like protein-9 (GLUT9): alternative splicing alters trafficking. *J Biol Chem, 279*(16), 16229-16236. doi: 10.1074/jbc.M312226200**

**Azar, S. T., Zalloua, P. A., Zantout, M. S., Shahine, C. H., & Salti, I. (2002). Leptin levels in patients with type 1 diabetes receiving intensive insulin therapy compared with those in patients receiving conventional insulin therapy. *J Endocrinol Invest, 25*(8), 724-726. doi: 5887 [pii]**

**Baccetti, B., La Marca, A., Piomboni, P., Capitani, S., Bruni, E., Petraglia, F., & De Leo, V. (2002). Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality. *Hum Reprod, 17*(10), 2673-2677.**

**Bajpai, M., Gupta, G., & Setty, B. S. (1998). Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. *Eur J Endocrinol, 138*(3), 322-327.**

**Ballester, J., Munoz, M. C., Dominguez, J., Rigau, T., Guinovart, J. J., & Rodriguez-Gil, J. E. (2004). Insulin-dependent diabetes affects testicular function by FSH- and LH-linked mechanisms. *J Androl, 25*(5), 706-719.**

**Barash, I. A., Cheung, C. C., Weigle, D. S., Ren, H., Kabigting, E. B., Kuijper, J. L., . . . Steiner, R. A. (1996). Leptin is a metabolic signal to the reproductive system. *Endocrinology, 137*(7), 3144-3147.**

**Barber, A. J. (2003). A new view of diabetic retinopathy: a neurodegenerative disease of the eye. *Prog Neuropsychopharmacol Biol Psychiatry, 27*(2), 283-290. doi: S0278-5846(03)00023-X [pii]**

**10.1016/S0278-5846(03)00023-X**

**Barr, V. A., Malide, D., Zarnowski, M. J., Taylor, S. I., & Cushman, S. W. (1997). Insulin stimulates both leptin secretion and production by rat white adipose tissue. *Endocrinology, 138*(10), 4463-4472.**

**Bhat, G. K., Sea, T. L., Olatinwo, M. O., Simorangkir, D., Ford, G. D., Ford, B. D., & Mann, D. R. (2006). Influence of a leptin deficiency on testicular morphology, germ cell apoptosis, and expression levels of apoptosis-related genes in the mouse.** ***J Androl, 27*****(2), 302-310. doi: 10.2164/jandrol.05133**

**Bobes, R. J., Castro, J. I., Miranda, C., & Romano, M. C. (2001). Insulin modifies the proliferation and function of chicken testis cells.** ***Poult Sci, 80*****(5), 637-642.**

**Boura-Halfon, S., & Zick, Y. (2009). Phosphorylation of IRS proteins, insulin action, and insulin resistance.** ***Am J Physiol Endocrinol Metab, 296*****(4), E581-591. doi: 10.1152/ajpendo.90437.2008**

**Bronson, R. A., Peresleni, T., & Golightly, M. (1999). Progesterone promotes the acrosome reaction in capacitated human spermatozoa as judged by flow cytometry and CD46 staining.** ***Mol Hum Reprod, 5*****(6), 507-512.**

**Bruning, J. C., Gautam, D., Burks, D. J., Gillette, J., Schubert, M., Orban, P. C., . . . Kahn, C. R. (2000). Role of brain insulin receptor in control of body weight and reproduction.** ***Science, 289*****(5487), 2122-2125.**

**Bucci, D., Isani, G., Spinaci, M., Tamanini, C., Mari, G., Zambelli, D., & Galeati, G. (2010). Comparative immunolocalization of GLUTs 1, 2, 3 and 5 in boar, stallion and dog spermatozoa.** ***Reprod Domest Anim, 45*****(2), 315-322. doi: 10.1111/j.1439-0531.2008.01307.x**

**Burks, D. J., Font de Mora, J., Schubert, M., Withers, D. J., Myers, M. G., Towery, H. H., . . . White, M. F. (2000). IRS-2 pathways integrate female reproduction and energy homeostasis.** ***Nature, 407*****(6802), 377-382. doi: 10.1038/35030105**

**Caminos, J. E., Nogueiras, R., Gaytan, F., Pineda, R., Gonzalez, C. R., Barreiro, M. L., . . . Tena-Sempere, M. (2008). Novel expression and direct effects of adiponectin in the rat testis.** ***Endocrinology, 149*****(7), 3390-3402. doi: en.2007-1582 [pii]**
**10.1210/en.2007-1582**

**Cammisotto, P. G., & Bukowiecki, L. J. (2002). Mechanisms of leptin secretion from white adipocytes.** ***Am J Physiol Cell Physiol, 283*****(1), C244-250. doi: 10.1152/ajpcell.00033.2002**

**Carayannopoulos, M. O., Chi, M. M., Cui, Y., Pingsterhaus, J. M., McKnight, R. A., Mueckler, M., . . . Moley, K. H. (2000). GLUT8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst.** ***Proc Natl Acad Sci U S A, 97*****(13), 7313-7318.**

**Carlson, A. E., Quill, T. A., Westenbroek, R. E., Schuh, S. M., Hille, B., & Babcock, D. F. (2005). Identical phenotypes of CatSper1 and CatSper2 null sperm.** ***J Biol Chem, 280*****(37), 32238-32244. doi: 10.1074/jbc.M501430200**

**Carruthers, A., DeZutter, J., Ganguly, A., & Devaskar, S. U. (2009). Will the original glucose transporter isoform please stand up!** ***Am J Physiol Endocrinol Metab, 297*****(4), E836-848. doi: 10.1152/ajpendo.00496.2009**

**Carvalheira, J. B., Torsoni, M. A., Ueno, M., Amaral, M. E., Araujo, E. P., Velloso, L. A., . . . Saad, M. J. (2005). Cross-talk between the insulin and leptin signaling systems in rat hypothalamus.** ***Obes Res, 13*****(1), 48-57. doi: 13/1/48 [pii]**
**10.1038/oby.2005.7**

**Chauvin, T., Xie, F., Liu, T., Nicora, C. D., Yang, F., Camp, D. G., 2nd, . . . Roberts, K. P. (2012). A systematic analysis of a deep mouse epididymal sperm proteome.** ***Biol Reprod, 87*****(6), 141. doi: 10.1095/biolreprod.112.104208**

**Cheeseman, C. (2008). GLUT7: a new intestinal facilitated hexose transporter. *Am J Physiol Endocrinol Metab, 295*(2), E238-241. doi: 10.1152/ajpendo.90394.2008**

**Dawson, P. A., Mychaleckyj, J. C., Fossey, S. C., Mihic, S. J., Craddock, A. L., & Bowden, D. W. (2001). Sequence and functional analysis of GLUT10: a glucose transporter in the Type 2 diabetes-linked region of chromosome 20q12-13.1. *Mol Genet Metab, 74*(1-2), 186-199. doi: 10.1006/mgme.2001.3212**

**Doblado, M., & Moley, K. H. (2009). Facilitative glucose transporter 9, a unique hexose and urate transporter. *Am J Physiol Endocrinol Metab, 297*(4), E831-835. doi: 10.1152/ajpendo.00296.2009**

**Doege, H., Bocianski, A., Scheepers, A., Axer, H., Eckel, J., Joost, H. G., & Schurmann, A. (2001). Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. *Biochem J, 359*(Pt 2), 443-449.**

**Douard, V., & Ferraris, R. P. (2008). Regulation of the fructose transporter GLUT5 in health and disease. *Am J Physiol Endocrinol Metab, 295*(2), E227-237. doi: 10.1152/ajpendo.90245.2008**

**El-Hefnawy, T., Ioffe, S., & Dym, M. (2000). Expression of the leptin receptor during germ cell development in the mouse testis. *Endocrinology, 141*(7), 2624-2630.**

**Florman, H. M., Jungnickel, M. K., & Sutton, K. A. (2008). Regulating the acrosome reaction. *Int J Dev Biol, 52*(5-6), 503-510. doi: 10.1387/ijdb.082696hf**

**Fraser, L. R., & Quinn, P. J. (1981). A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. *J Reprod Fertil, 61*(1), 25-35.**

**Fujikawa, T., Chuang, J. C., Sakata, I., Ramadori, G., & Coppari, R. (2010). Leptin therapy improves insulin-deficient type 1 diabetes by CNS-dependent mechanisms in mice. *Proc Natl Acad Sci U S A, 107*(40), 17391-17396. doi: 10.1073/pnas.1008025107**

**Gawlik, V., Schmidt, S., Scheepers, A., Wennemuth, G., Augustin, R., Aumuller, G., . . . Schurmann, A. (2008). Targeted disruption of Slc2a8 (GLUT8) reduces motility and mitochondrial potential of spermatozoa. *Mol Membr Biol, 25*(3), 224-235. doi: 10.1080/09687680701855405**

**German, J. P., Wisse, B. E., Thaler, J. P., Oh, I. S., Sarruf, D. A., Ogimoto, K., . . . Morton, G. J. (2010). Leptin deficiency causes insulin resistance induced by uncontrolled diabetes. *Diabetes, 59*(7), 1626-1634. doi: 10.2337/db09-1918**

**Gluud, C., Madsbad, S., Krarup, T., & Bennett, P. (1982). Plasma testosterone and androstenedione in insulin dependent patients at time of diagnosis and during the first year of insulin treatment. *Acta Endocrinol (Copenh), 100*(3), 406-409.**

**Gomez, O., Ballester, B., Romero, A., Arnal, E., Almansa, I., Miranda, M., . . . Terrado, J. (2009). Expression and regulation of insulin and the glucose transporter GLUT8 in the testes of diabetic rats. *Horm Metab Res, 41*(5), 343-349. doi: 10.1055/s-0028-1128146**

**Gomez, O., Romero, A., Terrado, J., & Mesonero, J. E. (2006). Differential expression of glucose transporter GLUT8 during mouse spermatogenesis. *Reproduction, 131*(1), 63-70. doi: 10.1530/rep.1.00750**

**Hamner, Charles E. (1973). *Sperm capacitation*. New York,: MSS Information Corp.**
**Hanaki, K., Becker, D. J., & Arslanian, S. A. (1999). Leptin before and after insulin therapy in children with new-onset type 1 diabetes. *J Clin Endocrinol Metab, 84*(5), 1524-1526.**
**Havrankova, J., Brownstein, M., & Roth, J. (1981). Insulin and insulin receptors in rodent brain. *Diabetologia, 20 Suppl*, 268-273.**
**Havrankova, J., Schmechel, D., Roth, J., & Brownstein, M. (1978). Identification of insulin in rat brain. *Proc Natl Acad Sci U S A, 75*(11), 5737-5741.**
**Huang, S., & Czech, M. P. (2007). The GLUT4 glucose transporter. *Cell Metab, 5*(4), 237-252. doi: 10.1016/j.cmet.2007.03.006**
**Im, S. S., Kang, S. Y., Kim, S. Y., Kim, H. I., Kim, J. W., Kim, K. S., & Ahn, Y. H. (2005). Glucose-stimulated upregulation of GLUT2 gene is mediated by sterol response element-binding protein-1c in the hepatocytes. *Diabetes, 54*(6), 1684-1691.**
**Jensen, M. B., & Publicover, S. J. (2012). Progesterone and CatSper dependency. *Int J Androl, 35*(5), 631-632. doi: 10.1111/j.1365-2605.2012.01294.x**
**Jutte, N. H., Grootegoed, J. A., Rommerts, F. F., & van der Molen, H. J. (1981). Exogenous lactate is essential for metabolic activities in isolated rat spermatocytes and spermatids. *J Reprod Fertil, 62*(2), 399-405.**
**Karimi, J., Goodarzi, M. T., Tavilani, H., Khodadadi, I., & Amiri, I. (2011). Increased receptor for advanced glycation end products in spermatozoa of diabetic men and its association with sperm nuclear DNA fragmentation. *Andrologia*. doi: 10.1111/j.1439-0272.2011.01178.x**
**Kim, S. T., & Moley, K. H. (2008). Paternal effect on embryo quality in diabetic mice is related to poor sperm quality and associated with decreased glucose transporter expression. *Reproduction, 136*(3), 313-322. doi: 10.1530/REP-08-0167**
**Kolaczynski, J. W., Nyce, M. R., Considine, R. V., Boden, G., Nolan, J. J., Henry, R., . . . Caro, J. F. (1996). Acute and chronic effects of insulin on leptin production in humans: Studies in vivo and in vitro. *Diabetes, 45*(5), 699-701.**
**Krishnamurthy, H., Babu, P. S., Morales, C. R., & Sairam, M. R. (2001). Delay in sexual maturity of the follicle-stimulating hormone receptor knockout male mouse. *Biol Reprod, 65*(2), 522-531.**
**Krishnamurthy, H., Danilovich, N., Morales, C. R., & Sairam, M. R. (2000). Qualitative and quantitative decline in spermatogenesis of the follicle-stimulating hormone receptor knockout (FORKO) mouse. *Biol Reprod, 62*(5), 1146-1159.**
**Lampiao, Du Plessis. (2010). insulin stimulates glut8 expression in human spermatozoa. *J Biosci Tech, 1*(2), 90-93.**
**Leese, H. J., Astley, N. R., & Lambert, D. (1981). Glucose and fructose utilization by rat spermatozoa within the uterine lumen. *J Reprod Fertil, 61*(2), 435-437.**
**Lei, Z. M., Mishra, S., Zou, W., Xu, B., Foltz, M., Li, X., & Rao, C. V. (2001). Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Mol Endocrinol, 15*(1), 184-200.**
**Lishko, P. V., Kirichok, Y., Ren, D., Navarro, B., Chung, J. J., & Clapham, D. E. (2012). The control of male fertility by spermatozoan ion channels. *Annu Rev Physiol, 74*, 453-475. doi: 10.1146/annurev-physiol-020911-153258**

**Lisinski, I., Schurmann, A., Joost, H. G., Cushman, S. W., & Al-Hasani, H. (2001). Targeting of GLUT6 (formerly GLUT9) and GLUT8 in rat adipose cells. *Biochem J, 358*(Pt 2), 517-522.**

**Lopez-Alvarenga, J. C., Zarinan, T., Olivares, A., Gonzalez-Barranco, J., Veldhuis, J. D., & Ulloa-Aguirre, A. (2002). Poorly controlled type I diabetes mellitus in young men selectively suppresses luteinizing hormone secretory burst mass. *J Clin Endocrinol Metab, 87*(12), 5507-5515.**

**M. Maneesh, * H. Jayalakshmi, ** T A Singh** Amit Chakrabarti***. (2006). IMPAIRED HYPOTHALAMIC-PITUITARY-GONADAL AXIS FUNCTION IN MEN WITH DIABETES MELLITUS. *Indian Journal of Clinical Biochemistry,, 21*(1), 165-168.**

**Ma, X., Dong, Y., Matzuk, M. M., & Kumar, T. R. (2004). Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proc Natl Acad Sci U S A, 101*(49), 17294-17299. doi: 0404743101 [pii]**

**10.1073/pnas.0404743101**

**Madsbad, S., Gluud, C., Bennett, P., & Krarup, T. (1986). Rapid changes in plasma androgens during insulin withdrawal in male type 1 (insulin-dependent) diabetics. *J Endocrinol Invest, 9*(1), 21-25.**

**Mallidis, C., Agbaje, I., Rogers, D., Glenn, J., McCullough, S., Atkinson, A. B., . . . McClure, N. (2007). Distribution of the receptor for advanced glycation end products in the human male reproductive tract: prevalence in men with diabetes mellitus. *Hum Reprod, 22*(8), 2169-2177. doi: dem156 [pii]**

**10.1093/humrep/dem156**

**Martikainen, P., Sannikka, E., Suominen, J., & Santti, R. (1980). Glucose content as a parameter of semen quality. *Arch Androl, 5*(4), 337-343.**

**Matsumoto, A. M., Paulsen, C. A., & Bremner, W. J. (1984). Stimulation of sperm production by human luteinizing hormone in gonadotropin-suppressed normal men. *J Clin Endocrinol Metab, 59*(5), 882-887.**

**Medrano, A., Garcia-Gil, N., Ramio, L., Montserrat Rivera, M., Fernandez-Novell, J. M., Ramirez, A., . . . Rodriguez-Gil, J. E. (2006). Hexose-specificity of hexokinase and ADP-dependence of pyruvate kinase play important roles in the control of monosaccharide utilization in freshly diluted boar spermatozoa. *Mol Reprod Dev, 73*(9), 1179-1194. doi: 10.1002/mrd.20480**

**Meduri, G., Bachelot, A., Cocca, M. P., Vasseur, C., Rodien, P., Kuttenn, F., . . . Misrahi, M. (2008). Molecular pathology of the FSH receptor: new insights into FSH physiology. *Mol Cell Endocrinol, 282*(1-2), 130-142. doi: 10.1016/j.mce.2007.11.027**

**Mounzih, K., Lu, R., & Chehab, F. F. (1997). Leptin treatment rescues the sterility of genetically obese ob/ob males. *Endocrinology, 138*(3), 1190-1193.**

**Mulholland, J., Mallidis, C., Agbaje, I., & McClure, N. (2011). Male diabetes mellitus and assisted reproduction treatment outcome. *Reprod Biomed Online, 22*(2), 215-219. doi: 10.1016/j.rbmo.2010.10.005**

**Nakamura, M., Okinaga, S., & Arai, K. (1984). Metabolism of pachytene primary spermatocytes from rat testes: pyruvate maintenance of adenosine triphosphate level. *Biol Reprod, 30*(5), 1187-1197.**

**Nogueiras, R., Barreiro, M. L., Caminos, J. E., Gaytan, F., Suominen, J. S., Navarro, V. M., . . . Tena-Sempere, M. (2004). Novel expression of resistin in rat testis: functional role and regulation by nutritional status and hormonal factors. *J Cell Sci, 117*(Pt 15), 3247-3257. doi: 10.1242/jcs.01196**

**117/15/3247 [pii]**

**O'Shaughnessy, P. J., Monteiro, A., Verhoeven, G., De Gendt, K., & Abel, M. H. (2010). Effect of FSH on testicular morphology and spermatogenesis in gonadotrophin-deficient hypogonadal mice lacking androgen receptors. *Reproduction, 139*(1), 177-184. doi: REP-09-0377 [pii]**

**10.1530/REP-09-0377**

**O'Toole, C. M., Arnoult, C., Darszon, A., Steinhardt, R. A., & Florman, H. M. (2000). Ca(2+) entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. *Mol Biol Cell, 11*(5), 1571-1584.**

**Obici, S., Zhang, B. B., Karkanias, G., & Rossetti, L. (2002). Hypothalamic insulin signaling is required for inhibition of glucose production. *Nat Med, 8*(12), 1376-1382. doi: 10.1038/nm798**

**nm798 [pii]**

**Pakarainen, T., Zhang, F. P., Makela, S., Poutanen, M., & Huhtaniemi, I. (2005). Testosterone replacement therapy induces spermatogenesis and partially restores fertility in luteinizing hormone receptor knockout mice. *Endocrinology, 146*(2), 596-606. doi: en.2004-0913 [pii]**

**10.1210/en.2004-0913**

**Porte, D., Jr., Baskin, D. G., & Schwartz, M. W. (2005). Insulin signaling in the central nervous system: a critical role in metabolic homeostasis and disease from C. elegans to humans. *Diabetes, 54*(5), 1264-1276.**

**Qi, H., Moran, M. M., Navarro, B., Chong, J. A., Krapivinsky, G., Krapivinsky, L., . . . Clapham, D. E. (2007). All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proc Natl Acad Sci U S A, 104*(4), 1219-1223. doi: 10.1073/pnas.0610286104**

**Quennell, J. H., Mulligan, A. C., Tups, A., Liu, X., Phipps, S. J., Kemp, C. J., . . . Anderson, G. M. (2009). Leptin indirectly regulates gonadotropin-releasing hormone neuronal function. *Endocrinology, 150*(6), 2805-2812. doi: 10.1210/en.2008-1693**

**Quill, T. A., Sugden, S. A., Rossi, K. L., Doolittle, L. K., Hammer, R. E., & Garbers, D. L. (2003). Hyperactivated sperm motility driven by CatSper2 is required for fertilization. *Proc Natl Acad Sci U S A, 100*(25), 14869-14874. doi: 10.1073/pnas.2136654100**

**Rato, L., Alves, M. G., Socorro, S., Duarte, A. I., Cavaco, J. E., & Oliveira, P. F. (2012). Metabolic regulation is important for spermatogenesis. *Nat Rev Urol, 9*(6), 330-338. doi: 10.1038/nrurol.2012.77**

**Ren, D., Navarro, B., Perez, G., Jackson, A. C., Hsu, S., Shi, Q., . . . Clapham, D. E. (2001). A sperm ion channel required for sperm motility and male fertility. *Nature, 413*(6856), 603-609. doi: 10.1038/35098027**

**Rigau, T., Rivera, M., Palomo, M. J., Fernandez-Novell, J. M., Mogas, T., Ballester, J., . . . Rodriguez-Gil, J. E. (2002). Differential effects of glucose and fructose on hexose metabolism in dog spermatozoa. *Reproduction, 123*(4), 579-591.**

**Said, G. (2007). Diabetic neuropathy--a review. *Nat Clin Pract Neurol, 3*(6), 331-340. doi: ncpneuro0504 [pii]**
**10.1038/ncpneuro0504**
**Sairam, M. R., & Krishnamurthy, H. (2001). The role of follicle-stimulating hormone in spermatogenesis: lessons from knockout animal models. *Arch Med Res, 32*(6), 601-608.**
**Sancho, S., Casas, I., Ekwall, H., Saravia, F., Rodriguez-Martinez, H., Rodriguez-Gil, J. E., . . . Bonet, S. (2007). Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs. *Reproduction, 134*(1), 111-121. doi: 10.1530/REP-07-0118**
**Scheepers, A., Schmidt, S., Manolescu, A., Cheeseman, C. I., Bell, A., Zahn, C., . . . Schurmann, A. (2005). Characterization of the human SLC2A11 (GLUT11) gene: alternative promoter usage, function, expression, and subcellular distribution of three isoforms, and lack of mouse orthologue. *Mol Membr Biol, 22*(4), 339-351. doi: 10.1080/09687860500166143**
**Schmidt, S., Joost, H. G., & Schurmann, A. (2009). GLUT8, the enigmatic intracellular hexose transporter. *Am J Physiol Endocrinol Metab, 296*(4), E614-618. doi: 10.1152/ajpendo.91019.2008**
**Schurmann, A., Axer, H., Scheepers, A., Doege, H., & Joost, H. G. (2002). The glucose transport facilitator GLUT8 is predominantly associated with the acrosomal region of mature spermatozoa. *Cell Tissue Res, 307*(2), 237-242. doi: 10.1007/s00441-001-0499-2**
**Selice, R., Ferlin, A., Garolla, A., Caretta, N., & Foresta, C. (2011). Effects of endogenous FSH on normal human spermatogenesis in adults. *Int J Androl, 34*(6 Pt 2), e511-517. doi: 10.1111/j.1365-2605.2010.01134.x**
**Shalev, A., Patterson, N. B., Hirshberg, B., Rother, K. I., & Harlan, D. M. (2004). Resistin serum levels in type 1 diabetes pre- and post-islet transplantation. *Metabolism, 53*(4), 403-404. doi: S0026049503005456 [pii]**
**Simpson, I. A., Dwyer, D., Malide, D., Moley, K. H., Travis, A., & Vannucci, S. J. (2008). The facilitative glucose transporter GLUT3: 20 years of distinction. *Am J Physiol Endocrinol Metab, 295*(2), E242-253. doi: 10.1152/ajpendo.90388.2008**
**Sowers, J. R., Epstein, M., & Frohlich, E. D. (2001). Diabetes, hypertension, and cardiovascular disease: an update. *Hypertension, 37*(4), 1053-1059.**
**Stuart, C. A., Howell, M. E., Zhang, Y., & Yin, D. (2009). Insulin-stimulated translocation of glucose transporter (GLUT) 12 parallels that of GLUT4 in normal muscle. *J Clin Endocrinol Metab, 94*(9), 3535-3542. doi: 10.1210/jc.2009-0162**
**Suarez, S. S., Katz, D. F., Owen, D. H., Andrew, J. B., & Powell, R. L. (1991). Evidence for the function of hyperactivated motility in sperm. *Biol Reprod, 44*(2), 375-381.**
**Thorens, B., & Mueckler, M. (2010). Glucose transporters in the 21st Century. *Am J Physiol Endocrinol Metab, 298*(2), E141-145. doi: 10.1152/ajpendo.00712.2009**
**Thorens, B., Wu, Y. J., Leahy, J. L., & Weir, G. C. (1992). The loss of GLUT2 expression by glucose-unresponsive beta cells of db/db mice is reversible and is induced**

**by the diabetic environment. *J Clin Invest, 90*(1), 77-85. doi: 10.1172/JCI115858**

**Travis, A. J., Jorgez, C. J., Merdiushev, T., Jones, B. H., Dess, D. M., Diaz-Cueto, L., . . . Moss, S. B. (2001). Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa. *J Biol Chem, 276*(10), 7630-7636. doi: 10.1074/jbc.M006217200**

**Uldry, M., Ibberson, M., Horisberger, J. D., Chatton, J. Y., Riederer, B. M., & Thorens, B. (2001). Identification of a mammalian H(+)-myo-inositol symporter expressed predominantly in the brain. *EMBO J, 20*(16), 4467-4477. doi: 10.1093/emboj/20.16.4467**

**van Dam, E. W., Dekker, J. M., Lentjes, E. G., Romijn, F. P., Smulders, Y. M., Post, W. J., . . . Krans, H. M. (2003). Steroids in adult men with type 1 diabetes: a tendency to hypogonadism. *Diabetes Care, 26*(6), 1812-1818.**

**Vigil, P., Orellana, R. F., & Cortes, M. E. (2011). Modulation of spermatozoon acrosome reaction. *Biol Res, 44*(2), 151-159. doi: /S0716-97602011000200007**

**Vinik, A. I., Holland, M. T., Le Beau, J. M., Liuzzi, F. J., Stansberry, K. B., & Colen, L. B. (1992). Diabetic neuropathies. *Diabetes Care, 15*(12), 1926-1975.**

**Visconti, P. E., Westbrook, V. A., Chertihin, O., Demarco, I., Sleight, S., & Diekman, A. B. (2002). Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J Reprod Immunol, 53*(1-2), 133-150.**

**Voglmayr, J. K., & White, I. G. (1979). Effects of rete testis and epididymal fluid on the metabolism and motility of testicular and post-testicular spermatozoa of the ram. *Biol Reprod, 20*(2), 288-293.**

**Wabitsch, M., Jensen, P. B., Blum, W. F., Christoffersen, C. T., Englaro, P., Heinze, E., . . . Hauner, H. (1996). Insulin and cortisol promote leptin production in cultured human fat cells. *Diabetes, 45*(10), 1435-1438.**

**Wang, M. Y., Chen, L., Clark, G. O., Lee, Y., Stevens, R. D., Ilkayeva, O. R., . . . Unger, R. H. (2010). Leptin therapy in insulin-deficient type I diabetes. *Proc Natl Acad Sci U S A, 107*(11), 4813-4819. doi: 10.1073/pnas.0909422107**

**Watanobe, H. (2002). Leptin directly acts within the hypothalamus to stimulate gonadotropin-releasing hormone secretion in vivo in rats. *J Physiol, 545*(Pt 1), 255-268.**

**Wu, X., & Freeze, H. H. (2002). GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics, 80*(6), 553-557.**

**Yamagata, K., Murayama, K., Okabe, M., Toshimori, K., Nakanishi, T., Kashiwabara, S., & Baba, T. (1998). Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. *J Biol Chem, 273*(17), 10470-10474.**

**Yoon, J. W., & Jun, H. S. (2005). Autoimmune destruction of pancreatic beta cells. *Am J Ther, 12*(6), 580-591. doi: 00045391-200511000-00015 [pii]**

**Zamorano, P. L., Mahesh, V. B., De Sevilla, L. M., Chorich, L. P., Bhat, G. K., & Brann, D. W. (1997). Expression and localization of the leptin receptor in endocrine and neuroendocrine tissues of the rat. *Neuroendocrinology, 65*(3), 223-228.**

**Zeidler, A., Gelfand, R., Tamagna, E., Marrs, R., Chopp, R., & Kletzky, O. (1982). Pituitary gonadal function in diabetic male patients with and without impotence. *Andrologia, 14*(1), 62-68.**

**Zhang, F. P., Poutanen, M., Wilbertz, J., & Huhtaniemi, I. (2001). Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice.** ***Mol Endocrinol, 15*****(1), 172-183.**

## Chapter 2: Insulin rescues impaired spermatogenesis via the hypothalamic-pituitary-gonadal axis in Akita diabetic mice and restores male fertility

The research presented in this chapter consists of data that are published in *Diabetes.*

Insulin rescues impaired spermatogenesis via the hypothalamic-pituitary-gonadal axis in Akita diabetic mice and restores male fertility. Schoeller EL, Albanna G, Frolova AI, Moley KH. Diabetes. 2012 Jul; 61(7): 1869-78. doi: 10.2337/db11-1527. Epub 2012 Apr 20.

**Abstract:**

The mechanism responsible for poor reproductive outcomes in male diabetics is not well understood. In light of new evidence that the Sertoli cells of the testis secrete insulin, it is now unclear whether diabetic subfertility is due to pancreatic insulin deficiency or abnormal insulin signaling within the testis (testicular insulin deficiency). In this study, a genetic diabetic mouse model was used to distinguish between systemic and local effects of insulin deficiency on the process of spermatogenesis and fertility. The Akita mouse model expresses a dominant negative mutation in the *ins2* gene, thus expressing mutant insulin in both the pancreas and the testis. A homozygous Akita model was used to study the implications of a lack of functional insulin on testicular composition and spermatogenesis. In this study, we show that Akita homozygous mice are infertile, have reduced testis size and abnormal morphology. Spermatogonial germ cells are still present, but are unable to differentiate into spermatocytes and spermatids. We show that exogenous insulin treatment regenerates testes and restores fertility, but that this plasma insulin cannot pass through the blood testis barrier. We conclude that does not rescue fertility through direct interaction with the testis, but instead by restoring function of the hypothalamic pituitary gonadal axis through restoring hormone levels of LH and testosterone. Although we show that the Sertoli cells of the testis secrete insulin protein, this insulin does not seem to be critical for fertility.

**Introduction:**

Type 1 diabetes has long been associated with human male infertility (Amaral, Oliveira, & Ramalho-Santos, 2008; Pacey, 2010). Mechanisms for this association are widely debated. Studies in rodent models suggest mechanisms including oxidative stress, DNA damage to sperm, altered hormonal profiles, and abnormal progression through spermatogenesis (Navarro-Casado et al., 2010). More recently, investigations into human sperm samples from diabetic males show an increase in nuclear and mitochondrial damage (Agbaje et al., 2007), suggesting that hyperglycemia may cause oxidative stress and free radical damage to sperm DNA. Clinical data from IVF clinics shows that sperm from diabetic patients are able to fertilize oocytes at similar rates compared to sperm from non-diabetic patients. However, pregnancy rates are significantly lower when these embryos are transplanted, suggesting that a diabetic environment damages sperm cells causing poor sperm quality (Mulholland, Mallidis, Agbaje, & McClure, 2011). It is still unclear whether diabetes affects male fertility at the early stages of spermatogenesis or at the level of mature sperm cells. In light of new data that show insulin expression by cells in the testis, it is now unclear whether the effects of diabetes on fertility are mediated through testicular insulin insufficiency or through systemic effects of diabetes.

In this study, we use the Akita mouse model to study the effects of insulin deficiency on gonadal function. The Akita mouse is a model of type 1 diabetes resulting from a mutation in the *ins2* gene. Unlike most other organisms, mice and rats have two functional insulin genes, located on separate chromosomes. *Ins1* is a retrotransposon of the ancestral *ins2* gene, and human insulin is

thus homologous to murine *ins2* (Shiao, Liao, Long, & Yu, 2008). Deletion of both genes results in pup lethality shortly after birth (Duvillie et al., 1997). The mutation of the *ins2* gene results in a misfolded protein product, which accumulates in the ER, causing ER stress, and ultimately death of the insulin-producing beta cells of the pancreas (Oyadomari et al., 2002). The diabetic Akita mouse is thus similar to humans with type 1 diabetes, which is caused from an autoimmune destruction of the beta cells of the pancreas (Yoon & Jun, 2005). The Akita model also displays a severe onset of diabetes, similar to untreated diabetes in human adults, and lacks drug-induced toxicity of a streptozotocin-induced model. Thus, the Akita diabetic mouse is a useful tool for studying the effects of untreated Type 1 diabetes on the male reproductive system.

The diabetic Akita mouse represents a novel way to study the role of insulin in the male reproductive tract. This work demonstrates the ability of the Sertoli cells within the testes to produce insulin in addition to the beta cells of the pancreas. The Akita model thus displays an interesting way to study the importance of insulin within the testes, as Akita homozygotes produce only mutant insulin within the testes. Unlike the pancreas, the Akita homozygote testes do not display any indication of ER stress, however they are completely infertile and the testis show progressive atrophy as the mice become increasingly diabetic. Exogenous insulin treatment improves Akita homozygote fertility, and thus, it is the goal of this study to determine that pathway of the insulin rescue, and differentiate between the significance of testicular insulin versus pancreatic insulin in the maintenance of spermatogenesis.

Although both heterozygous and homozygous mice develop hyperglycemia, Akita homozygotes develop severe hyperglycemia by 3 weeks of age, and thus develop a reproductive phenotype by 8-9 weeks of age, as opposed to Akita heterozygotes, which become infertile at approximately 6 months of age.

Previous studies on the Akita mice have shown that sperm from Akita mice fertilize fewer embryos and those embryos that do fertilize are developmentally impaired at the blastocyst stage, indicating a paternal effect of diabetes on sperm quality (Kim & Moley, 2008). While there exists an association with type 1 diabetes and infertility, the significance of insulin and normoglycemia to the reproductive system has not been fully characterized.

More recent studies have begun to characterize the function of insulin and insulin receptor in testes and sperm. Murine testes and sperm have both been shown to produce transcripts of insulin, suggesting a significant function for insulin in the reproductive tract and an increased potential for diabetes to impact male fertility. Additionally, human sperm have been shown to release insulin in a dose-dependent manner in response to glucose (Aquila, Gentile, Middea, Catalano, & Ando, 2005). Insulin receptor has also been detected in the midpiece of sperm cells, implicating an autocrine function for the secretion of insulin by sperm cells and demonstrating the importance of proper insulin signaling in the reproductive tract. (Carpino, Rago, Guido, Casaburi, & Aquila, 2010). Here we show that Akita mice homozygous for a mutation in the *ins2* gene are completely infertile, have a reduced testis size, and abnormal testis and sperm morphology. In this study, we are able to rescue Akita homozygous infertility with exogenous insulin pellets. We also see that plasma insulin cannot cross the blood testis barrier, showing that the exogenous insulin does not directly rescue fertility.

**Methods:**

**Akita Mice:** All mouse studies were approved by the Animal Studies Committee at Washington University School of Medicine and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. FVB.B6-Ins2Akita/MlnJ mice were obtained from Jackson labs. Ins2Akita is a dominant, spontaneous, point mutation that introduces a Cys to Tyr substitution at the seventh amino acid in the A chain of mature insulin (amino acid 96 in the preproinsulin II sequence), and results in a major conformational change in the insulin 2 molecule. The Ins2Akita spontaneous mutation on the C57BL/6 background (Jackson Laboratories, Stock No. 003548) was backcrossed to FVB/NJ for 9 generations. Speed congenic analysis confirmed the N6 generation was <0.2% C57BL/6J and the sex chromosomes were fixed at generations N6 and N7. In 2007, the Type 1 Diabetes Resource received this strain at N9 from Dr. Mary Loeken at the Joslin Diabetes Center. This mouse was mated to FVB/NJ for 1 generation prior to initiating sibling matings. For this study heterozygous males and females were mated to obtain homozygous males. Heterozygous and wild type males were similarly obtained from heterozygote parents.

**qPCR of Testes:** Total RNA was extracted from whole testes using Trizol following manufacturers directions. RNA was DNase treated (Turbo DNAfree, Ambion). 1 ug of RNA was converted into cDNA using Qiagen Quantitect kit and qRT-PCR was performed using intron-spanning primers. qRT-PCR was performed in triplicate using cDNA from 100 ng of RNA for *ins1* and *ins2* reactions and cDNA from 50 ng of RNA for all other reactions. SYBR

Green detection system was used for all qRT-PCR assays (Applied Biosystems Fast SYBR Green). All primers were designed using Integrated DNA Technologies software and validated for efficiency. PCR products were cloned and validated for specificity. All qRT-PCR reactions were performed on ABI cycler 7500 Fast system and samples were normalized to actin. The ddCT method was used to calculate fold changes.

**Histopathology and Immunohistology of Testes:** Whole mouse testes were fixed overnight in 4% paraformaldehyde, after which they were dehydrated in graded series of alcohol and embedded in paraffin. Paraffin sections were processed for immune-peroxidase staining as described (Bielinska, Seehra, Toppari, Heikinheimo, & Wilson, 2007) using goat anti-GATA4 (sc-1237, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:200 dilution and donkey anti-goat biotinylated IgG (Jackson Immunoresearch, West Grove, PA) at a 1:1000 dilution. The avidin–biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine (Sigma–Aldrich Corp., St. Louis, MO) were used to visualize the bound antibody; slides were counterstained with toluidine blue.

**Electron Microscopy of Testes and Sperm:** For immunolocalization at the ultrastructural level, samples were fixed in 4% paraformaldehyde/0.05% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100mM PIPES/0.5mM MgCl2, pH 7.2 for 1 hr at 4˚C. Samples were then embedded in 10% gelatin and infiltrated overnight with 2.3M sucrose/20% polyvinyl pyrrolidone in PIPES/MgCl2 at 4˚C. Samples were trimmed, frozen in liquid nitrogen, and sectioned with a Leica Ultracut UCT cryo-ultramicrotome (Leica Microsystems Inc., Bannockburn, IL). 50 nm sections were blocked with 5% FBS/5% NGS for 30 min and subsequently incubated with rabbit

anti-Sox9 (1:100) and guinea pig anti-insulin (1:200), followed by anti-rabbit 12 nm and anti-guinea pig 18 nm colloidal gold-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove PA). Sections were washed in PIPES buffer followed by a water rinse, and stained with 0.3% uranyl acetate/2% methyl cellulose. Samples were viewed with a JEOL 1200EX transmission electron microscope (JEOL USA Inc., Peabody, MA). All labeling experiments were conducted in parallel with isotype primary antibody controls. These controls were consistently negative at the concentration of colloidal gold conjugated secondary antibodies used in these studies.

**Serum Hormone ELISAs:** Blood serum samples were isolated from 8-12 week-old individually housed mice. Blood was collected into serum separator tubes (BD microtainer # 365967), centrifuged at 6000 x g for 90 seconds, and serum supernatant was frozen at -80ºC. Serum hormone levels were assayed for follicle-stimulating hormone, luteinizing hormone, or testosterone using rodent ELISA kits (Endocrine Technologies, USA, ERK R7007, R7010, R7016). Results were read spectrophotometrically on a Molecular Devices VersaMax Microplate reader at a 450nm wavelength.

**FITC-insulin injection of Testes:** Mice were anaesthetized with a mixture of Xylazene and Ketamine (final concentration 86.9 mg/kg ketamine: 13.4 mg/kg xylazene), after which both testicles were exposed and FITC-insulin was injected just under the tunica albuginea of one testis, while the other was injected with PBS. After 30 minutes, mice were sacrificed and testes were removed and fixed overnight in 4% paraformaldehyde. Testes were then dehydrated in a graded series of alcohol and embedded in paraffin. 5um sections were deparaffinizied, rehydrated, permeabilized and blocked in PBS/2% BSA/0.5% tritonX-100 for 1 hour at room

temperature. Slides were washed three times in PBS/2%BSA and then incubated for 20 minutes in ToPro nuclear dye (1:500 Molecular Probes in PBS). Slides were washed three times in PBS and mounted with vectashield. Immunofluorescent images were recovered using a Nikon Eclipse E800 confocal microscope.

**TUNEL Detection in Testes:** Testes were fixed in 4% paraformaldehyde overnight. Tissues were then embedded in paraffin and 5um section were cut and dried onto slides. Slides were then deparaffinized in xylene and rehydrated in a graded series of ethanol. Tissue sections were then permeabilized in 0.5% TritonX-100 in PBS for 20 minutes, rinsed twice in PBS, and incubated in TUNEL reaction mixture for 1 hour at room temperature. Tissue sections were then rinsed twice in PBS and counterstained with To-Pro-3 Iodide (1:250) (Invitrogen). Confocal immunofluorescent microscopy of stained testes sections was performed with an Olympus laser-scanning microscope.

**Computer Assisted Semen Analyzer (CASA):** Caudal epididymal sperm was released into HTF Embryomax media (Irvine Scientific, Inc.) equilibrated overnight in 5% $CO_2$ and 37˚C. Sperm were allowed to disperse in an at 5% $CO_2$ and 37˚C incubator for 15 minutes and then sperm cells were loaded into a sperm analysis chamber and inserted into a Computer Assisted Semen Analyzer machine (Hamilton-Thorne, Beverly, MA). At least 1000 cells were counted and sperm cells were evaluated for total motility.

**Insulin Treatment and Orchiectomy:** Akita homozygous mice were treated with insulin implants (Linshin Canada). At 9 weeks of age, Akita homozygous mice were anesthetized and

one testis was removed for histological examination and confirmation of spermatogenic arrest. The other testis was left in the mouse for evaluation after insulin treatment. Then,13 mg insulin pellets were inserted subcutaneously under the dorsal skin. Additional pellets were inserted until blood glucose achieved a level of <200 mg/dl. Mice were treated with insulin for eight weeks after which the remaining testis was removed, and half was fixed in Bouin's solution for histology while the other half was snap frozen in liquid nitrogen for RNA extraction.

**Statistical Analysis:** Statistical analysis and evaluation were performed using unpaired Students t-test for equal variance. Significance was defined as a p-value $<0.05$.

**Results:**

**The Akita Mouse Model**: A genetic mutation in the *ins2* gene of Akita mice results in a misfolded insulin protein product which is retained in the ER, causing ER stress and subsequent destruction in the insulin-producing beta cells of the pancreas (Oyadomari et al., 2002). As a result, Akita mice develop low plasma insulin levels as well as elevated blood glucose. Heterozygous males are similar in weight to wild type and develop blood glucose levels above 300 mg/dl by 5 weeks of age **(Fig 2-1D)**. Akita homozygous males develop blood glucose levels above 300 mg/dl by 3 weeks of age, display runted growth, and have a lifespan of 8-12 weeks, unless treated with insulin. Testes were examined for the presence of ER stress by assaying for the presence of spliced XBP-1 as well as an increase in BiP protein levels. In response to ER stress, cells upregulate transcription of chaperone proteins such as BiP/GRP78 to assist in protein folding. XBP-1 mRNA is also spliced in response to the ER stress to produce a functional protein which acts as a transcription factor for many molecules involved in the unfolded protein response. (Lee, Iwakoshi, & Glimcher, 2003) (Back, Lee, Vink, & Kaufman, 2006). Neither assay indicated that the testes of Akita homozygous mice had an increased level of ER stress **(Sup. Fig. 2-4)**, suggesting that the testicular phenotype of the Akita homozygous mouse is caused by diabetes and not by ER stress.

**Testicular atrophy of insulin-deficient Akita mice:** Homozygous Akita male mice are completely infertile. At 7 weeks of age, these mice show a reduction in testis size as well as a reduction in seminal vesicle weight, suggesting a decrease in testosterone levels. Histological examination of the testes at 8-9 weeks of age shows marked atrophy of the seminiferous tubules,

characterized by a loss of germ cell populations and an increase in the presence of large, multinucleated cells and vacuolization (**Fig. 2-1c**). This phenotype is 100% penetrant and was observed in every Akita homozygote examined at approximately 9 weeks of age. This atrophy is progressive and the testes start to undergo atrophy at approximately 8 weeks of age **(Sup. Fig 2-2)**. To address the possibility that the testicular atrophy is simply a result of systemic illness of the Akita homozygous mice, a full necropsy was performed on wild type, heterozygous, and homozygous Akita mice. An outside researcher examined tissue sections, and no severe defects in any organs were observed aside from the testes and seminal vesicles **(Sup. Fig. 2-3)**. Pancreatic islets were slightly reduced in size and the kidneys appeared distended in both Akita heterozygotes and homozygotes.

TUNEL analysis reveals that homozygous Akita testes have an increased amount of DNA damage among the cells in the seminiferous tubules compared to both wild type and heterozygotes (**Fig. 2-1d**). Akita heterozygous mice also display infertility later in life, and cease to sire pups after 4-6 months of age.

**Akita mice have impaired sperm motility and morphology**: Akita homozygotes have significantly lower motile sperm, as well as lower progressively and rapidly moving sperm (**Fig. 2-2a)**. Akita homozygotes display fewer sperm cells in the epididymis and sperm cells obtained from the cauda epididymis undergo degeneration. Electron micrograph images of caudal epididymal sperm display sperm lacking acrosomes, some cells with membrane blebbing, and numerous detached sperm heads as well as abnormal mitochondria (**Fig. 2-2b**).

**Insulin mRNA increases in Akita testes and sperm**: Insulin mRNA expression was examined in Akita mice using quantitative RT-PCR. RT-PCR shows that while *ins1* and *ins2* mRNA are both present in the pancreas, wild type testes only contain detectable levels of *ins2* and lack any detectable expression of *ins1*. (**Fig. 2-3a**). Insulin expression levels were decreased in Akita pancreases relative to wild type, as detected by qRT-PCR. In contrast, Akita homozygous testes show increased insulin expression, suggesting either a compensatory mechanism or an enrichment of the cell types responsible for insulin production in the testes. Additionally, *Pdx-1*, a known transcription factor for insulin in the pancreas, is also present in the testes and also increases expression significantly in the Akita homozygous testes (**Fig. 2-3b**).

**Insulin Localizes to the Endoplasmic Reticulum of Sertoli Cell Nuclei:** Electron microscopy images of Sertoli cell nuclei of Akita homozygous mice were obtained by immunogold labeling with SOX9, a prominent Sertoli cell nuclear marker (**Fig. 2-5**). Since Akita homozygotes accumulate misfolded *ins2* protein in the endoplasmic reticulum, immunogold co-labeling with insulin was done in these mice. Insulin particles localize predominantly to the endoplasmic reticulum surrounding the Sertoli cell nuclei, which are labeled with SOX9, showing that Sertoli cells are capable of *ins2* production in the testes. Electron microscopy shows an increased concentration of insulin labeling in the endoplasmic reticulum of Akita homozygous testes, indicative of the accumulation of misfolded insulin **(Sup. Fig. 2-1)**.

**Insulin Impermeability of the Blood Testes Barrier**: The blood testes barrier is a dynamic barrier between the interstitium and lumen of the testes that restricts passage of many substances into the lumen of the seminiferous tubule. To determine whether plasma insulin is able to cross

the barrier from the bloodstream into the seminiferous tubules, a biologically active FITC-labeled insulin was injected into the interstitium of the testes. The labeled insulin is completely retained in the interstitium of both wild type and Akita homozygous testes, suggesting that the insulin produced by the testes is the only source in the lumen of the seminiferous tubules (**Fig. 2-5a and 2-5b**). PBS-injected testes were used as a negative control (**Fig. 2-5c**). As a positive control, neonatal male mice were injected with FITC-insulin. Since the blood testes barrier in mice forms at P12, we injected p7, p9, and p12 testes with FITC-insulin and this experiment showed an increasing restriction to the interstitium as the barrier forms **(Fig. 2-5d**). We concluded that the blood testis barrier was present even in the Akita homozygous mice and prevented systemic insulin from entering the seminiferous tubules.

**Exogenous Insulin Treatment rescues testicular phenotype:** Following orchiectomy and initial confirmation of arrested spermatogenesis, Akita homozygous mice treated with subcutaneous insulin pellets for eight weeks display a regeneration of the seminiferous tubules of the testes **(Fig. 2-6a and 2-6b)**. Testis weight as well as whole animal weight increase in response to insulin treatment **(Fig. 2-6c)**. All cell types of the testes were restored upon insulin treatment, detected by qPCR analysis (**Fig. 2-6b**) and CASA revealed a restoration of sperm parameters such that there was no significant difference in any genotype compared to control (**Fig. 2-6d**). In addition, insulin-treated males were able to successfully sire normal size litters, when mated to wild type females (data not shown).

**Cell types affected by lack of functional insulin**: To evaluate which cell types in the testis are affected in the Akita mouse, both a histological analysis as well as a qPCR approach was used.

Histological analysis using GATA4, a Sertoli cell marker, shows that Akita homozygotes still retained a Sertoli cell population (**Fig. 2-7a**). qPCR analysis of whole testes shows that relative levels of a *Sox9*, a Sertoli cell marker, are increased in the Akita homozygotes compared to wild type or Akita heterozygotes (**Fig. 2-7b).** Additionally, *Magea4* (germ cell marker) was increased in Akita homozygotes compared to control or heterozygotes. Akita homozygous testes, however, display significantly absent populations of both *CytC* (spermatocyte marker) and *Tnp1* (spermatid marker), suggesting that these mice may have either a meiotic block or a targeted apoptosis of post-meiotic germ cells (**Fig. 2-7b**). Treatment with insulin pellets partially normalized expression levels of all cell types examined.

**Serum testosterone and luteinizing hormone are decreased in Akita homozygous mice.** Serum levels of both testosterone and luteinizing hormone (LH) are severely decreased in Akita homozygous mice, whereas serum levels of follicular stimulating hormone are not significantly different (**Fig. 2-8a, 2-8b and 2-8c**). Exogenous insulin treatment partially rescues both testosterone and luteinizing hormone levels. Given the resumption of spermatogenesis in Akita homozygotes treated with exogenous insulin pellets and the impermeability of the blood testes barrier to insulin, we conclude that the insulin-mediated rescue is due to normalized levels of the LH and testosterone. The increased levels of LH in insulin-treated Akita homozygotes may promote testosterone synthesis in the Leydig cells of the testes, allowing spermatogenesis to resume.

**Discussion:**

Since the recent discovery that insulin is produced in murine testes, the association between diabetes and infertility becomes more complex. In this study, we used an Akita diabetic mouse model, which has a mutation in the *ins2* gene. This mouse retains wild type *ins1*, but because the misfolded *ins2* protein product causes significant ER stress, this leads to overall beta cell dysfunction and the impairment of all insulin secretion by the pancreas. Considering that the murine testes solely produce *ins2*, as opposed to both *ins1* and *ins2* by the pancreas, the Akita testis should represent a complete knockout of insulin in the testis. However, we saw that the application of exogenous insulin was able to rescue the fertility of Akita mice. Given the impermeability of the blood-testis barrier to insulin, we speculate that the fertility-related defects seen in these mice are a result of the systemic effects of diabetes and potentially a less severe and reversible effect of *ins2* deficiency within the seminiferous tubules.

Insulin is known to influence the hypothalamic-pituitary axis (Bucholtz et al., 2000), which can alter serum hormone levels important in spermatogenesis. Brain specific knockout of the insulin receptor is known to impair spermatogenesis by decreasing circulating amounts of luteinizing hormone (Bruning et al., 2000). LH interacts with the Leydig cells in the interstitium of the testes to promote testosterone production. Indeed, mice lacking the luteinizing hormone receptor display an arrest in spermatogenesis, which is rescued by the administration of exogenous testosterone (Pakarainen, Zhang, Makela, Poutanen, & Huhtaniemi, 2005). Our studies indicate that LH and testosterone are significantly decreased in Akita mice. We suspect that the lack of circulating insulin in the Akita mouse reduces LH secretion by the pituitary gland, impacting the

ability of the Leydig cells of the testes to produce testosterone, leading to the observed arrest in spermatogenesis.

It is also known that insulin may be able to interact with receptors on the Leydig cells directly to mediate the process of steroidogenesis, so we cannot exclude the possibility that plasma insulin also directly interacts with Leydig cells in addition to the indirect signaling through the pituitary. Leydig cells contain insulin receptors (Kimura, Lukinius, Ericsson, & Grimelius, 1992), and the administration of insulin to Leydig primary cultures causes an increase in testosterone formation (Lin, Haskell, Vinson, & Terracio, 1986). This suggests that low plasma insulin levels can significantly decrease testosterone formation, which is known to limit the process of spermatogenesis (Spaliviero, Jimenez, Allan, & Handelsman, 2004). In this case, it is fitting that administering insulin via the bloodstream should rescue the process spermatogenesis in the Akita mouse model, as Leydig cells reside outside the blood-testis barrier and thus have access to plasma proteins.

The role of the insulin produced by the testes is still unclear. Our study demonstrates that exogenous insulin can rescue fertility despite the presence of only mutant insulin within the seminiferous tubules. This indicates that Sertoli-derived insulin does not have a critical role in spermatogenesis. However, the production of insulin within the seminiferous tubule remains an interesting question. It seems unlikely that the testis would secrete insulin without providing any function. It is possible that Akita homozygotes are able to upregulate *ins1* in the Sertoli cells when mutant *ins2* is produced. Given that the Sertoli cells also produce other hormones important in the regulation of spermatogenesis (Griswold, 1988), it is reasonable to ascertain that

these cells might also produce insulin to maintain the process of spermatogenesis. Indeed, we show by electron microscopy that the Sertoli cells are responsible for insulin production in the testes. Insulin may be important both as a growth factor/survival signal as well as a mechanism for glucose homeostasis in the testes.

Since the Sertoli cells span the blood testes barrier, it is foreseeable that the insulin produced by these cells may be used by the developing spermatocytes and spermatids that lie beyond the barrier. In addition to indirectly affecting spermatogenesis through interactions with the hypothalamic-pituitary axis, the low levels of plasma insulin in the Akita may also affect the testes directly by interacting with insulin receptors on the Sertoli cell (Ballester et al., 2004). The presence of insulin receptors on Sertoli cells suggests that these cells may be able to directly respond to circulating levels of insulin, considering that the basal compartment of the seminiferous tubule is in contact with the blood and lymph. There is evidence to suggest that insulin has a stimulatory effect on spermatogenesis. Treatment of seminiferous tubule segments in culture with insulin stimulates spermatogonial DNA synthesis in rats (Soder, Bang, Wahab, & Parvinen, 1992)and insulin treatment also promotes spermatogonia differentiation of primary spermatocytes in the newt (Nakayama, Yamamoto, & Abe, 1999)These experiments indicate that in additional to insulin's effect on the hypothalamic-pituitary axis, insulin may have a direct role in the process of spermatogenesis.

Additionally, it is important to note that the Akita testes may not represent a complete lack of insulin in the testes. In the Akita pancreas, *ins1* is not able to compensate for the lack of functional *ins2*, as the high levels of mutant *ins2* protein cause ER stress that damages the

insulin-producing beta cells of the pancreas. In the testes, there does not seem to be an increase ER stress (supplemental figure 4). Thus, although *ins1* is not normally present at high levels, the lack of functional *ins2* may induce an upregulation of *ins1* and provide enough local insulin to facilitate spermatogenesis. The data presented here demonstrates that a lack of systemic insulin results in infertility due to defects in the hypothalamic pituitary gonadal axis. In order to determine the function of insulin produced by the testes, a complete knockout of both ins1 and ins2 in the testis must be achieved.

The Akita mouse provides a new model for studying the effects of type 1 diabetes on fertility. The maintenance of proper sperm formation depends on a functional hypothalamic pituitary gonadal axis and thus depends of proper insulin regulation.

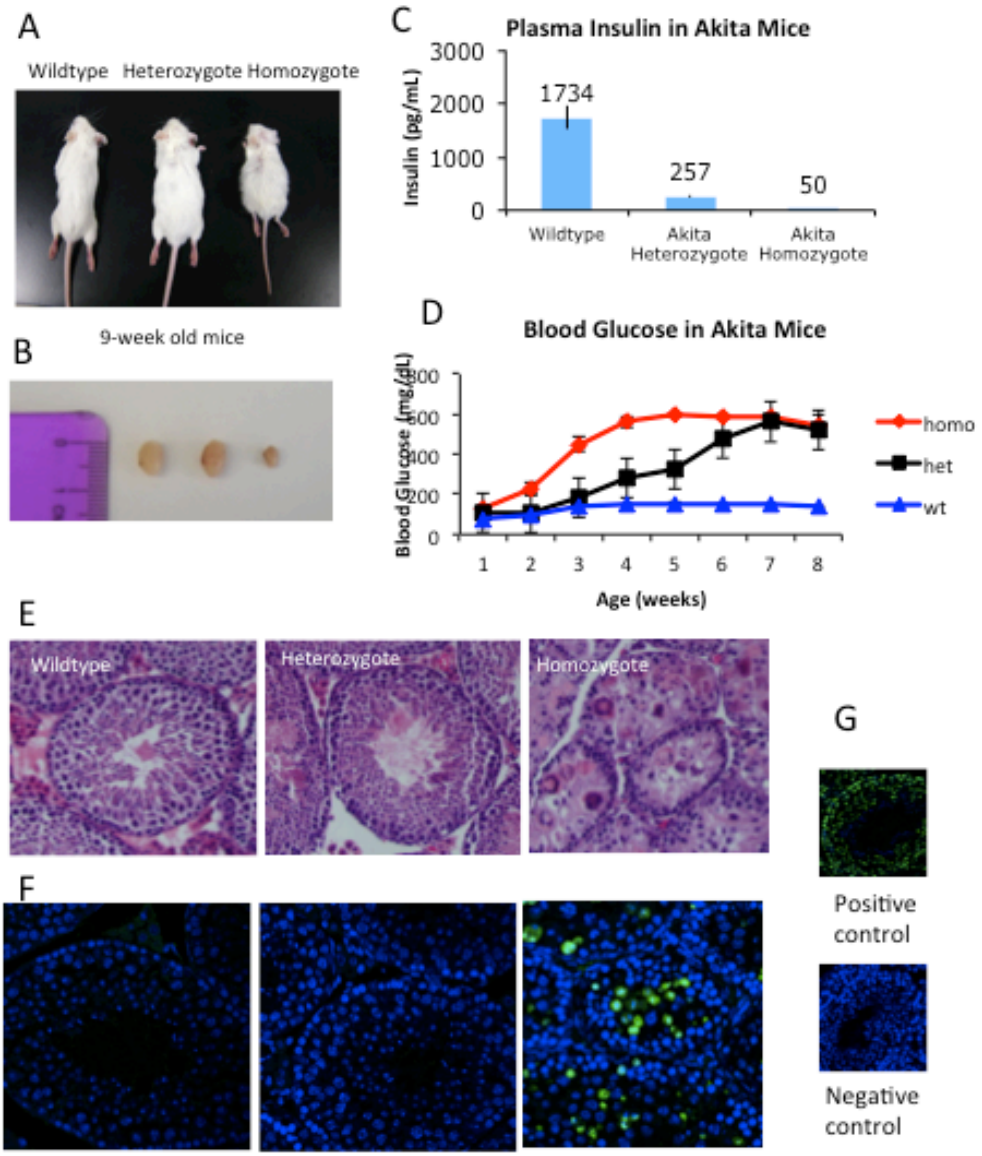



**Figure 2-1**. **Testicular atrophy in the male diabetic Akita mouse model**. A) 9-week old Wildtype, Akita heterozygous, and Akita homozygous mice (left to right) and B) corresponding testis decrease in size; C) plasma insulin decreases in Akita heterozygotes and Akita homozygotes (n=6) (9-week old mice); D) Blood glucose levels in Akita mice.; E) H&E stain of testes section. Akita homozygous testes (9-week old) lack mature sperm cells and display vacuolization and large multinucleated cells; F) TUNEL analysis on Akita homozygous testes shows a greater degree of DNA damage in the seminiferous tubules; G) negative and positive controls

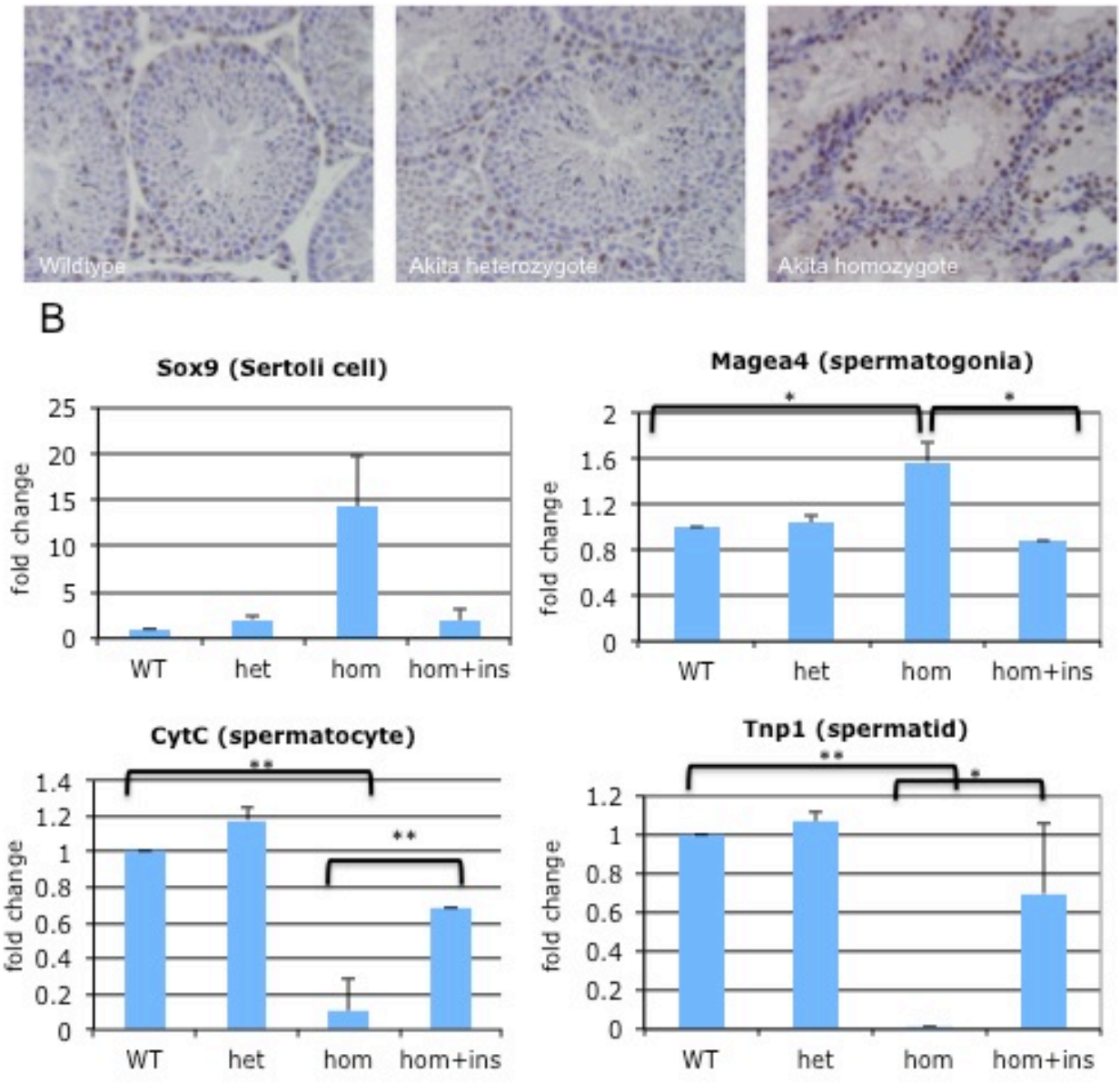



**Figure 2-2. Testicular cell types affected by lack of functional insulin**. A) Testes sections from 9-week old control, Akita heterozygote and homozygote males. GATA4 staining of Akita homozygous testes reveals a retained a population of Sertoli cells (n=3); B) qRT-PCR analysis of cell types in control and Akita testes suggests increased Sertoli cell and spermatogonia cell populations in homozygous Akita testes, while both spermatocytes and spermatids are virtually absent compared to controls and heterozygotes. Insulin treatment reversed this trend and restored all cell populations in the testes to normal levels. (n=5 for wt, Akita heterozygote, Akita homozygote; n=2 for Akita homozygote + insulin)(* p-value <

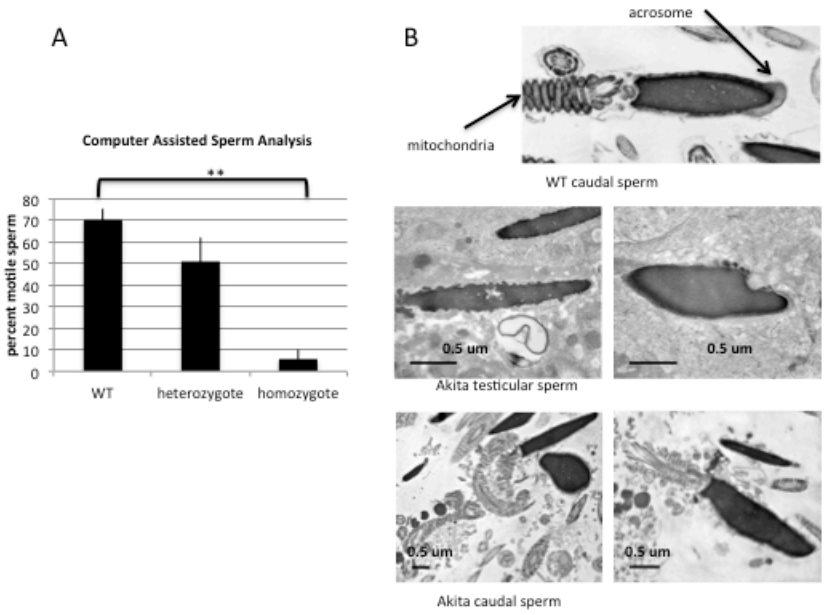



**Figure 2-3. Akita mice have impaired sperm motility and morphology.** A) Computer assisted sperm analysis of caudal sperm from wildtype, Akita heterozygotes, and Akita homozygotes reveals that Akita homozygous sperm has a lower percentage of motile sperm, progressively moving sperm as well as rapidly moving sperm. Akita heterozygous mice also display poorer CASA parameters (n=3 experiments; **$p<0.01$); B) Electron micrographs of wildtype and Akita homozygous sperm. Sperm cells obtained from either testicular sections or the cauda epidiymus lack acrosomes, often display head detachment, membrane blebbing, and abnormal mitochondria.

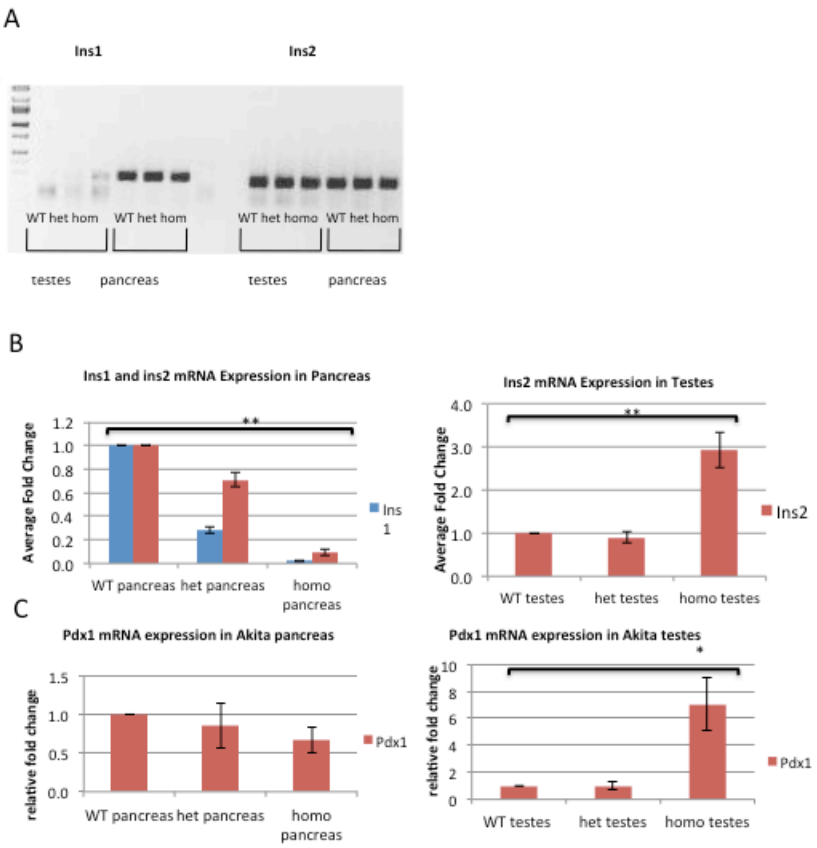



**Figure 2-4. Insulin transcription increases in Akita testes and sperm.** A) Differential expression of *ins1* and *ins2* by RT-PCR. Both transcripts are present in the pancreas, while only *ins2* is present in the testes; b) qRT-PCR demonstrates expression of both insulin genes is decreases in the Akita pancreas, yet increases in Akita homozygous testes; C) mRNA for *pdx-1*, a transcription factor for both insulin genes, decreases expression in Akita pancreas, yet also increases in Akita homozygous testes, following the same trend as *ins2* (n=3; *p-value <0.05, ** p-value <0.01) (9-week old mice).

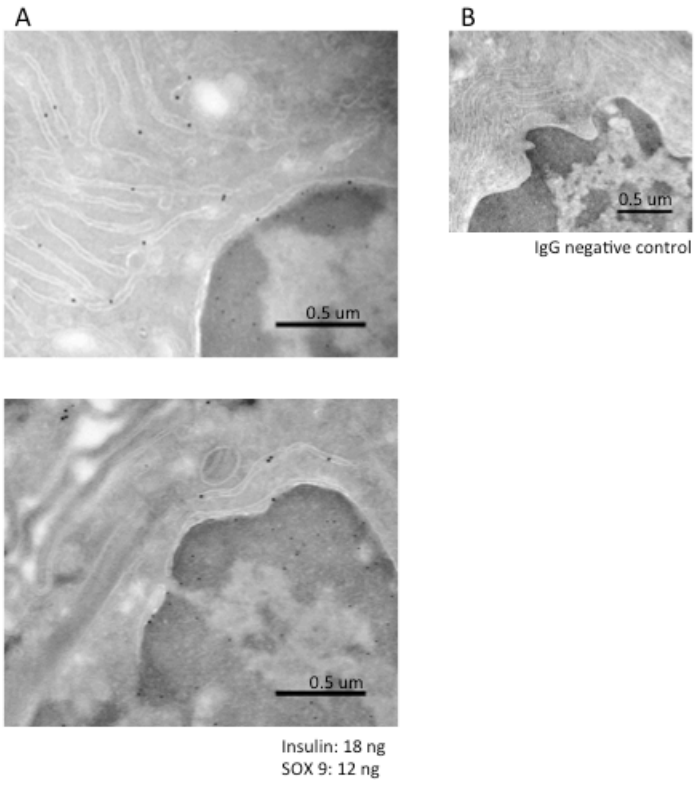



**Figure 2-5**. **Insulin localizes to the endoplasmic reticulum of Sertoli cell nuclei**. A and B) Akita homozygous mouse testes were fixed for electron microscopy and immunogold labeled for insulin (18ng-large gold particle) and the Sertoli cell nuclear marker, Sox9 (12ng-small gold particle). Insulin localizes to the endoplasmic reticulum surrounding the Sertoli cell nuclei, labeled with Sox9. C) IgG negative control antibody. Bars represent 0.5 um

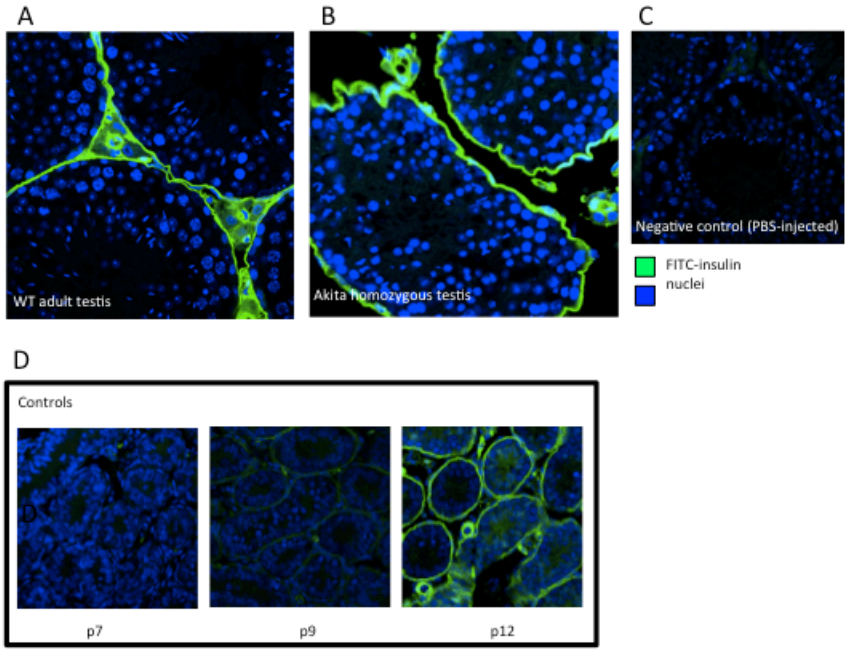



**Figure 2-6. Insulin is impermeable to the blood testes barrier.** A) FITC-labeled insulin injected into the interstitium of wildtype mice does not penetrate the blood testes barrier. (n=3, green=FITC-insulin, blue=nuclei). B) The blood testis barrier in Akita homozygotes also restricts passage of insulin across the barrier. C) PBS-injected control demonstrates no autofluorescence; D) Experimental controls: FITC-insulin is increasingly restricted to the interstitium as the blood testes barrier forms in developing mice; interstitial staining is seen at postnatal day 7 and 9 (p7 and 9). By p12, the barrier has formed and is intact, thus preventing passage of interstitially injected FITC-labeled insulin.

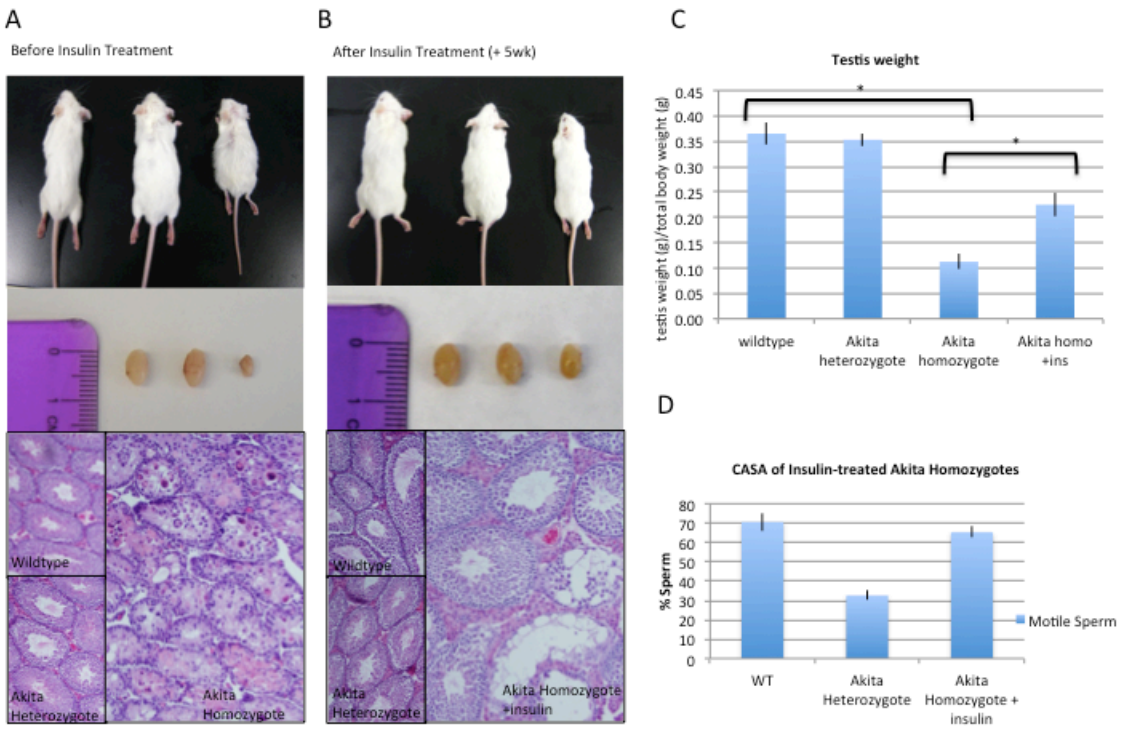



**Figure 2-7. Exogenous insulin treatment rescues testicular phenotype following orchiectomy.** A) Wildtype and Akita mice before surgery and removal of one testis and insulin pellet treatment (n=7 experiments with at least 3 mice in each group); B) Mice after treatment with insulin for 8 weeks. Note the regeneration of the seminiferous tubules as well as the presence of all germ cell populations (n=3 experiments) histology and testes images represent the remaining testis from the same mouse after removal of the first testis and subsequent insulin treatment; C) Mouse testis weight increases after insulin treatment. (Pre-ins treatment n=7 experiments; post-ins treatment n=3 experiments); D) CASA parameters normalize after treatment (n=3 experiments).

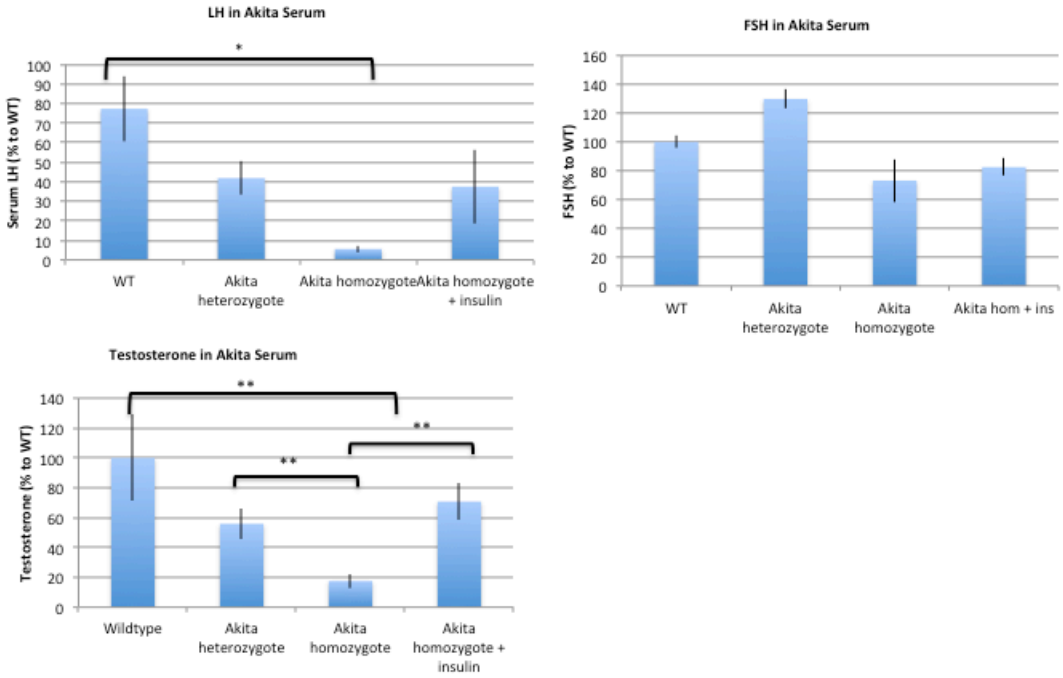
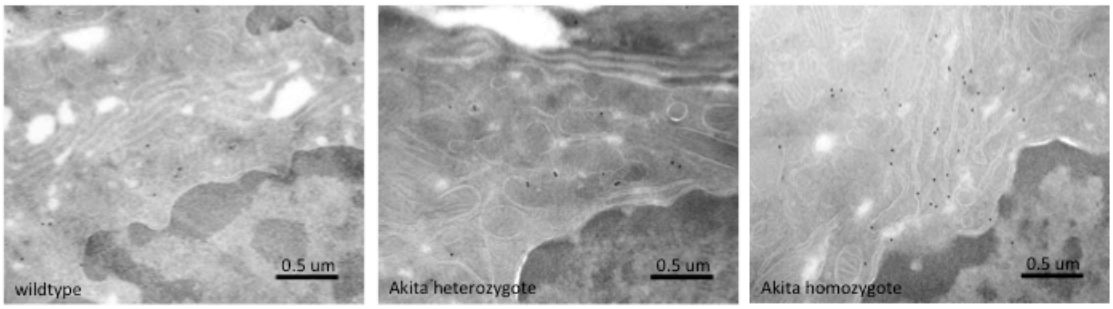



**Figure 2-8. Serum testosterone and luteinizing hormone are decreased in Akita**.

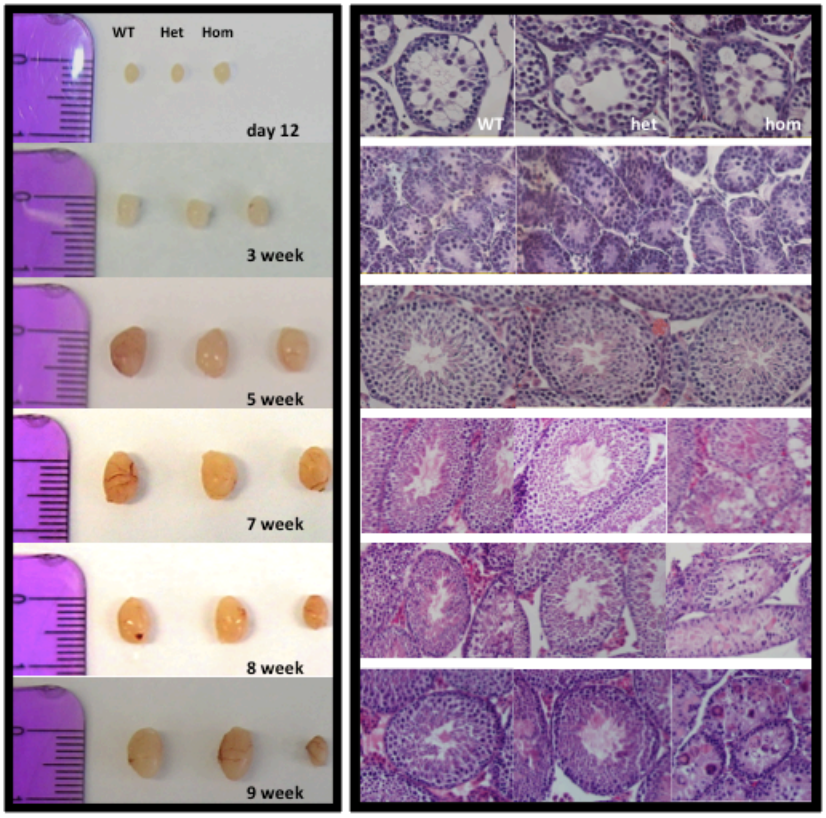
Luteinizing hormone and testosterone are significantly decreased in Akita heterozygote and homozygotes. Insulin pellet treatment reversed this trend. (n=4, * p-value <0.05, ** p-value <0.01).

| Gene name | Forward Primer | Reverse Primer |
|---|---|---|
| Magea4 | AGA GGA AGC CAC TCC CAC CTC AA | GAA GCT TCC TCA GAT GGG CCT TCA |
| Sox9 | CAA GAC TCT GGG CAA GCT C | GGG CTG GTA CTT GTA ATC GG |
| Cytochrome C | GCA TCA GTG GAC CAA GGG TGG C | CAC CGT GTG GCA CTG AGC ACA TT |
| Tnp1 | AGC TCC TCA CAA GGG CGT CAA | TCA TGC TCC TGC CCC GTC TTG |
| Ins1 | CCT GCT GGC CCT GCT TGC | ACT GAT CCA CAA TGC CAC GC |
| Ins2 | CCT GCT GGC CCT GCT CTT | CAA GGT CTG AAG GTC ACC TG |

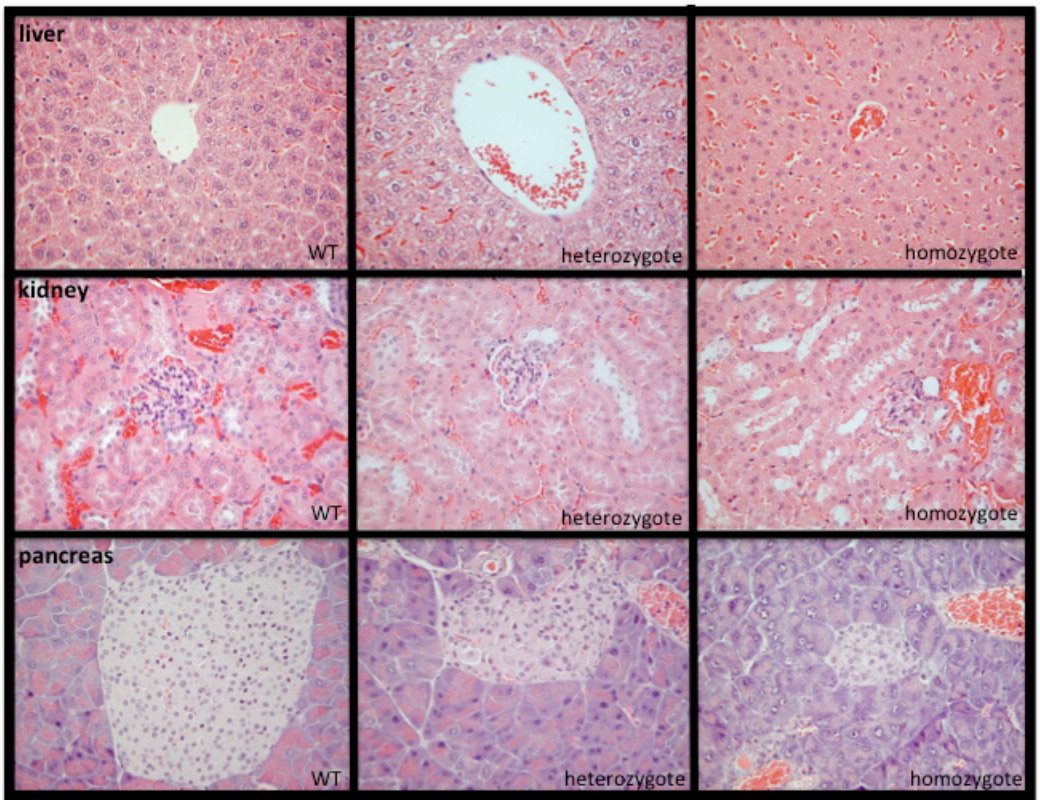
**Table 1. Primer sets**



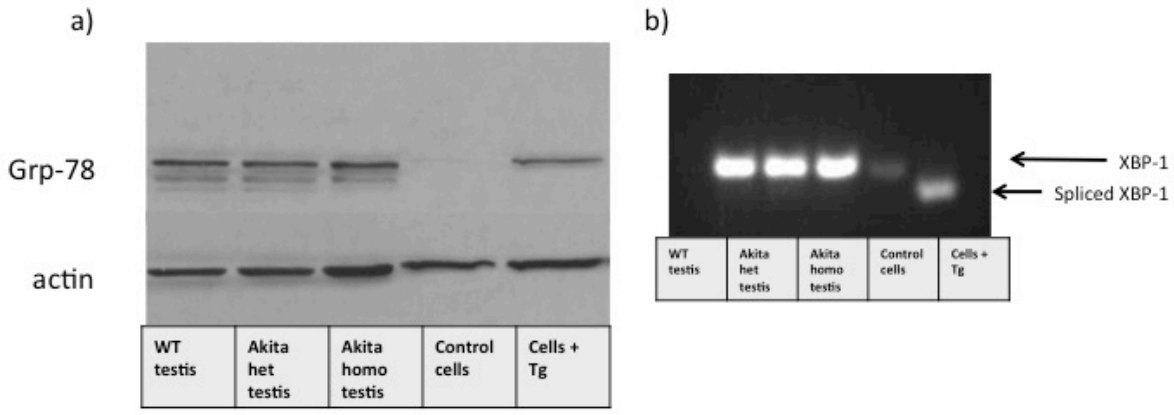

**Supplemental Figure 2-1: Immunogold localization of insulin to the endoplasmic reticulum in Akita mice.** Immunogold labeling of insulin shows a higher density of insulin localizing to the endoplasmic reticulum in Akita homozygous mice. This demonstrates the accumulation of the misfolded mutant *ins2* protein product in homozygotes.




**Supplemental Figure 2-2: Progressive atrophy of Akita homozygous testes.** The testes of Akita homozygous mice progressively atrophy with age in the Akita homozygous mouse corresponding to increased vacuolization and loss of mature sperm cell populations.




**Supplemental Figure 2-3**: **Histological analysis of Akita tissues.** Full necropsy of wildtype, Akita heterozygous, and Akita homozygous mice show no severe defects in any other organ aside from the testes.




**Supplemental Figure 2-4: ER stress.** (a) GRP-78, a chaperone protein involved in ER stress in not upregulated in Akita homozygous testes compared to wildtype or heterozygous Akita testes (Lanes 1-3). Lanes 4 and 5: Control cells treated with thapsigargin, an inducer of ER-stress, had increased expression of Grp-78. (b) XBP-1 mRNA, which is spliced in response to ER-stress, remains unspliced in Akita homozygous testes. Control cells treated with thapsigargin have spliced XBP-1 mRNA.

**References:**


**Agbaje, I. M., Rogers, D. A., McVicar, C. M., McClure, N., Atkinson, A. B., Mallidis, C., & Lewis, S. E. (2007). Insulin dependant diabetes mellitus: implications for male reproductive function. *Hum Reprod, 22*(7), 1871-1877. doi: 10.1093/humrep/dem077**

**Amaral, S., Oliveira, P. J., & Ramalho-Santos, J. (2008). Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive oxygen species. *Curr Diabetes Rev, 4*(1), 46-54.**

**Aquila, S., Gentile, M., Middea, E., Catalano, S., & Ando, S. (2005). Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology, 146*(2), 552-557. doi: 10.1210/en.2004-1252**

**Back, S. H., Lee, K., Vink, E., & Kaufman, R. J. (2006). Cytoplasmic IRE1alpha-mediated XBP1 mRNA splicing in the absence of nuclear processing and endoplasmic reticulum stress. *J Biol Chem, 281*(27), 18691-18706. doi: M602030200 [pii]**
**10.1074/jbc.M602030200**

**Ballester, J., Munoz, M. C., Dominguez, J., Rigau, T., Guinovart, J. J., & Rodriguez-Gil, J. E. (2004). Insulin-dependent diabetes affects testicular function by FSH- and LH-linked mechanisms. *J Androl, 25*(5), 706-719.**

**Bielinska, M., Seehra, A., Toppari, J., Heikinheimo, M., & Wilson, D. B. (2007). GATA-4 is required for sex steroidogenic cell development in the fetal mouse. *Dev Dyn, 236*(1), 203-213. doi: 10.1002/dvdy.21004**

**Bruning, J. C., Gautam, D., Burks, D. J., Gillette, J., Schubert, M., Orban, P. C., . . . Kahn, C. R. (2000). Role of brain insulin receptor in control of body weight and reproduction. *Science, 289*(5487), 2122-2125. doi: 8841 [pii]**

**Bucholtz, D. C., Chiesa, A., Pappano, W. N., Nagatani, S., Tsukamura, H., Maeda, K. I., & Foster, D. L. (2000). Regulation of pulsatile luteinizing hormone secretion by insulin in the diabetic male lamb. *Biol Reprod, 62*(5), 1248-1255.**

**Carpino, A., Rago, V., Guido, C., Casaburi, I., & Aquila, S. (2010). Insulin and IR-beta in pig spermatozoa: a role of the hormone in the acquisition of fertilizing ability. *Int J Androl, 33*(3), 554-562. doi: IJA971 [pii]**
**10.1111/j.1365-2605.2009.00971.x**

**Duvillie, B., Cordonnier, N., Deltour, L., Dandoy-Dron, F., Itier, J. M., Monthioux, E., . . . Bucchini, D. (1997). Phenotypic alterations in insulin-deficient mutant mice. *Proc Natl Acad Sci U S A, 94*(10), 5137-5140.**

**Griswold, M. D. (1988). Protein secretions of Sertoli cells. *Int Rev Cytol, 110*, 133-156.**

**Kim, S. T., & Moley, K. H. (2008). Paternal effect on embryo quality in diabetic mice is related to poor sperm quality and associated with decreased glucose transporter expression. *Reproduction, 136*(3), 313-322. doi: 10.1530/REP-08-0167**

**Kimura, M., Lukinius, A., Ericsson, J. L., & Grimelius, L. (1992). Distribution of insulin binding sites on Leydig cells of rat testes using insulin-coated gold particles. *Histochemistry, 97*(3), 213-220.**

**Lee, A. H., Iwakoshi, N. N., & Glimcher, L. H. (2003). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol Cell Biol, 23*(21), 7448-7459.**

**Lin, T., Haskell, J., Vinson, N., & Terracio, L. (1986). Characterization of insulin and insulin-like growth factor I receptors of purified Leydig cells and their role in steroidogenesis in primary culture: a comparative study. *Endocrinology, 119*(4), 1641-1647.**

**Mulholland, J., Mallidis, C., Agbaje, I., & McClure, N. (2011). Male diabetes mellitus and assisted reproduction treatment outcome. *Reprod Biomed Online, 22*(2), 215-219. doi: S1472-6483(10)00652-8 [pii]**

**10.1016/j.rbmo.2010.10.005**

**Nakayama, Y., Yamamoto, T., & Abe, S. I. (1999). IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes. *Int J Dev Biol, 43*(4), 343-347.**

**Navarro-Casado, L., Juncos-Tobarra, M. A., Chafer-Rudilla, M., de Onzono, L. I., Blazquez-Cabrera, J. A., & Miralles-Garcia, J. M. (2010). Effect of experimental diabetes and STZ on male fertility capacity. Study in rats. *J Androl, 31*(6), 584-592. doi: 10.2164/jandrol.108.007260**

**Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E., & Mori, M. (2002). Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest, 109*(4), 525-532. doi: 10.1172/JCI14550**

**Pacey, A. A. (2010). Environmental and lifestyle factors associated with sperm DNA damage. *Hum Fertil (Camb), 13*(4), 189-193. doi: 10.3109/14647273.2010.531883**

**Pakarainen, T., Zhang, F. P., Makela, S., Poutanen, M., & Huhtaniemi, I. (2005). Testosterone replacement therapy induces spermatogenesis and partially restores fertility in luteinizing hormone receptor knockout mice. *Endocrinology, 146*(2), 596-606. doi: en.2004-0913 [pii]**

**10.1210/en.2004-0913**

**Shiao, M. S., Liao, B. Y., Long, M., & Yu, H. T. (2008). Adaptive evolution of the insulin two-gene system in mouse. *Genetics, 178*(3), 1683-1691. doi: 10.1534/genetics.108.087023**

**Soder, O., Bang, P., Wahab, A., & Parvinen, M. (1992). Insulin-like growth factors selectively stimulate spermatogonial, but not meiotic, deoxyribonucleic acid synthesis during rat spermatogenesis. *Endocrinology, 131*(5), 2344-2350.**

**Spaliviero, J. A., Jimenez, M., Allan, C. M., & Handelsman, D. J. (2004). Luteinizing hormone receptor-mediated effects on initiation of spermatogenesis in gonadotropin-deficient (hpg) mice are replicated by testosterone. *Biol Reprod, 70*(1), 32-38. doi: 10.1095/biolreprod.103.019398**

**biolreprod.103.019398 [pii]**

**Yoon, J. W., & Jun, H. S. (2005). Autoimmune destruction of pancreatic beta cells. *Am J Ther, 12*(6), 580-591. doi: 00045391-200511000-00015 [pii]**

# Chapter 3: Leptin Monotherapy Rescues Spermatogenesis in Akita Type 1 Diabetic Mice

**Abstract**

Type 1 diabetes is associated with subfertility in humans. The current treatment for type 1 diabetes, insulin monotherapy, is suboptimal to fully stabilize glycemia, potentially leading to this subfertility. Recent work with mouse models of type 1 diabetes has demonstrated that treatment with the energy-regulating hormone leptin, alone or in combination with insulin, can more effectively control glycemia. Here we sought to determine whether the fertility defects in a type 1 diabetic mouse model, the Akita mouse, can be rescued with leptin monotherapy, in the absence of any exogenous insulin. Akita homozygous mice treated with leptin alone had a larger total body size, larger testes, and larger seminal vesicles than their untreated siblings. Leptin-treated mice also produced motile sperm cells that could successfully fertilize oocytes *in vitro*. Despite completely rescuing spermatogenesis, the critical reproductive hormones luteinizing hormone and testosterone were only modestly higher than in untreated mice, suggesting a threshold effect. Cumulatively, these findings implicate the importance of leptin in maintaining fertility and support the use of leptin therapy in the treatment of type 1 diabetes.

## Introduction

Untreated type 1 diabetes causes disruptions in the hypothalamic pituitary gonadal (HPG) axis, which integrates signals from the central nervous system and the reproductive organs. As a combined result of endocrine disorders and damage to sperm nuclear and mitochondrial DNA from increased oxidative stress, men with type 1 diabetes are subfertile (Agbaje et al., 2007; Callaghan, Little, Feldman, & Hughes, 2012; La Vignera, Condorelli, Vicari, D'Agata, & Calogero, 2012; Mallidis, Agbaje, O'Neill, & McClure, 2009). Similarly, mouse models of untreated type 1 diabetes exhibit decreased levels of reproductive hormones, including luteinizing hormone, follicle-stimulating hormone, testosterone, insulin, and leptin, all of which affect proper function of the HPG axis (Ballester et al., 2004; Schoeller, Albanna, Frolova, & Moley, 2012).

The Akita mouse is a useful model for studying the mechanism of type 1 diabetes-related male infertility. Akita heterozygous and homozygous mice are diabetic due to a mutation in the *ins2* gene, which codes for insulin. This mutated gene produces a misfolded protein that is retained in the endoplasmic reticulum (ER), causing ER stress and subsequent beta cell death (Oyadomari et al., 2002). The homozygous Akita mouse is severely diabetic, with blood glucose levels surpassing 600 mg/dL by three weeks of age, and has a lifespan of only 8-12 weeks. In contrast, wild-type mice have blood glucose levels of 100-150 mg/dL and generally live 2-3 years. The Akita mouse is infertile and displays reduced testis size, arrested spermatogenesis, and an absence of any mature sperm cells (Schoeller et al., 2012). Serum levels of critical reproductive

hormones, including luteinizing hormone, testosterone, and leptin, are significantly reduced, indicating that the infertility is due to disruptions in the HPG axis.

Leptin, an important signaling hormone involved in energy expenditure, is decreased in newly diagnosed type 1 diabetic human patients prior to receiving insulin treatment and in mouse models of type 1 diabetes (Hanaki, Becker, & Arslanian, 1999; Havel et al., 1998). Leptin is secreted by fat cells, and its concentration in serum is directly proportional to the amount of adipose tissue present in an individual. (Banerji, Faridi, Atluri, Chaiken, & Lebovitz, 1999; Dua et al., 1996). Consistent with this, untreated type 1 diabetic animals lacking insulin have depleted leptin levels that correspond with diminished fat stores (Havel et al., 1998). Leptin signaling has a wide range of effects, including regulation of appetite, maintenance of body weight and normoglycemia, as well as many neuroendocrine effects. Furthermore, low levels of leptin are known to have detrimental effects on fertility (Goumenou, Matalliotakis, Koumantakis, & Panidis, 2003). One explanation for this is that leptin stimulates the secretion of gonadotropin-releasing hormone from the hypothalamus, which then causes the pituitary to release luteinizing hormone and testosterone (Watanobe, 2002). Without luteinizing hormone or testosterone, spermatogenesis is impaired, resulting in infertility. Leptin levels are also markedly decreased in cases of nutritional deprivation, such as anorexia nervosa, in humans. Fertility can be restored in anorexic patients with hypothalamic amenorrhea by administration of exogenous leptin, suggesting that the maintenance of fertility is leptin dependent (Chou et al., 2011). Additionally, mice lacking either leptin or its receptor (*ob/ob* or *db/db* mice, respectively) are infertile due to a decrease in gonadotropins (Swerdloff, Batt, & Bray, 1976). Cumulatively, these data demonstrate the critical nature of leptin in the maintenance of fertility.

Type 1 diabetes in humans is currently treated with recombinant insulin, but a number of co-morbidities, such as atherosclerosis, hypertension, nephropathy, and neuropathy, can persist despite treatment (Callaghan et al., 2012; Krolewski & Bonventre, 2012; Rathsman, Rosfors, Sjoholm, & Nystrom, 2012). Recently, leptin monotherapy has been shown to reverse hyperglycemia in type 1 diabetic mice as well as ameliorate many other symptoms of diabetes, including hyperglucagonemia, hyperketonemia, and polyuria (Fujikawa, Chuang, Sakata, Ramadori, & Coppari, 2010). Also, leptin inhibits lipogenesis (Buettner et al., 2008), whereas insulin promotes lipogenesis (Kersten, 2001); thus leptin monotherapy may prevent some of the metabolic risks associated with the increased fat deposition that occurs as a result of insulin monotherapy. Current data in mice suggest that leptin therapy, alone or in combination with insulin, may result in better glycemic control than is achieved with insulin alone. Furthermore, such treatments may prevent many of the type 1 diabetes complications that are due to blood glucose fluctuations.

Previous studies in our lab demonstrated that exogenous insulin treatment restored the HPG axis in Akita mice, rescuing testicular atrophy and germ cell loss (Schoeller et al., 2012). However, we hypothesized that the ultimate cause of infertility was not lack of insulin, but rather the lack of leptin due to the decreased adipose tissue in these mice. Here we tested the hypothesis that leptin monotherapy can rescue the fertility of the Akita homozygous mouse. We found that a two-week course of leptin treatment, in the absence of any insulin, was able to prevent the testicular atrophy in Akita homozygous mice, resulting in the presence of functional mature sperm cells. These data demonstrate that leptin monotherapy can prevent the infertility of Akita type 1 diabetic mice.

**Methods:**

**Mice:** All studies were approved by the animal studies committee at Washington University School of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. FVB.B6-Ins2Akita/MlnJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Heterozygous males and females were mated to obtain homozygous, heterozygous, and wild-type males.

**Leptin treatment:** Akita homozygous sibling pairs were used for leptin treatment. At 5 weeks of age, leptin was administered subcutaneously to one mouse at a dose of 100 μg (chosen on the basis of preliminary studies in our lab) twice daily for two weeks; the sibling Akita homozygote received PBS.

**Histology:** Testes were fixed overnight in Bouin's solution, dehydrated in ethanol, and paraffin-embedded. Tissue sections (5 μm) were cut, dried overnight at 60 °C, rehydrated, and then stained with hemotoxylin and eosin.

**Sperm Motility Analysis:** Cauda epididymal sperm were collected in HTF Embryomax (Millipore) and allowed to disperse for 10 minutes at 37 °C in 5% $CO_2$. Computer Assisted Sperm Analysis software (Hamilton-Thorne) was used to analyze sperm motility.

**In Vitro Fertilization:** Wild-type FVB females were injected with PMSG and then HcG 48 hours later. Twelve hours post-HcG injection, ovulated cumulus oocyte complexes (COCs) were collected in Research Vitro Fert media (Cook Medical). After capacitation for 90 minutes, 1 million to 5 million sperm were added to COCs in 500 μl drops of media under oil. Sperm and COCs were incubated at 37 °C and 5% $CO_2$ for 6 hours after which oocytes were washed free of

sperm and then incubated overnight. Fertilization rate was calculated as the number of 2-cell embryos at 24-hours post-fertilization. Blastocyst rate was calculated as the number of 2-cell embryos reaching blastocyst stage after four days.

**Serum Hormone Measurements:** Mice were housed individually for 1 week before serum collection into serum separator tubes (BD, Franklin Lakes, NJ). Serum testosterone (radioimmunoassay) and luteinizing hormone (Mouse LH Sandwich Assay) were measured by the UVA Center for Research in Reproduction Ligand Assay and Analysis Core. Leptin was measured by ELISA (Millipore, Billerica, MA) and analyzed on a Molecular Devices VersaMax Microplate reader at 450 and 590 nm. Glucose was measured as described previously (Lowry, 1993)

**Body fat measurement:** Body fat measurements were made by EchoMRI at the Diabetes Phenotyping Core at Washington University in Saint Louis.

**Statistics:** Data were analyzed by using one-way ANOVA followed by a Tukey post-test.

**Results:**

**Leptin treatment of Akita homozygous mice:** To determine whether leptin monotherapy could prevent infertility in type 1 diabetic Akita mice, homozygotes were injected subcutaneously with 100 μg of leptin twice per day for two weeks. Homozygous littermates were injected with an equal volume of PBS as a control. On average, Akita homozygotes treated with leptin had a larger overall body size (17.4 g vs. 10.2 g), larger testes (.093 g vs. .012 g), and larger seminal vesicles (.089 g vs. .045 g) than untreated Akita homozygote siblings **(Fig. 3-1)**. Importantly, the rescue of body, testis, and seminal vesicle sizes occurred in the absence of exogenous insulin. The rescue of the seminal vesicle size suggests a greater production of testosterone, and the larger overall body size suggests that leptin treatment may regulate growth hormones. Indeed, a recent study by Yu et al. showed that leptin-treated type 1 diabetic mice had higher levels of the growth factor IGF-1 than the untreated controls (Yu, Park, Wang, Wang, & Unger, 2008). Additionally, leptin- and insulin-signaling pathways are known to converge at the level of PI3-kinase, so leptin may be able to induce many of the same biochemical reactions as insulin.

**Fertility of leptin-treated Akita homozygotes**: Akita homozygous mice are deficient in spermatocytes and spermatids, resulting in infertility (Schoeller et al., 2012). To determine whether leptin monotherapy could prevent infertility in type 1 diabetic Akita mice, homozygotes were injected with 100 μg of leptin (or PBS as a control) twice per day for two weeks. Akita homozygotes treated with leptin had a larger overall body size (17.4 g vs. 10.2 g), larger testes (0.093 g vs. 0.012 g), and larger seminal vesicles (0.089 g vs. 0.045 g) than untreated Akita homozygote siblings **(Fig. 3-1)**. The testes of PBS-treated Akita homozygotes contained no mature spermatozoa, a high-degree of vacuolization, and large, multinucleated cells. By contrast, leptin-treated Akita homozygous mice possessed all of the cell types within the testes, including

mature spermatozoa (**Fig 3-2A**). Additionally, sperm from leptin-treated mice were significantly more motile than those from untreated mice (**Fig 3-2B**). Although leptin-treated Akita homozygous mice could not successfully sire pups, sperm obtained from leptin-treated Akita homozygotes could fertilize wild-type oocytes at a similar rate as wild-type sperm (57% +/- 4% vs. 45.5% +/- 15.5%) (**Fig. 3-2C**). Of those oocytes that fertilized, (75 +/- 10) % of those from leptin-treated sperm developed to the blastocyst stage, whereas (76 +/- 2) % of those from wild-type sperm developed to the blastocyst stage (**Fig 3-2D**). These findings suggest that the sperm from leptin-treated Akita homozygotes were functional, but that mating behavior was abnormal. In support of this idea, video footage of co-housed mice indicated that leptin-treated Akita homozygotes did not mount female mice.

**Serum leptin and fat mass in leptin-treated Akita Mice:** Despite injecting high concentrations of leptin (ten times that used to rescue leptin-deficient *ob/ob* males(Mounzih, Lu, & Chehab, 1997)) into Akita homozygous mice, serum leptin levels of these mice remained quite low. Akita homozygous leptin levels were frequently below the detectable limit of the assay, so they were probably even lower than 0.2 ng/mL reported here. After two weeks of treatment, leptin levels rose to 1.04 ng/ml (+/- 0.37) compared to 0.409 ng/ml (+/- 0.13) for untreated Akita homozygotes. Serum leptin was 1.55 ng/ml (+/- 0.39) in Akita heterozygotes and 6.13 ng/ml (+/- 1.14) in wild-type mice (**Fig 3-3A**). Thus, a low threshold of leptin is required to prevent the arrest of spermatogenesis. These data are in accordance with previous literature showing that high serum leptin levels do not seem to be required for the reversal of hyperglycemia in a streptozotocin-induced mouse model of type 1 diabetes (Fujikawa et al., 2010). Similarly, Akita heterozygotes have low serum leptin levels but maintain spermatogenesis. Peripheral fat storage also did not increase significantly in leptin-treated Akita homozygous mice, and perigonadal fat

pads remained very small or absent in leptin-treated Akita homozygotes (**Fig. 3-3B**), indicating that fat storage was not drastically increased in these mice. Analysis by EchoMRI indicated that leptin-treated Akita homozygotes had a slightly higher percentage of body fat than the untreated siblings (14.4% vs. 11.48%), (+/- 1.33 and 1.30, respectively) but their percent body fat was still much lower than that of wild-type mice (19.16%) (+/- 0.89) (**Fig. 3-3C**). Thus, it is not the presence of adipose tissue that is critical for the maintenance of fertility, but the presence of sufficiently high leptin levels.

**Gonadotropins in leptin-treated Akita homozygous mice:** To test whether HPG axis activity was restored in the presence of leptin, we measured the gonadotropins LH and testosterone. Although these values increased in leptin-treated Akita homozygotes, the differences did not reach significance. Untreated homozygous Akita mice had (0.045 +/- 0.01) ng/ml LH and (47.7 +/- 7.4) ng/dl testosterone, whereas leptin-treated Akita homozygotes had (0.079 +/- 0.02) ng/ml LH and (197.4 +/- 101) ng/dl testosterone (**Fig. 3-3E and 3-3F**). Because leptin treatment completely rescued spermatogenesis, this suggests that a low threshold of gonadotropins is necessary to maintain spermatogenesis. However, the low level of testosterone may explain why the leptin-treated Akita homozygotes did not mount female mice, as the testosterone levels may be too low to support mating behavior.

As with leptin, measurements of LH and testosterone in Akita homozygotes were frequently below the threshold of detectability. Notably, Akita heterozygotes also have decreased levels of gonadotropin, leptin, and insulin and have extreme hyperglycemia, yet maintain fertility. Although we used a large dose of leptin in these studies, it was still insufficient to completely

rescue the Akita homozygote gonadotropin levels to those of wild type. The requirement for a relatively large dose of leptin to rescue fertility is not surprising considering that the Akita homozygous mice also remain hyperglycemic when treated with doses of insulin that rescue spermatogenesis (**Fig 3-3D**) (Schoeller et al., 2012); this is most likely due to the severity of diabetes in the Akita mouse. It is possible that even larger doses of leptin could further rescue the Akita homozygous diabetic mouse.

**Testosterone Treatment of Akita Homozygous Mice:** Since Akita homozygous mice have depleted levels of testosterone, a hormone critical in the maintenance of spermatogenesis, we hypothesized that testosterone treatment may be sufficient to rescue spermatogenesis in these mice. Slow-release insulin pellets were inserted into Akita homozygous mice at five weeks of age, and left in for four weeks. At the end of the time course, Akita homozygotes had increased seminal vesicle size, indicating higher levels of testosterone, but the testis size did not increase, the seminiferous tubules appeared degenerated, and no mature sperm cells were present. This experiment demonstrates that testosterone alone may not be sufficient to rescue spermatogenesis in the severely insulin and leptin depleted Akita homozygous mice.

**Insulin and glucagon expression in the pancreas:** There is some evidence that leptin treatment improves glucose levels in type 1 diabetic mice through suppression of glucagon. Glucagon is a hormone secreted by the alpha cells of the pancreas in response to low insulin levels, which causes the liver to release glucose into the bloodstream. Previous studies show that glucagon levels are elevated in type 1 diabetic mice, and that leptin treatment lowers glucagon levels back to wild type levels (Yu et al., 2008). Additionally, when glucagon is knocked out, mice do not develop hyperglycemia in response to streptozotocin administration (Lee, Wang, Du, Charron, & Unger, 2011). This has led to the hypothesis that leptin-mediated rescue of type 1 diabetes acts

through glucagon suppression. Thus, we examined histological sections of pancreata from wild type, Akita, and leptin-treated Akita mice to determine whether glucagon levels are reduced in leptin-treated Akita mice. Insulin levels are markedly decreased in Akita heterozygous mice, and severely depleted in homozygous mice. This decrease in insulin is accompanied by an increase in glucagon **(Fig. 3-4)**. Leptin-treated mice have slightly higher expression of insulin, but do not display a decrease in glucagon. This indicates that elevated levels of glucagon persist even with leptin treatment in Akita homozygous mice. This increased glucagon may be responsible for the hyperglycemia in leptin-treated mice. This suggests that the rescue of spermatogenesis is not due to restoration of glucagon levels or normoglycemia.

**Discussion:**

We have previously shown that high doses of insulin can rescue the fertility of Akita homozygous mice, and here we show that high doses of leptin can prevent their testicular atrophy. Leptin treatment was able to completely prevent degeneration of the testes and maintain a functional sperm cell population. It appears that the causative factor of Akita infertility is not a lack of insulin, but a lack of leptin. This is in agreement with data reporting subfertility in leptin-deficient and lipoatrophic mice (Moitra et al., 1998). Uncontrolled diabetes in Akita homozygous mice leads to a severe depletion of adipose tissue, which, in turn, depletes leptin levels. The lack of leptin signals a lack of energy availability to the central nervous system, leading to hypogonadism and infertility (Chan & Mantzoros, 2001; Hausman, Barb, & Lents, 2012). It is likely that supplementing Akita mice with leptin at least partially restores the HPG axis, thereby increasing LH and testosterone above the thresholds necessary to maintain spermatogenesis.

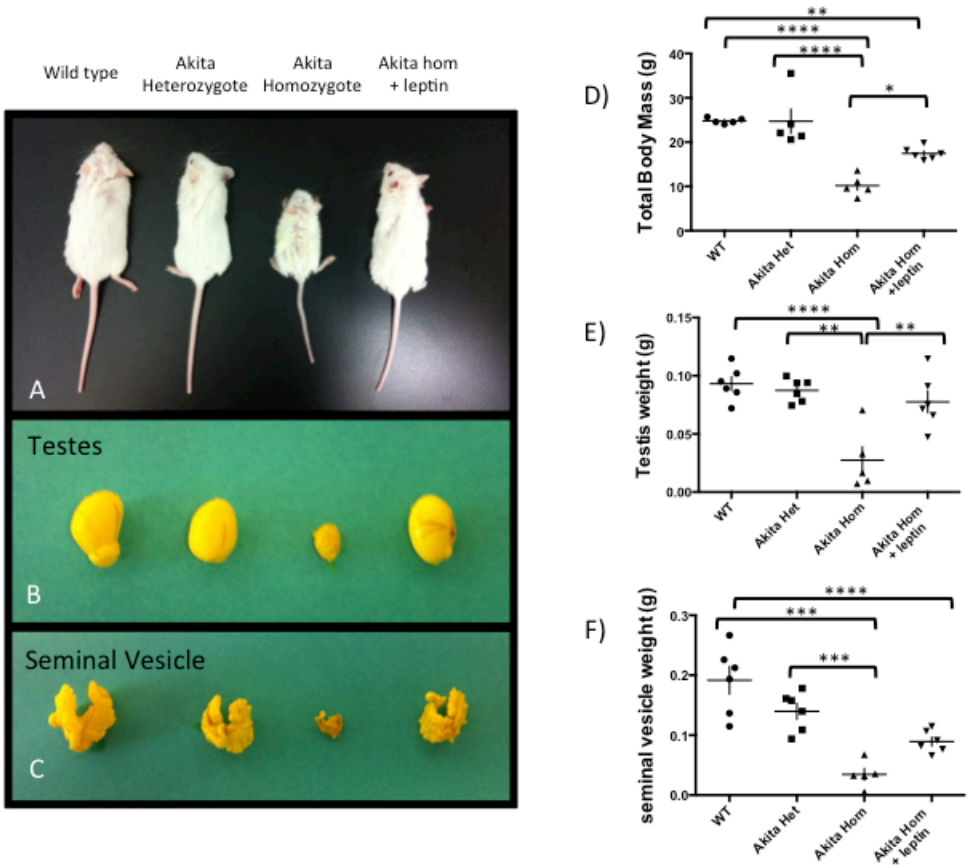



**Figure 3-1:** Leptin treatment rescues body size (A and D), testis size (B and E), and seminal vesicle size (C and F) of Akita homozygous mice. Error bars: standard error of the mean (SEM). ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$. n = 5 for wild type, Akita het, and Akita hom; n = 6 for Akita hom+leptin.

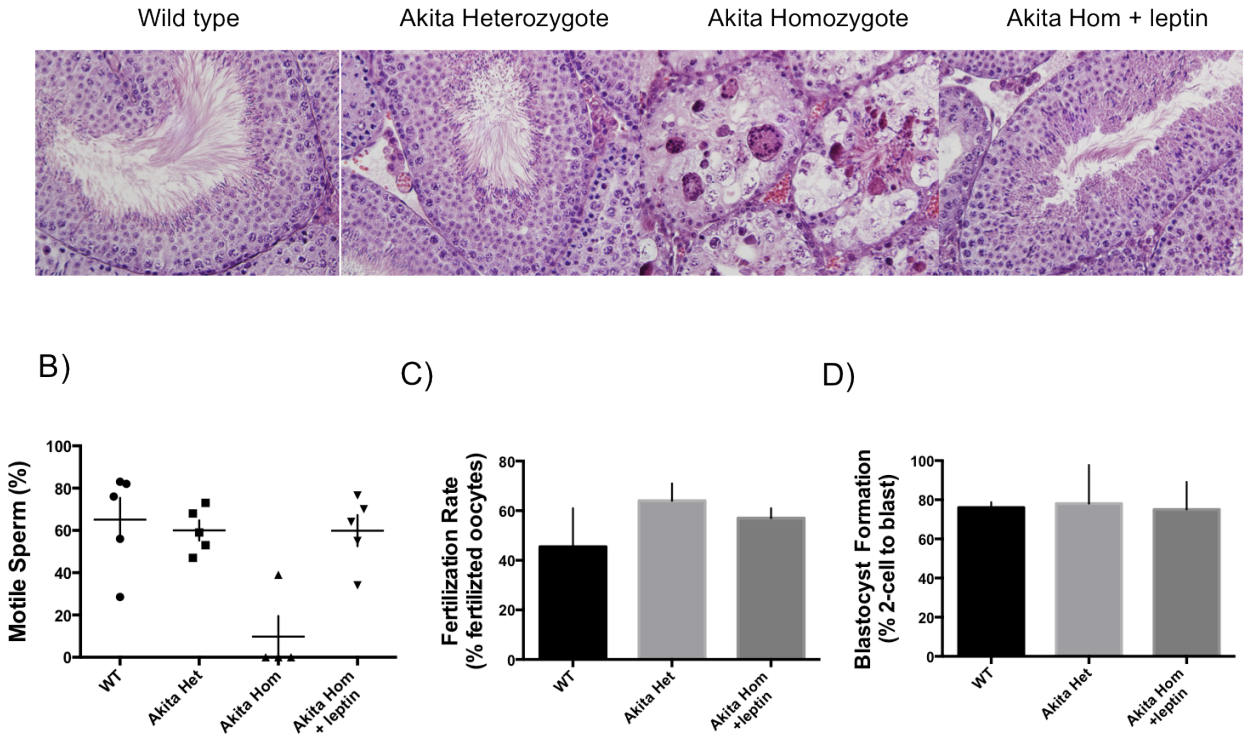



**Figure 3-2:** Leptin treatment of Akita homozygous mice prevents loss of germ cells, increases sperm motility, and results in production of fertilization-competent sperm. H&E sections of the testes (A), computer assisted sperm motility analysis (B), fertilization rate (C), and two-cell-to-blastocyst development rate (D). Arrows in A indicate mature spermatozoa. Error bars: SEM. n = 5 for A and B; n = 2 rounds of IVF (100-200 oocytes each group) for C; n = 2 mice (50-100 two-cell embryos each group) for D. ** $p<0.01$

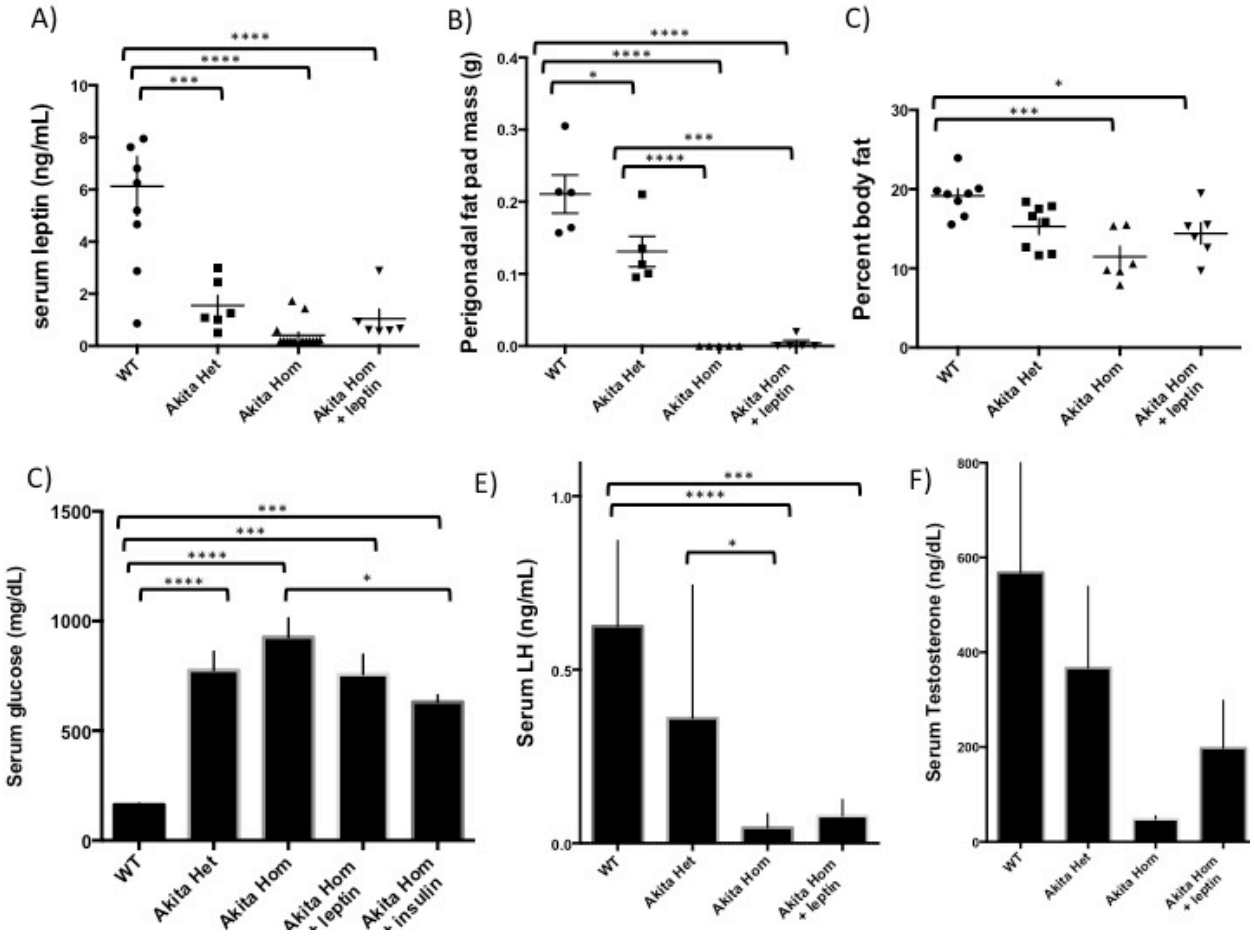



**Figure 3-3:** Serum leptin levels (A), perigonadal fat pad mass (B), total body fat (C), serum blood glucose (D), serum LH (E), and serum testosterone (F) in wild type and Akita mice. Error bars: SEM. $n \geq 6$ for A and C; $n \geq 5$ for B and D; $n \geq 7$ for E. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$

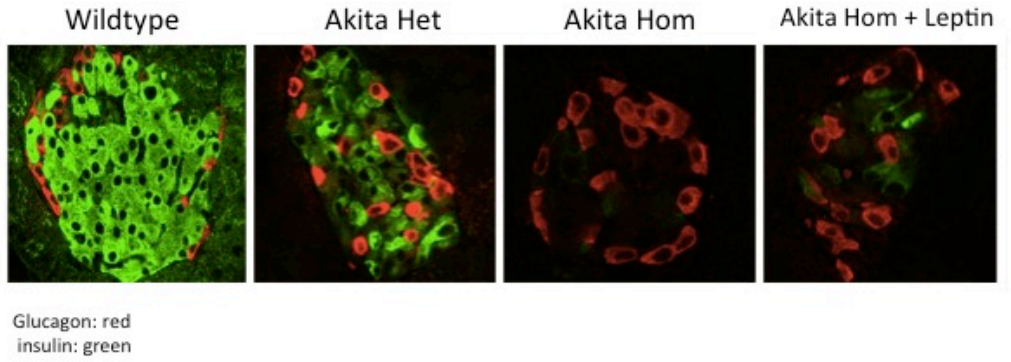



**Figure 3-4:** Pancreatic insulin and glucagon in leptin-treated Akita diabetic mice. Akita heterozygous mice have lower levels of insulin (green) and higher levels of glucagon (red). Akita homozygous mice have drastically reduced levels on pancreatic insulin and increased levels of glucagon. Treatment of Akita homozygous mice with leptin mildly increases insulin expression but does not decrease glucagon expression. Green=insulin, Red=glucagon.

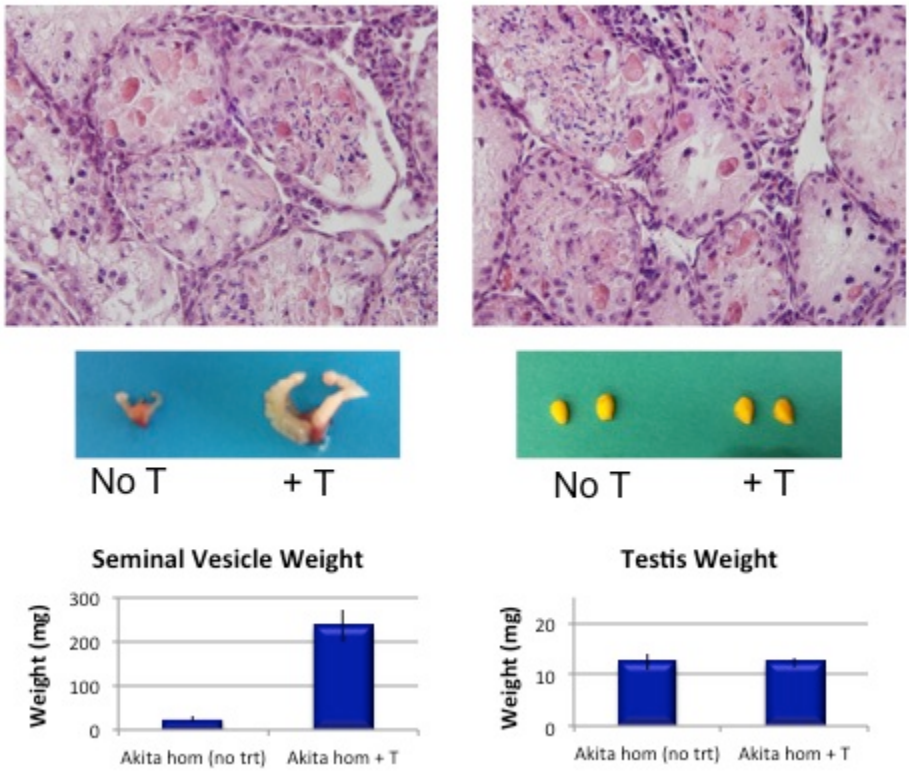



**Figure 3-5:** Testosterone treatment did not rescue spermatogenesis. a) histology of seminiferous tubules. Testosterone treatment had no effect on preventing testicular atrophy in Akita homozygous mice. B) Testosterone increases seminal vesicle weight, an indicator of testosterone production, but not testis size, indicating germ cell depletion.

**References:**


**Agbaje, I. M., Rogers, D. A., McVicar, C. M., McClure, N., Atkinson, A. B., Mallidis, C., & Lewis, S. E. (2007). Insulin dependant diabetes mellitus: implications for male reproductive function. *Hum Reprod, 22*(7), 1871-1877. doi: 10.1093/humrep/dem077**

**Ballester, J., Munoz, M. C., Dominguez, J., Rigau, T., Guinovart, J. J., & Rodriguez-Gil, J. E. (2004). Insulin-dependent diabetes affects testicular function by FSH- and LH-linked mechanisms. *J Androl, 25*(5), 706-719.**

**Banerji, M. A., Faridi, N., Atluri, R., Chaiken, R. L., & Lebovitz, H. E. (1999). Body composition, visceral fat, leptin, and insulin resistance in Asian Indian men. *J Clin Endocrinol Metab, 84*(1), 137-144.**

**Buettner, C., Muse, E. D., Cheng, A., Chen, L., Scherer, T., Pocai, A., . . . Rossetti, L. (2008). Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. *Nat Med, 14*(6), 667-675. doi: nm1775 [pii] 10.1038/nm1775**

**Callaghan, B. C., Little, A. A., Feldman, E. L., & Hughes, R. A. (2012). Enhanced glucose control for preventing and treating diabetic neuropathy. *Cochrane Database Syst Rev, 6*, CD007543. doi: 10.1002/14651858.CD007543.pub2**

**Chan, J. L., & Mantzoros, C. S. (2001). Leptin and the hypothalamic-pituitary regulation of the gonadotropin-gonadal axis. *Pituitary, 4*(1-2), 87-92.**

**Chou, S. H., Chamberland, J. P., Liu, X., Matarese, G., Gao, C., Stefanakis, R., . . . Mantzoros, C. S. (2011). Leptin is an effective treatment for hypothalamic amenorrhea. *Proc Natl Acad Sci U S A, 108*(16), 6585-6590. doi: 10.1073/pnas.1015674108**

**Dua, A., Hennes, M. I., Hoffmann, R. G., Maas, D. L., Krakower, G. R., Sonnenberg, G. E., & Kissebah, A. H. (1996). Leptin: a significant indicator of total body fat but not of visceral fat and insulin insensitivity in African-American women. *Diabetes, 45*(11), 1635-1637.**

**Fujikawa, T., Chuang, J. C., Sakata, I., Ramadori, G., & Coppari, R. (2010). Leptin therapy improves insulin-deficient type 1 diabetes by CNS-dependent mechanisms in mice. *Proc Natl Acad Sci U S A, 107*(40), 17391-17396. doi: 10.1073/pnas.1008025107**

**Goumenou, A. G., Matalliotakis, I. M., Koumantakis, G. E., & Panidis, D. K. (2003). The role of leptin in fertility. *Eur J Obstet Gynecol Reprod Biol, 106*(2), 118-124.**

**Hanaki, K., Becker, D. J., & Arslanian, S. A. (1999). Leptin before and after insulin therapy in children with new-onset type 1 diabetes. *J Clin Endocrinol Metab, 84*(5), 1524-1526.**

**Hausman, G. J., Barb, C. R., & Lents, C. A. (2012). Leptin and reproductive function. *Biochimie, 94*(10), 2075-2081. doi: 10.1016/j.biochi.2012.02.022**

**Havel, P. J., Uriu-Hare, J. Y., Liu, T., Stanhope, K. L., Stern, J. S., Keen, C. L., & Ahren, B. (1998). Marked and rapid decreases of circulating leptin in streptozotocin diabetic rats: reversal by insulin. *Am J Physiol, 274*(5 Pt 2), R1482-1491.**

**Kersten, S. (2001). Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep, 2*(4), 282-286. doi: 10.1093/embo-reports/kve071 2/4/282 [pii]**

**Krolewski, A. S., & Bonventre, J. V. (2012). High risk of ESRD in type 1 diabetes: new strategies are needed to retard progressive renal function decline. *Semin Nephrol, 32*(5), 407-414. doi: S0270-9295(12)00126-X [pii] 10.1016/j.semnephrol.2012.07.002**

**La Vignera, S., Condorelli, R., Vicari, E., D'Agata, R., & Calogero, A. E. (2012). Diabetes mellitus and sperm parameters. *J Androl, 33*(2), 145-153. doi: 10.2164/jandrol.111.013193**

**Lee, Y., Wang, M. Y., Du, X. Q., Charron, M. J., & Unger, R. H. (2011). Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice. *Diabetes, 60*(2), 391-397. doi: 10.2337/db10-0426**

**Lowry, OH, Passonneau, JV. (1993). *Enzymatic Analysis: A Practical Guide* (1 ed.): Springer-Verlag, New York, LLC.**

**Mallidis, C., Agbaje, I., O'Neill, J., & McClure, N. (2009). The influence of type 1 diabetes mellitus on spermatogenic gene expression. *Fertil Steril, 92*(6), 2085-2087. doi: 10.1016/j.fertnstert.2009.06.006**

**Moitra, J., Mason, M. M., Olive, M., Krylov, D., Gavrilova, O., Marcus-Samuels, B., . . . Vinson, C. (1998). Life without white fat: a transgenic mouse. *Genes Dev, 12*(20), 3168-3181.**

**Mounzih, K., Lu, R., & Chehab, F. F. (1997). Leptin treatment rescues the sterility of genetically obese ob/ob males. *Endocrinology, 138*(3), 1190-1193.**

**Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E., & Mori, M. (2002). Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest, 109*(4), 525-532. doi: 10.1172/JCI14550**

**Rathsman, B., Rosfors, S., Sjoholm, A., & Nystrom, T. (2012). Early signs of atherosclerosis are associated with insulin resistance in non-obese adolescent and young adults with type 1 diabetes. *Cardiovasc Diabetol, 11*, 145. doi: 1475-2840-11-145 [pii] 10.1186/1475-2840-11-145**

**Schoeller, E. L., Albanna, G., Frolova, A. I., & Moley, K. H. (2012). Insulin rescues impaired spermatogenesis via the hypothalamic-pituitary-gonadal axis in Akita diabetic mice and restores male fertility. *Diabetes, 61*(7), 1869-1878. doi: 10.2337/db11-1527**

**Swerdloff, R. S., Batt, R. A., & Bray, G. A. (1976). Reproductive hormonal function in the genetically obese (ob/ob) mouse. *Endocrinology, 98*(6), 1359-1364.**

**Watanobe, H. (2002). Leptin directly acts within the hypothalamus to stimulate gonadotropin-releasing hormone secretion in vivo in rats. *J Physiol, 545*(Pt 1), 255-268.**

**Yu, X., Park, B. H., Wang, M. Y., Wang, Z. V., & Unger, R. H. (2008). Making insulin-deficient type 1 diabetic rodents thrive without insulin. *Proc Natl Acad Sci U S A, 105*(37), 14070-14075. doi: 10.1073/pnas.0806993105**

# Chapter 4: Glucose Transporters in Sperm cells

**Abstract:**

Sperm cells use a variety of glucose transporters (GLUTs) to regulate hexose transport. A total of 14 members of the GLUT family have been described, but there is still a lack of complete understanding of the identity and function of the various GLUTs present in sperm cells. We show that GLUT1 and GLUT4 are present in both human and mouse sperm cells by western blot and by confocal microscopy. In mouse sperm cells, GLUT1 and GLUT4 are localized to the acrosome, and in human sperm cells, GLUT1 localizes to the acrosome while GLUT4 localizes to the tailpiece. We additionally examined whether GLUT4 expression would change with the induction of capacitation or acrosome reaction. Capacitation had no effect on either the abundance or localization of glucose transporter expression. We then show that both GLUT1 and GLUT4 are critical for regulating glucose uptake and fertilization. Sperm cells treated with inhibitors of GLUT1 and GLUT4 have a decreased ability to take up glucose and a decreased ability to fertilize oocytes *in vitro.* We conclude that GLUT1 and GLUT4 are critical for maintaining glucose influx and providing energy for the events leading to fertilization.

**Introduction:**

Mature sperm cells have high metabolic demands to support motility, and thus the regulation of glucose and fructose entry into these cells is critical to fertility. Hexose transport during the life of a sperm cell in not fixed, but rather a dynamic process dependent on the environment of the sperm cell. Spermatogenesis begins in the seminiferous tubules of the testis, where sperm cells utilize primarily lactate provided by the Sertoli cells. Sperm cells are then released from the testis and enter the epididymis, where they use glucose and lactic acid (Voglmayr & White, 1979), and then move into the vas deferens and finally are ejaculated with the seminal fluid, which contains a high percentage of fructose as well as glucose (Martikainen, Sannikka, Suominen, & Santti, 1980). Once the sperm cell enters the uterine environment, it uses primary glucose as well as lactose (Hamner, 1973). The female tract triggers dramatic changes in sperm cell metabolism, and a sperm cell must undergo a specific set of reactions before it is capable of fertilization. These reactions include capacitation, hyperactivation, and the acrosome reaction. Capacitation refers collectively to a number of known changes in sperm cell metabolism, including tyrosine phosphorylation, cholesterol efflux, calcium influx, an increase in intracellular cAMP, and an increase in pH. Interestingly, the fluids in the uterine and oviduct contain compounds that inhibit capacitation, seemingly to extend the lifespan of the sperm until ovulation (Hunter & Rodriguez-Martinez, 2004). Upon ovulation, signals from the oocyte and cumulus cell trigger capacitation and hyperactivation and activate the sperm cells for fertilization (Pavlok & McLaren, 1972; Rodriguez-Martinez, 2007). In this way, sperm metabolism is tightly regulated so that sperm cells are prepared to fertilize when an oocyte is present. The metabolic changes involved in sperm activation coupled with the availability of different substrates in different environments

requires the regulation of energy influx, which can be mediated by changes in utilization of the various glucose transporters.

Prior to fertilization, sperm cells must undergo capacitation, hyperactivation and the acrosome reaction. Sperm cells also initiate hyperactivated motility once in the female tract in response to signals from the egg. This hyperactivated motility is thought to help the sperm cell penetrate the cumulus cell layer surrounding the egg as well as help penetrate the zona pellucida. Sperm cells also must undergo the acrosome reaction prior to fertilization. The acrosome reaction involves the release of digestive enzymes, such as hyaluronidase and acrosin, from the head of the sperm that help to break through the cumulus layer of the egg The acrosome reaction occurs near the site of fertilization and can be induced by signals from the egg, including progesterone and ZP3, or by calcium ionophores (Florman, Jungnickel, & Sutton, 2008). It is possible that changes in glucose transport expression can help modulate the rapid metabolic changes experienced by sperm cells preceding fertilization.

Sperm cells encounter a variety of cellular and metabolic environments on their journey from the testes where they develop, to the oviduct where they fertilize an egg. In the testis, sperm cells utilize primarily lactate and pyruvate as energy substrates. When the sperm move into the epididymis, they also use glucose as an energy substrate. As they mix with seminal fluid seminal, sperm cells use primarily fructose as an energy source as well as glucose, sorbitol, and mannose. (Cao, Aghajanian, Haig-Ladewig, & Gerton, 2009; Frenkel, Peterson, & Freund, 1975). Finally, once sperm cells enter the female reproductive tract, the environment contains largely glucose and fructose, as well as lactate and pyruvate, though they preferentially use glucose as an energy

substrate (Leese, Astley, & Lambert, 1981). Each GLUT has a different affinity for various substrates, so the unique set of transporters present within sperm cells help the cell to adapt to these changing environments.

Sperm cells contain a specific set of glucose transporters (GLUTs) that facilitatively transport hexoses and other substrates into the cells. Each different glucose transporter has the ability to move different hexoses into cells, not only glucose but also fructose, inositol, urate, and certain vitamins. Each GLUT transports a unique subset of substrates with differing affinities. Glucose transporters fall into one of three classes. Class I transporters include GLUTs 1, 2, 3, 4, 5 and 14, and are the most well characterized GLUTs as they were the first to be identified. GLUT14 was more recently discovered, but it is included in Class I as it is a result of a gene duplication of GLUT3 and shares 95% homology to the GLUT3 protein (Wu X, Freeze HH 2002). Class II transporters include GLUTs 5, 7, 9, and 11 and they are known primarily as fructose transporters, though they are able to transport other substrates as well. Class III transporters include GLUTs 6, 8, 10, and 12 and are notable for an intracellular localization motif. By varying the expression of these different glucose transporters, cells can regulate their metabolism by utilizing the various available substrates. Sperm cells are unique in that they traverse through a variety of environments from their development in the testis to the site of fertilization in the oviduct, and thus they require a unique set of glucose transporters to regulate the influx of the different substrates they use throughout their journey from the testis to the egg.

Several glucose transporters have been identified in sperm cells. GLUTs 1, 2, 3, and 5 have been previously identified in sperm cells, as demonstrated by RT-PCR, immunoblot and immunolocatization, while low levels of GLUT4 were detected only by RT-PCR, and not seen

by immunoblot or immunolocalization (Galeati 2008)(Angulo et al., 1998). In dog sperm, GLUT3 and GLUT5 were identified as the main hexose transporters, and GLUT3 displayed localization to the midpiece while GLUT5 localized to the acrosome (Rodríguez-Gil 2002). GLUTs 1, 2, 3, and 5 were reexamined in dog, boar, and stallion spermatozoa (Galeati 2008), with GLUTs 3 and 5 exhibiting the strongest signals and only GLUTs in dog spermatozoa displaying redistribution of glucose transporters upon capacitation. More recently, Kim and Moley reported the presence of GLUT 8 and GLUT9 in mouse sperm and show that both GLUT isoforms decreased expression in diabetic mice while the expression of GLUTs 1, 3, and 5 remained the same. (Kim and Moley 2008). GLUT8 was shown to translocate from the midpiece to the acrosome upon stimulation with insulin, suggesting that GLUT may be an insulin-sensitive transporter in sperm cells (du Plessis 2010). Additionally, GLUT8 knockout mice have sperm cells with decreased motility, decreased APT, and decreased mitochondrial membrane potential, suggesting an important role for this glucose transporter in sperm cell metabolism (Annette Schürmann 2008.) While a significant body of work shows a variety of glucose transporters in sperm cells, a complete evaluation of all the glucose transporters has yet to be completed.

The primary goal of this research is to provide a more thorough evaluation of two of the glucose transporters present in mouse and human sperm cells. We focus on GLUT1 because it is an important basal glucose transporter in all tissues and glucose is critical for sperm hyperactivated motility (Williams & Ford, 2001). We then focus on GLUT4 as it has not been previously identified in sperm cells, but its traditionally insulin-sensitive nature makes it intriguing, especially considering new evidence of insulin transcription and function within the sperm and testes (Aquila 2005, Gomez 2009, Aquila 2009).

**Methods:**

**Sperm cell collection:** Human sperm samples were collected, allowed to liquefy at room temperature for at least 30 minutes. Mouse sperm cells were dissected from the cauda epididymis and collected in Ham's F10 media. Sperm cells were placed over a 60% Percoll gradient and centrifuged for 30 minutes at 800g to obtain a pure sperm pellet. Sperm cells were then washed with Ham's F10 media.

**RNA isolation and cDNA synthesis:** Human sperm cell RNA was extracted using RNeasy mini kit (Qiagen) with the following modifications to manufacturer's instructions: Cells were incubated with RLT buffer plus BME for 10 minutes prior to passage through a 26-guage needle. Additionally, nuclease-free water was heated to 37 °C and then incubated on the membrane for eight minutes prior to RNA elution. Total RNA from mouse sperm cells was extracted from using Trizol Reagent (Invitrogen) according to manufacturers instructions.

**Immunoblot:** Sperm cells were lysed in RIPA buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1%, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl) and sonicated for 10 seconds. Extracted protein was quantified by BCA assay (Pierce), and 30 ug of protein was loaded into an SDS page gel at 120V. Protein was transferred to a nitrocellulose membrane and blocked with 5% milk in TBST. Membranes were probes with primary antibody (GLUT1 1:2000; GLUT4 1:1000) (GLUT1 and GLUT4 antibodies were kindly provided by Dr. Mike Mueckler) overnight at 4°C. Membranes were washed in TBST, probed with goat-anti rabbit secondary (Jackson 1:10,000) for one hour at room temperature, washed, and visualized using enhanced chemiluminescence

reagent (Amersham, Piscataway, NJ) or West Pico Chemiluminescent Substrate (Thermo Scientific).

**Immunofluorescence:** Sperm cells were fixed in 3.2% paraformaldehyde and then permeabilized and blocked for one hour at room temperature in a solution of 2% bovine serum albumin (BSA), 0.5% Triton X-100, 5% normal goat serum in PBS. Cells were then incubated overnight in primary antibody (1:200) (GLUT1 and GLUT4 antibodies were kindly provided by Dr. Mike Mueckler). Cells were then washed in PBS/2% BSA, incubated for one hour in goat anti-rabbit secondary antibody conjugated to an Alexa fluorophore (Invitrogen), and ToPro nuclear stain (Molecular Probes). PNA-lectin (50 uM) (Invitrogen) was incubated with the secondary antibody. Cells were mounted with Vectashield and visualized using a Nikon Eclipse E800 confocal microscope.

**Glucose Uptake**: Sperm cells were collected and purified, and incubated in Krebs Ringer/1% BSA with 100 um Indinavir, 50 um ritonavir, or vehicle control for 30 minutes. Cells were then centrifuged at 800g and then resuspended in 50ul of Krebs Ringer with 2uc of 3-H deoxyglucose. Uptake was measure for 5 minutes and then stopped by the addition of ice-cold PBS and cytochalasin b. Cells were then washed 2X in PBS and then lysed in RIPA buffer. CPMs in lysate were counted in econosafe liquid scintillation fluid (Fisher Scientific, Waltham, MA).

**Results:**

**GLUT1 Protein Expression:** GLUT1 protein expression was detected by western blot. To determine whether GLUT1 expression changed as a result of capacitation or acrosome reaction, sperm cells were incubated in noncapacitating conditions (media without bovine serum albumin or sodium bicarbonate), capacitating conditions (media with bovine serum albumin and sodium bicarbonate), or acrosome reaction inducing conditions (capacitating media followed by the addition of a calcium ionophore, A23187). GLUT1 expression is upregulated under capacitating conditions compared to noncapacitating (**Fig. 4-5 and Fig. 4-6)**. This is not surprising, considering that sperm cells acquire hyperactivated motility under capacitating conditions and need more ATP to support this increased motility. Sperm cells also increase glycolytic activity during sperm fusion and decondensation, indicating an increased need for glucose during fertilization (Urner & Sakkas, 1999).

**GLUT4 Protein Expression:** GLUT4 protein expression was unchanged in response to noncapacitating, capacitating, or acrosome reacting conditions **(Fig. 4-5 and Fig. 4-6).** This would suggest that GLUT4 does not play a role in mediating hexose utilization in response to these metabolic changes. Since GLUT4 is known for its ability to translocate in response to insulin, it is also possible that while the amount of total protein changes, GLUT 4 translocates in response to either capacitation of the acrosome reaction.

**GLUT1 localization**: Confocal microscopy was used to localize GLUT1 on human **(Fig. 4-1)** and mouse sperm cells **(Fig. 4-2)**. In human sperm, GLUT1 localizes strongly to the acrosomal

region. One of the more interesting features was the lack of uniform staining in a field of sperm cells. Only a percentage of sperm cells displayed positive signal for GLUT1. To further elucidate the reason for this, we incubated sperm cells under three different conditions to determine whether capacitation or acrosomal reaction may play a role in GLUT1 regulation. To examine whether GLUT1 is important for the acrosome reaction, we co-immunostained for GLUT1 and PNA-lectin, an acrosomal marker. The absence of PNA stain corresponds to sperm cells that have undergone the acrosome reaction. In human sperm cells, GLUT1 expression appeared primarily in the absence of an acrosome, though a few sperm cells with intact acrosomes still displayed GLUT1 expression. In mouse sperm cells, GLUT1 co-localized with acrosome-intact sperm. This disparity suggests species-specific roles for GLUT1in sperm cells.

**GLUT4 localization:** In humans, GLUT4 is restricted to the midpiece of the sperm cell **(Fig. 4-3)**. In mice, GLUT 4 localizes to the acrosome of the sperm cell as well as the tail **(Fig. 4-4)**. It is important to note that not all acrosome-intact sperm cells display acrosomal GLUT4, but no acrosome-reacted sperm cells displayed acrosomal GLUT4.

**Glucose Uptake:** Since the presence of GLUT4 in mouse and human sperm cells conflicts with previously published data, we next sought to establish the effects of blocking GLUT4 function in sperm cells with indinavir. Indinavir is a rapid and reversible specific inhibitor of GLUT4 (Hresko & Hruz, 2011). The treatment of sperm cells with indinavir significantly decreased the uptake of radio-labeled glucose into sperm cells. In human sperm cells, treatment with 100um indinavir reduced glucose uptake by 27%, while in mouse 100um indinavir reduced uptake by 54% **(Fig. 4-7)**. We also tested glucose uptake in the presence of ritonavir, an inhibitor of both

GLUT1 and GLUT4. Ritonavir inhibited glucose uptake significantly more than indinavir alone, suggesting that GLUT1 also plays an important role in glucose metabolism by sperm cells. Treatment of sperm cells with 50um of ritonavir reduced glucose uptake by 60% in human and 77% in mouse **(Fig. 4-7)**.

**In Vitro Fertilization:** To determine whether GLUT1 or GLUT4 are important in fertilization, we performed *in vitro* fertilization in the presence of indinavir or ritonavir. Indinavir treatment (200um) reduced fertilization by 27%. Interestingly, indinavir also inhibited blastocyst formation, indicating a potential role for GLUT4 in embryo development. Culture with 100um indinavir reduced blastocyst formation by 29%, while culture with 200um indinavir resulted in no blastocyst formation **(Fig. 4-8)**. This is a perplexing result as the oocyte has minimal capacity for glucose transport, as GLUT4 is not known to be expressed in the murine preimplantation embryo (Hogan *et al.*1991, Aghayan *et al.* 1992). Santos 2004 Contrasting studies show GLUT4 expression in the preimplantation embryo Santos 2004. Our data suggest that GLUT4 is important for preimplantation embryo development, and its inhibition either causes paternal damage to the sperm cell or directly affects the growing embryo. To test this, we designed an experiment to determine whether blastocyst formation was inhibited by the effect of indinavir on sperm cells or on embryo culture. *In vitro* fertilization was performed using three separate culture conditions, (1) sperm cells capacitation with indinavir, (2) sperm capacitation and fertilization with indinavir, and (3) sperm capacitation, fertilization, and embryo culture with indinavir. Our data show that culture of embryos with indinavir does not allow for the development of blastocysts from 2-cell embryos, indicating that the inhibition of GLUT4 is adversely affects embryo development **(Sup. Fig. 4-1)**.

**Discussion:**

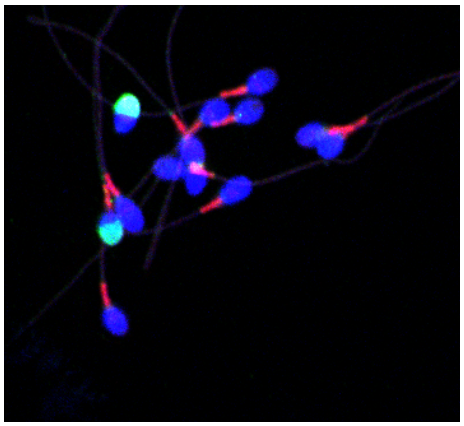
**Importance of Glucose to sperm cells:** The importance of glucose to sperm cell metabolism is well characterized. Sperm cells require glucose to maintain motility and to be competent for fertilization. Glucose enhances the motility and number of acrosome reactions in human sperm cells (Williams & Ford, 2001). Glucose has also been determined to facilitate tyrosine phosphorylation (cooper 1984), bind to the zona pellucida of the oocyte (Urner & Sakkas, 1996b), and penetration of the zona pellucida for fertilization (Urner 2001). However, glucose does not appear to be required for zona pellucida-induced acrosome reactions (Urner & Sakkas, 1996a), signifying that glucose is not a requirement for induction of the acrosome reaction, though it may play a role. A recent study characterized the substrates required for sperm cells to maintain ATP, hyperactivation, and tyrosine phosphorylation. (Goodson et al., 2012). The authors found that glucose was required for hyperactivation. Thus, it is clear that glucose is critical for many of the steps leading to sperm competence and fertilization. The unique set of glucose transporters in sperm cells may be an important way in which sperm cells modulate their metabolism to promote successful fertilization reactions.

**Sperm cell metabolism pathways:** Glucose in sperm cells is metabolized through the pentose phosphate pathway and glycolysis. These metabolic pathways appear to be compartmentalized in sperm cells, with glycolysis occurring in principal piece, the location of the mitochondrial sheath, and the pentose phosphate pathway restricted to the midpiece (Urner & Sakkas, 2005; Westhoff & Kamp, 1997). Additionally, a recent proteomic analysis of the sperm tail revealed a significant number of proteins involved in carbohydrate metabolism, but also those of fatty acid

oxidation, demonstrating that sperm cells may utilize another source of energy. Indeed, treatment of sperm cells with Extomir, an inhibitor of mitochondrial fatty acid oxidation, significantly decreased sperm motility (Amaral et al., 2013). Collectively, these data demonstrate the ability of sperm cells to utilize a variety of substrates as energy sources during their journey from the testis to the egg.

**Insulin in sperm cells:** The presence of GLUT4 on sperm cells is a novel finding and may be important in regulating key events during sperm capacitation and fertilization. The presence of an insulin-responsive glucose transporter in sperm cells provides a potential role for insulin within the sperm cell. This is especially interesting given the growing body of research detailing insulin production and secretion by sperm cells. Aquila et al. first identified sperm protein and mRNA transcript in human sperm cells (Aquila, Gentile, Middea, Catalano, & Ando, 2005). Additionally, sperm cells secrete insulin in response to increasing glucose concentrations, and this secretion is increased in capacitating conditions, indicating that sperm cells may regulate their metabolism during capacitation. In pig spermatozoa, insulin increases the cholesterol efflux, a key characteristic of capacitation that remodels the plasma membrane. This effect seems to be mediated by insulin receptor, as incubation with an insulin receptor antibody abolishes the effect of insulin on cholesterol efflux (Carpino, Rago, Guido, Casaburi, & Aquila, 2010). Interestingly, the amount of cholesterol efflux of noncapacitated sperm treated with insulin is similar to that of capacitated sperm, suggesting that insulin may play an important role in portions of the capacitation process. Insulin secretion is also greater in capacitated sperm than noncapacitated sperm, supporting this idea (Aquila et al., 2005). The presence GLUT4 in sperm cells provides a potential mechanism by which insulin may regulate these processes. Insulin and glucose

produced by the sperm cell may work together to potentiate capacitation, hyperactivation, or the acrosome reaction.



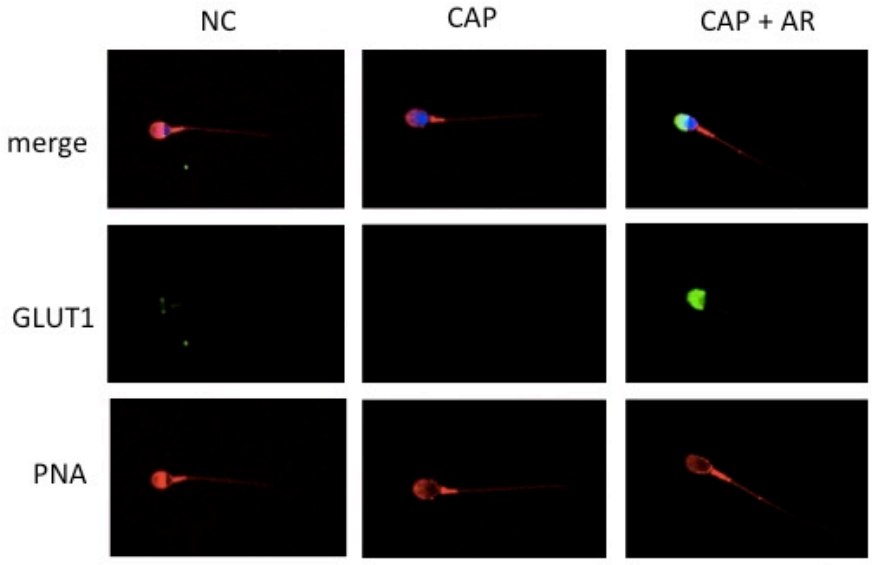



**Figure 4-1: GLUT1 localization in human sperm cells.** GLUT1 localizes strongly to the acrosome of human sperm cells, though only a percentage of sperm cells stain positively for GLUT1. GLUT1 appearance correlates to the absence of an acrosome. Green=GLUT1; red=PNA-lectin; blue=nuclei

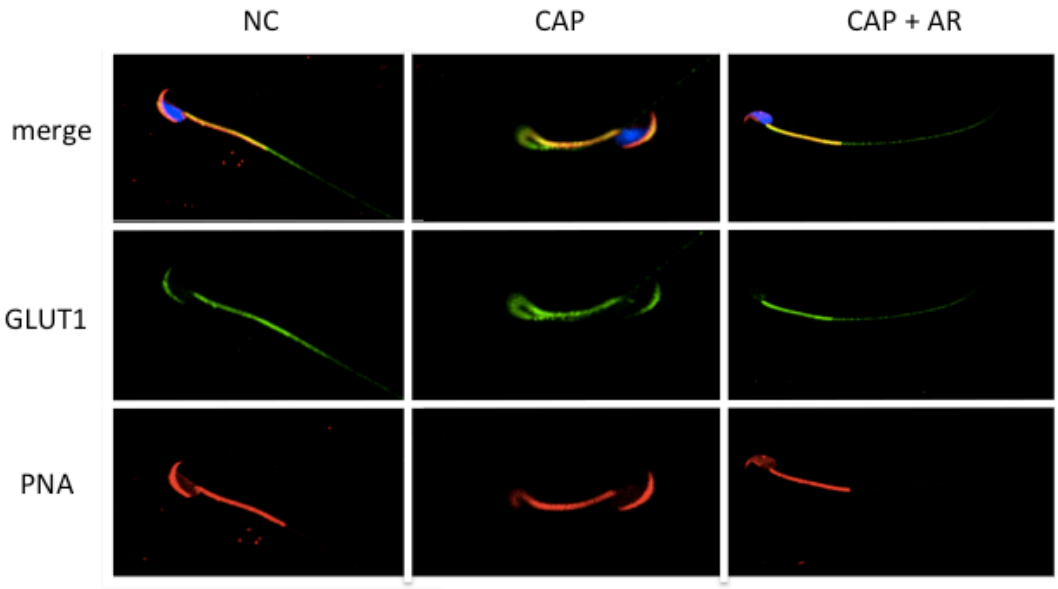



**Figure 4-2: GLUT1 Localization in mouse sperm cells**. GLUT1 localizes strongly to the acrosome. Upon acrosome reaction, GLUT1 dissipates from the acrosome yet remains present in the midpiece. Green = GLUT1; red = PNA-lectin; blue=nuclei

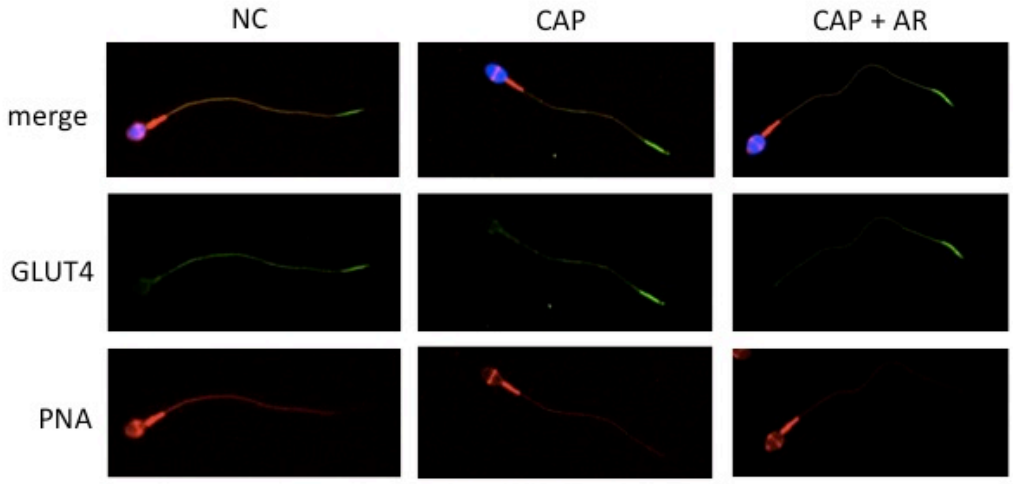



**Figure 4-3: GLUT4 localization in human sperm cells.** GLUT4 localizes to the tailpiece. GLUT4 expression remains unchanged upon capacitation or induction of the acrosome reaction. Green = GLUT1; red = PNA-lectin; blue=nuclei

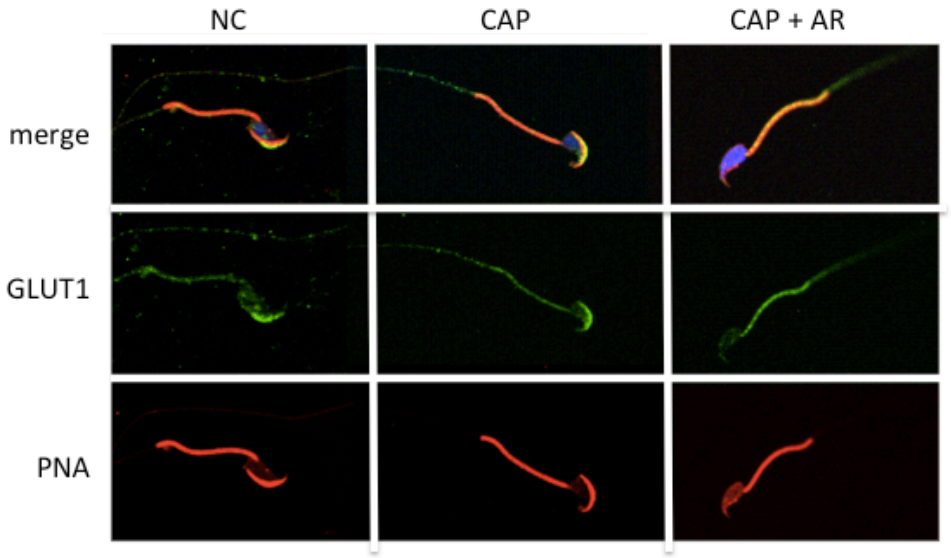



**Figure 4-4: GLUT4 localization in mouse sperm cells.** GLUT4 also localized to the acrosome. After acrosome reaction, GLUT4 expression remains in the midpiece but is absent in the acrosome. Green = GLUT1; red = PNA-lectin; blue=nuclei

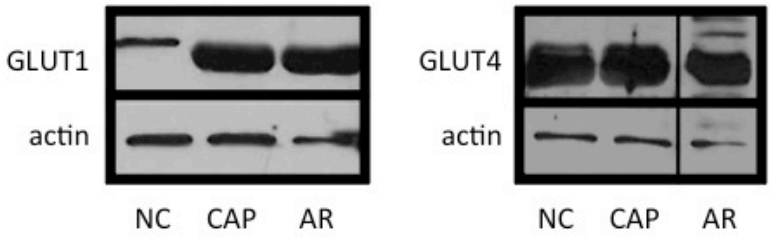



**Figure 4-5: Protein expression of GLUT 1 and GLUT4 in human sperm cells.** GLUT1 expression increases during capacitating conditions and after acrosome reaction. GLUT4 expression remained unchanged upon capacitation and acrosome reaction.


**Figure 4-6: Protein expression of GLUT1 and GLUT4 in mouse sperm cells.** GLUT1 expression increases during capacitating conditions and after acrosome reaction. GLUT4 expression remained unchanged upon capacitation and acrosome reaction.

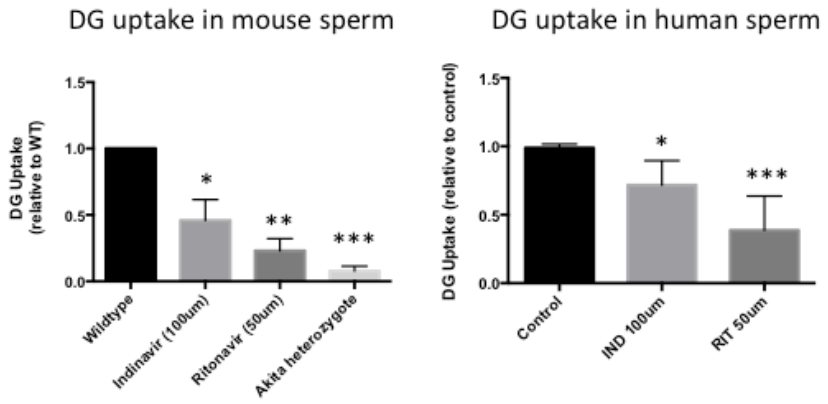



**Figure 4-7: Glucose uptake in mouse and human sperm cells.** Treatment of both human and mouse sperm cells with indinavir (Glut4 inhibitor) decreased the uptake of [3(H)]2-deoxy-D-glucose. Treatment of sperm cells with ritonavir, an inhibitor of both GLUT1 and GLUT4 further decreased glucose uptake in sperm cells. Sperm cells from Akita heterozygous type 1 diabetic mice took up significantly less glucose than control sperm cells. Human sperm n=5, mouse sperm n= 3. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001

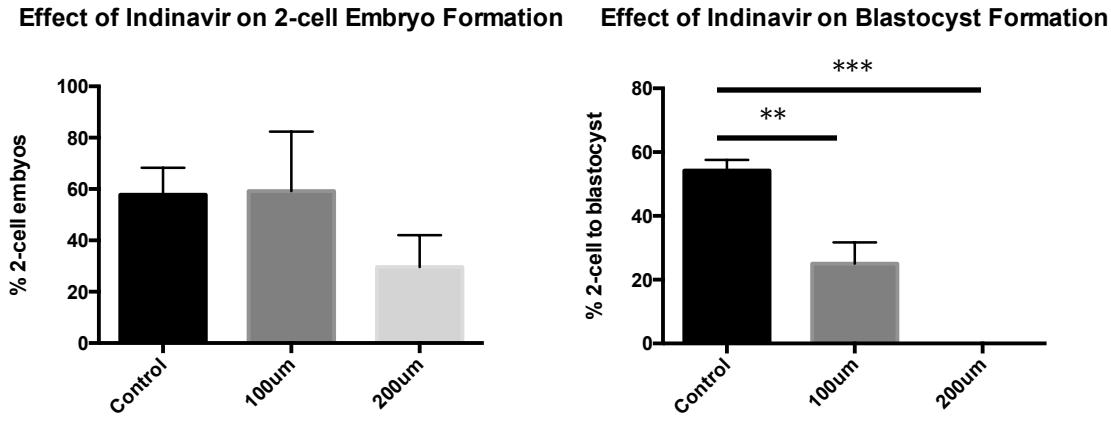
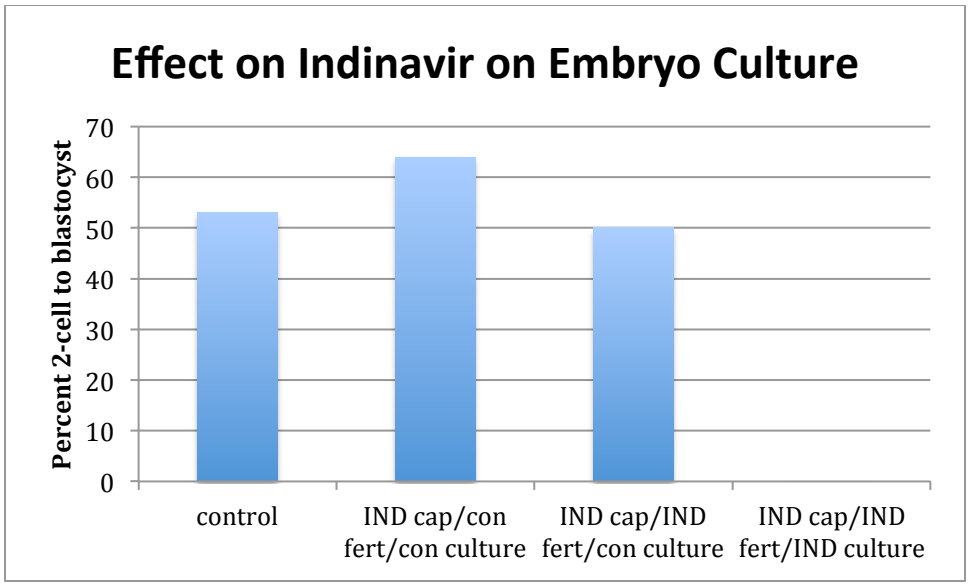



**Figure 4-8: The Effect of GLUT4 inhibition on *in vitro* fertilization and blastocyst formation.** Treatment of sperm cells with 200um of GLUT4 inhibitor, indinavir, significantly reduced fertilization rate. Treatment of 2-cell embryos with indinavir decreased the percentage of 2-cell embryos that developed to blastocysts.




**Supplemental Figure 4-1: Effects of indinavir on 2-cell to blastocyst rate.** 2-cell embryos that were fertilized in the presence of indinavir were able to successfully develop blastocysts, while those that were cultured in the presence of indinavir failed to progress. Column 1 = control; Column 2 = Sperm cells treated with IND during capacitation only; Column 3 = Sperm cells treated with IND during capacitation and during fertilization. Fertilized 2-cell embryos were then moved to IND-free culture medium; Column 4 = Sperm cells treated with IND during capacitation, fertilization, and 2-cell embryos were cultured with IND.

**References:**


**Amaral, A., Castillo, J., Estanyol, J. M., Ballesca, J. L., Ramalho-Santos, J., & Oliva, R. (2013). Human sperm tail proteome suggests new endogenous metabolic pathways. *Mol Cell Proteomics, 12*(2), 330-342. doi: 10.1074/mcp.M112.020552**

**Angulo, C., Rauch, M. C., Droppelmann, A., Reyes, A. M., Slebe, J. C., Delgado-Lopez, F., . . . Concha, II. (1998). Hexose transporter expression and function in mammalian spermatozoa: cellular localization and transport of hexoses and vitamin C. *J Cell Biochem, 71*(2), 189-203.**

**Aquila, S., Gentile, M., Middea, E., Catalano, S., & Ando, S. (2005). Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology, 146*(2), 552-557. doi: 10.1210/en.2004-1252**

**Cao, W., Aghajanian, H. K., Haig-Ladewig, L. A., & Gerton, G. L. (2009). Sorbitol can fuel mouse sperm motility and protein tyrosine phosphorylation via sorbitol dehydrogenase. *Biol Reprod, 80*(1), 124-133. doi: 10.1095/biolreprod.108.068882**

**Carpino, A., Rago, V., Guido, C., Casaburi, I., & Aquila, S. (2010). Insulin and IR-beta in pig spermatozoa: a role of the hormone in the acquisition of fertilizing ability. *Int J Androl, 33*(3), 554-562. doi: 10.1111/j.1365-2605.2009.00971.x**

**Florman, H. M., Jungnickel, M. K., & Sutton, K. A. (2008). Regulating the acrosome reaction. *Int J Dev Biol, 52*(5-6), 503-510. doi: 10.1387/ijdb.082696hf**

**Frenkel, G., Peterson, R. N., & Freund, M. (1975). Oxidative and glycolytic metabolism of semen components by washed guinea pig spermatozoa. *Fertil Steril, 26*(2), 144-147.**

**Goodson, S. G., Qiu, Y., Sutton, K. A., Xie, G., Jia, W., & O'Brien, D. A. (2012). Metabolic substrates exhibit differential effects on functional parameters of mouse sperm capacitation. *Biol Reprod, 87*(3), 75. doi: 10.1095/biolreprod.112.102673**

**Hamner, Charles E. (1973). *Sperm capacitation*. New York,: MSS Information Corp.**

**Hresko, R. C., & Hruz, P. W. (2011). HIV protease inhibitors act as competitive inhibitors of the cytoplasmic glucose binding site of GLUTs with differing affinities for GLUT1 and GLUT4. *PLoS One, 6*(9), e25237. doi: 10.1371/journal.pone.0025237**

**Hunter, R. H., & Rodriguez-Martinez, H. (2004). Capacitation of mammalian spermatozoa in vivo, with a specific focus on events in the Fallopian tubes. *Mol Reprod Dev, 67*(2), 243-250. doi: 10.1002/mrd.10390**

**Leese, H. J., Astley, N. R., & Lambert, D. (1981). Glucose and fructose utilization by rat spermatozoa within the uterine lumen. *J Reprod Fertil, 61*(2), 435-437.**

**Martikainen, P., Sannikka, E., Suominen, J., & Santti, R. (1980). Glucose content as a parameter of semen quality. *Arch Androl, 5*(4), 337-343.**

**Pavlok, A., & McLaren, A. (1972). The role of cumulus cells and the zona pellucida in fertilization of mouse eggs in vitro. *J Reprod Fertil, 29*(1), 91-97.**

**Rodriguez-Martinez, H. (2007). Role of the oviduct in sperm capacitation.** ***Theriogenology, 68 Suppl 1*****, S138-146. doi: 10.1016/j.theriogenology.2007.03.018**

**Urner, F., & Sakkas, D. (1996a). Glucose is not essential for the occurrence of sperm binding and zona pellucida-induced acrosome reaction in the mouse.** ***Int J Androl, 19*****(2), 91-96.**

**Urner, F., & Sakkas, D. (1996b). Glucose participates in sperm-oocyte fusion in the mouse.** ***Biol Reprod, 55*****(4), 917-922.**

**Urner, F., & Sakkas, D. (1999). Characterization of glycolysis and pentose phosphate pathway activity during sperm entry into the mouse oocyte.** ***Biol Reprod, 60*****(4), 973-978.**

**Urner, F., & Sakkas, D. (2005). Involvement of the pentose phosphate pathway and redox regulation in fertilization in the mouse.** ***Mol Reprod Dev, 70*****(4), 494-503. doi: 10.1002/mrd.20222**

**Voglmayr, J. K., & White, I. G. (1979). Effects of rete testis and epididymal fluid on the metabolism and motility of testicular and post-testicular spermatozoa of the ram.** ***Biol Reprod, 20*****(2), 288-293.**

**Westhoff, D., & Kamp, G. (1997). Glyceraldehyde 3-phosphate dehydrogenase is bound to the fibrous sheath of mammalian spermatozoa.** ***J Cell Sci, 110 ( Pt 15)*****, 1821-1829.**

**Williams, A. C., & Ford, W. C. (2001). The role of glucose in supporting motility and capacitation in human spermatozoa.** ***J Androl, 22*****(4), 680-695.**

# Chapter 5: Thesis Discussion

**Oxidative Stress and DNA damage in the Testes of Akita Diabetic Mice**

The relationship between diabetes and the study of spermatogenesis and sperm cell metabolism remains controversial. In severe models of type 1 diabetes, such as the Akita mouse, the HPG axis is severely disrupted, resulting in the blockade of spermatogenesis. This infertility can be rescued with insulin treatment. In fact, prevention of testicular atrophy can be achieved with insulin treatment despite the persistence of hyperglycemia. In humans, individuals with type 1 diabetes are treated with insulin, preventing these disruptions in spermatogenesis. However, there are more subtle changes in fertility associated with type 1 diabetes even in insulin-treated individuals. Sperm cells from humans with type 1 diabetes have higher amounts of DNA damage. Interestingly, there do not appear to be overt defects in the HPG axis of humans with type 1 diabetes, though there are more subtle subfertility complications. Our data suggest that fertility can be maintained with a threshold of insulin or leptin, and that spermatogenesis can be maintained despite persistence of hyperglycemia and lower than wild type levels of insulin, leptin, testosterone, and luteinizing hormone. Thus, diabetes-related subfertility in males may be a result of the oxidative stress related to episodic hyperglycemia experienced by these patients, and not simply a disruption of the HPG axis, though subtle hormonal variations may still play a role.

**Is leptin rescue a central effect or a peripheral effect?**

We have shown that leptin injections into Akita diabetic mice prevent testicular atrophy. However, we do not know whether these effects are mediated centrally or peripherally. Leptin is known to cross the blood-testis barrier (Banks, McLay, Kastin, Sarmiento, & Scully, 1999), though the authors of this study were unable to detect leptin receptor or a saturable transport

system across the testis barrier. This indicates that leptin does not act directly at the level of the testis, and instead, exerts its effects at the level of the central nervous system. More recent studies, however, have detected leptin receptors in both the testes and sperm (Jope, Lammert, Kratzsch, Paasch, & Glander, 2003) (Zamorano et al., 1997). In mouse testes, leptin receptors are expressed on spermatocytes in stages IX and X seminiferous tubules, indicating that there may be a direct role for leptin within the testes (El-Hefnawy, Ioffe, & Dym, 2000). Interestingly, leptin has been shown *in vitro* to inhibit testosterone secretion from cultured rat testes sections (Tena-Sempere et al., 1999). Leptin decreases the expression of the steroidogenic enzymes in sections of hCG-stimulated testes, including mRNA expression levels of SF-1, StAR, P450scc and 17B-HSD. (Tena-Sempere et al., 2001). The authors hypothesize that this may be a mechanism for the inverse relationship between hyperleptinemia and testosterone in obese individuals. Thus, we do not know whether leptin treatment of type 1 diabetes acts entirely through the HPG axis, or whether there are also direct effects on the testes. Further experiments need to be performed to elucidate the mechanism of leptin treatment on the rescue of spermatogenesis in Akita diabetic mice.

One experiment that could serve to differentiate between centrally and peripherally mediated leptin effects would be to administer leptin via central infusion to the brain. This experiment has already been performed in streptozotocin-induced type 1 diabetic mice to show that intracerebroventricular (icv) leptin infusion can rescue hyperglycemia (Fujikawa, Chuang, Sakata, Ramadori, & Coppari, 2010). This suggests that the mechanism of leptin action is central, though the authors of this study did not address fertility. An icv infusion of leptin into Akita homozygous mice could serve to address the effects of leptin in the central nervous system on

spermatogenesis. This could isolate any confounding effects of leptin diffusion into the seminiferous tubules of the testes, as well as address the issue of peripheral versus central rescue of fertility.

**The Role of Insulin Within the Testes**

Recent data has demonstrated that both the testes and sperm contain the insulin transcript. This indicates that insulin plays a direct role in spermatogenesis or sperm maturation. In our studies, we determined that while *ins1* is absent from the mouse testes, *ins2* is localized to the endoplasmic reticulum of the mouse testes, indicating that the Sertoli cells are producing and secreting insulin. This is not entirely surprising, given that Sertoli cells are known to secrete a variety of hormones necessary for germ cell survival, including IGF-I (insulin-like growth factor), IGF-II, AMH (anti-mullerian hormone), estrogen, and inhibin (Alves et al., 2013; Skinner & Griswold, 2005). We also found that insulin secreted by the pancreas into the bloodstream is unable to penetrate the blood testes barrier, so that the only source for insulin beyond the blood testes barrier would be Sertoli cells or germ cells. The function of insulin within the testis is yet unknown, so to elucidate this, we attempted to knock out insulin within the Sertoli cells using the Cre-lox recombination system. We obtained a transgenic mouse that was homozygous null for the *ins1* gene, and homozygous for a floxed copy of *ins2*. *Ins1* null mice have previously been characterized, and are viable and fertile as they still have functional copies of the *ins2* gene, which is able to fully compensate for the lack of *ins1* (Leroux et al., 2001). We crossed this mouse with a Sertoli cell-specific Amh-Cre expressing mouse, to obtain mice that were homozygous null for *ins1,* homozygous for a floxed allele of *ins2*, and expressed Cre recombinase driven by the Amh promoter ($ins1^{-/-}$; $ins2^{fl/fl}$;Amh-Cre mice). These mice were fertile and appeared normal. qRT-PCR analysis of the testes revealed that *ins2* transcript levels

were not decreased in the testes of ins1$^{-/-}$; ins2$^{fl/fl}$;Amh-Cre mice **(Sup. Fig 5-1)**. This indicates that either the Cre recombinase did not efficiently knockout out gene expression, or that *ins2* is not solely expressed in the Sertoli cells, but also in the germ cells or the Leydig cells. Future experiments could include a more thorough evaluation of *ins2* transcript levels in the testes by in situ hybridization to localize all the cell types in the testes capable of producing insulin transcripts. We could then choose a more appropriate Cre mouse line to cross to the ins1$^{-/-}$; ins2$^{fl/fl}$ mouse. Efficiency of the Amh-Cre mouse line could be tested by crossing Amh-Cre expressing mice to a reporter mouse, such as a Rosa mT/mG mouse, that expresses tomato red fluorescence prior to Cre-mediated excision, and GFP fluorescence after excision. It is possible that if insulin was not completely deleted from the Sertoli cells, that even a reduced amount of insulin was able to support spermatogenesis.

**Addressing the mechanism of leptin and insulin action in the central nervous system**

From our work, we know that both luteinizing hormone and testosterone are lower in Akita homozygous diabetic males. This result is recapitulated in other models of type 1 diabetes, such as streptozotocin-induced diabetes (Ballester et al., 2004). Additionally, LH secretion is decreased in humans with poorly controlled type 1 diabetes, indicating that the HPG axis is also perturbed in humans when insulin and glucose levels are not stable (Lopez-Alvarenga et al., 2002). We hypothesize that the lack of leptin and insulin in these mice inhibits the release of luteinizing hormone from the pituitary via signaling through the central nervous system. However, we do not know the levels of the intermediates in this pathway, so the molecules in the brain that are specifically affected by type 1 diabetes are unknown, and it is unclear whether these defects can be attributed to a lack of insulin or to a lack of leptin.

To determine which portion of the HPG axis is affected by type 1 diabetes, we propose to measure levels of hormones in the central nervous system known to be important in the maintenance of fertility. We know that in mice and humans, a host of signaling peptides act within the central nervous system to generate the GnRH pulse that results in the LH surge necessary for testosterone production. Defects within any of these populations could have the inhibitory effect seen at the level of LH. It would be useful to determine whether Akita homozygous mice have normal GnRH pulses. We have preliminarily begun to address this issue by injecting Akita mice with a Lupron, a GnRH agonist and measuring subsequent plasma LH levels **(Sup. Fig. 5-2)**. We observed that both Akita heterozygous mice and homozygous mice have a decreased LH surge in response to a GnRH agonist. It is of note that the basal levels of LH are decreased in Akita heterozygotes and homozygotes as well. However, despite a lower response to Lupron, Akita homozygotes still displayed a robust surge in serum LH. This indicates that these mice retain function of the GnRH system and that the defect in Akita mice may lie upstream of GnRH neurons.

We next propose to investigate the neural populations within the arcuate nucleus of the hypothalamus that control the GnRH pulse. The arcuate nucleus of the hypothalamus integrates the signals for nutritionals status and controlling reproductive function (Hill, Elmquist, & Elias, 2008). The arcuate nucleus contains a subset of neurons that are important for mediating the connection between energy status and reproductive capacity. There are both insulin and leptin receptors in the arcuate nucleus that may serve to integrate system energy status and appropriately regulate reproductive capacity (Baskin, Breininger, & Schwartz, 1999; Marks, Porte, Stahl, & Baskin, 1990). Leptin and insulin sensing takes place within the hypothalamus,

by the proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) neurons, which suppress food intake, and the neuropeptide Y/agouti-related protein (NPY/AgRP) neurons, which increase food intake (Hill et al., 2008). Insulin and leptin bind to their receptors on the surface of POMC neurons and activate a signaling cascade that decreases the expression of POMC, while binding of insulin and leptin to receptors on the surface of AgRP neurons initiate a signaling cascade that decreases the expression of AgRP (Plum, Belgardt, & Bruning, 2006). A loss of insulin receptors on POMC neurons reduces fertility, suggesting an important role for these neurons in mediating nutritional status and reproductive capacity in the central nervous system (Hill et al., 2010). These neurons may be critical in causing the infertility of the Akita homozygous mice. We therefore propose to measure POMC and AgRP protein levels in the homozygous mice, and if there is a downregulation, whether this can be rescued by leptin or insulin treatment.

More recently, a subset of neurons has been identified in the arcuate nucleus that colocalize with three different peptides, neurokinin B, kisspeptin, and dynorphin. (Castellano et al., 2006; Rodriguez-Martinez, 2007). Since our data suggest that Akita diabetic mice retain the ability to respond to GnRH agonists by producing luteinizing hormone **(Sup Fig 5-2)**, we hypothesize the defect in signaling lies upstream of GnRH. Kisspeptin is an essential hormone required for proper GnRH pulse generation. Humans and mice with mutations in kisspeptin or its receptor experience infertility due to a lack of GnRH pulses and subsequent low levels of luteinizing hormone. (Garcia-Galiano et al., 2012; Silveira, Tusset, & Latronico, 2010) Type 1 diabetic mice also express lower levels of kisspeptin, corresponding to decreased expression of luteinizing hormone and testosterone. The administration of intracerebroventricular kisspeptin, in the

absence of insulin therapy, can restore luteinizing hormone and testosterone in these mice, suggesting that this hormone is an important player in diabetes-related infertility (Castellano et al., 2006). It would be valuable to first investigate the levels of kisspeptin hormone in the hypothalamus of Akita homozygous mice. Preliminary experiments do not show a significant difference in Kiss1 mRNA expression in whole-brain homogenates of Akita homozygous diabetic mice **(Sup. Fig 5-3)**. However, it is necessary to validate these results by closer examination of Kiss1 expression by immunohistochemistry and also by using more precise protein and mRNA measurements in the hypothalamus to validate these results. Measuring mRNA from whole-brain homogenates may obscure any subtle changes in kisspeptin function. It is possible that long-term kisspeptin therapy may be sufficient to rescue spermatogenesis in Akita homozygous mice, by increasing luteinizing hormone and testosterone levels.

We also propose to examine levels of neurokinin B and dynorphin in these mice to determine whether these hormones are affected in Akita mice. Though there is no known link between diabetes and neurokinin B or dynorphin function, the hormones kisspeptin, dynorphin, and neurokinin B all colocalize to the same neuronal population and are thought to act together to generate the GnRH pulse (Lehman, Coolen, & Goodman, 2010). Neurokinin B has a recently identified important role in the hypothalamic pituitary gonadal axis, and mutations in this gene or its receptor in humans are associated with infertility and inhibited GnRH secretion (Ruiz-Pino et al., 2012; Topaloglu et al., 2009). Similarly, dynorphin localizes to this same neuronal population and has been implicated in the negative feedback that regulates GnRH release (Goodman et al., 2004). The interplay between the kisspeptin system and metabolic input has yet to be clearly defined, so it is important to examine the levels of all three of these hormones as

they may be important mediators of metabolic cues and reproductive outcomes (Navarro & Tena-Sempere, 2012).

**Glucose Transporters in Sperm**

While the majority of our work focused on GLUTs 1 and 4 in human and mouse sperm cells, we initially aimed to complete a more comprehensive study of the GLUTs present in sperm cells. In human sperms cells, we used reverse transcription approach to identify which of the 14 glucose transporters were present in sperm cells. We were able to identify GLUTs 1, 2, 3, 4, 5, 8, 9, and 12 in human sperm cells **(Sup. Fig. 5-4)**. We also verified the presence of these transporters by western blot. We propose to replicate the results of these experiments in mouse sperm cells to determine whether the same glucose transporters are present. By confocal microscopy, we have localized GLUT 8 and GLUT 12 in both human and mouse sperm. GLUT8 localized to the acrosome and the connecting piece **(Sup. Fig. 5-5 and Sup. Fig. 5-6)** while GLUT12 localized to the acrosome (**Sup. Fig. 5-7 and Sup. Fig 5-8)**. GLUT8 was of particular interest because its mRNA transcript appeared to be highly expressed in human sperm cells. GLUT8 transcript is also highly expressed in testes, giving it the potential to be important in sperm cell metabolism (Schmidt, Joost, & Schurmann, 2009). However, GLUT8 knockout mice appear to have only a mild subfertility phenotype, with one group reporting lower ATP levels in testicular sperm cells and slightly lower motility. Two groups have reported less than mendelian ratios of homozygous offspring from heterozygous matings (Gawlik et al., 2008; Membrez et al., 2006). GLUT8 knockout males are capable of siring pups, indicating that either GLUT8 is not crucial to fertility or that another glucose transporter can compensate for its loss. Interestingly, a recently published proteomic analysis of human sperm conspicuously lacked GLUT8 detection, while GLUT3 and GLUT5 were identified in this same study (Chauvin et al., 2012). Additionally, while we could

detect GLUT8 on both human and mouse sperm cells by confocal microscopy, we were unable to detect GLUT8 expression using the same antibody by western blot, despite robust expression in testis control samples.

GLUT12 expression also proved to be an interesting transporter in sperm cells. Like GLUT4, GLUT12 is able to translocate to the cell surface upon insulin stimulation (Stuart, Howell, Zhang, & Yin, 2009). We detected GLUT12 transcript first by RT-PCR in human sperm cells, and then protein by western blot and confocal microscopy **(Sup. Fig 5-4**). GLUT12 localized strongly to the acrosomal region of human and mouse sperm **(Sup. Fig 5-7 and Sup. Fig 5-8)** , though it was present in a small percentage of sperm cells. This is similar to the expression of GLUT1, which appeared on just a percentage of sperm cells, though we are yet unsure of the significance of this. Our studies of GLUT1 co-localization with the acrosome show that in humans, GLUT1 expression increases upon completion of the acrosome reaction, though in mouse, GLUT1 co-localizes with the acrosome. It is possible that GLUT12 expression is also mediated by the acrosome reaction, and further studies characterizing the conditions that regulate GLUT12 would be useful. While sperm cells are not thought to actively transcribe mRNA, they do have the ability to translate existing mRNA into protein, and it is possible that one of the reactions during capacitation triggers the translation and expression of GLUT12. One way to test this hypothesis would be to use flow cytometry to isolate GLUT12 positive sperm cells using an antibody generated to an extracellular loop of the transporter. Once sorted, we could evaluate the GLUT12 positive for hyperactivated motility, intracellular calcium levels, intracellular cAMP, or protein tyrosine phosphorylation.

One of the more interesting aspects of our glucose transporter studies on sperm is the presence of the insulin-responsive GLUTs 4 and 12. The presence of these transporters provides the potential for these cells to be regulated by insulin. This is especially interesting in light of recent discovery of insulin transcripts in sperm and testes (Aquila, Gentile, Middea, Catalano, & Ando, 2005; Schoeller, Albanna, Frolova, & Moley, 2012). This provides the potential for insulin regulation within the testis and sperm that could modulate sperm motility and capacitation.

**General Conclusions:**

From these data, we can make some broad conclusions about the impact of type 1 diabetes on male fertility, as well as the characterization of glucose transporters in human sperm. This work strengthens the link between type 1 diabetes and the function of the hypothalamic pituitary gonadal axis, highlighting especially the importance of leptin in maintaining fertility. We conclude that untreated type 1 diabetes leads to a decrease in fat stores, and consequently, low levels of leptin. This lack of leptin signals to the hypothalamus that there is a lack of systemic energy availability. While the precise hypothalamic mechanism mediating leptin and fertility remains unresolved, it is possible that neurons within the arcuate nucleus mediate the secretion of reproductive hormones, and further study in this area is necessary.

In sperm cells, the continued identification and characterization of glucose transporters will help to elucidate the mechanism of hexose movement during the maturation and fertilization reactions. Here we demonstrate important roles for both GLUT1 and GLUT4 in glucose uptake and fertilization capacity in both human and mouse sperm cells. The inhibition of GLUT1 and GLUT4 together results in a dramatic decrease in the ability of sperm cells to take up glucose, defining a clear role for these transporters in the glucose metabolism. GLUT4 is a novel glucose transporter in sperm cells and since it is an insulin-responsive transporter, it may provide a potential role for insulin regulation in sperm cell metabolism. The inhibition of GLUT4 alone results in decreased glucose uptake, demonstrating a functional role for this glucose transporter in sperm cells, though its potential for insulin regulation remains to be defined. These findings demonstrate the importance of hexose utilization to sperm cell competence. As more glucose transporters in sperm cells continue to be identified, a comprehensive characterization of these

transporters and their roles in capacitation and fertilization will further clarify the importance of hexose utilization to sperm cell competence.

**References:**


**Alves, M. G., Rato, L., Carvalho, R. A., Moreira, P. I., Socorro, S., & Oliveira, P. F. (2013). Hormonal control of Sertoli cell metabolism regulates spermatogenesis. *Cell Mol Life Sci, 70*(5), 777-793. doi: 10.1007/s00018-012-1079-1**

**Aquila, S., Gentile, M., Middea, E., Catalano, S., & Ando, S. (2005). Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology, 146*(2), 552-557. doi: 10.1210/en.2004-1252**

**Ballester, J., Munoz, M. C., Dominguez, J., Rigau, T., Guinovart, J. J., & Rodriguez-Gil, J. E. (2004). Insulin-dependent diabetes affects testicular function by FSH- and LH-linked mechanisms. *J Androl, 25*(5), 706-719.**

**Banks, W. A., McLay, R. N., Kastin, A. J., Sarmiento, U., & Scully, S. (1999). Passage of leptin across the blood-testis barrier. *Am J Physiol, 276*(6 Pt 1), E1099-1104.**

**Baskin, D. G., Breininger, J. F., & Schwartz, M. W. (1999). Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. *Diabetes, 48*(4), 828-833.**

**Castellano, J. M., Navarro, V. M., Fernandez-Fernandez, R., Roa, J., Vigo, E., Pineda, R., . . . Tena-Sempere, M. (2006). Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. *Diabetes, 55*(9), 2602-2610. doi: 10.2337/db05-1584**

**Chauvin, T., Xie, F., Liu, T., Nicora, C. D., Yang, F., Camp, D. G., 2nd, . . . Roberts, K. P. (2012). A systematic analysis of a deep mouse epididymal sperm proteome. *Biol Reprod, 87*(6), 141. doi: 10.1095/biolreprod.112.104208**

**El-Hefnawy, T., Ioffe, S., & Dym, M. (2000). Expression of the leptin receptor during germ cell development in the mouse testis. *Endocrinology, 141*(7), 2624-2630.**

**Fujikawa, T., Chuang, J. C., Sakata, I., Ramadori, G., & Coppari, R. (2010). Leptin therapy improves insulin-deficient type 1 diabetes by CNS-dependent mechanisms in mice. *Proc Natl Acad Sci U S A, 107*(40), 17391-17396. doi: 10.1073/pnas.1008025107**

**Garcia-Galiano, D., van Ingen Schenau, D., Leon, S., Krajnc-Franken, M. A., Manfredi-Lozano, M., Romero-Ruiz, A., . . . Tena-Sempere, M. (2012). Kisspeptin signaling is indispensable for neurokinin B, but not glutamate, stimulation of gonadotropin secretion in mice. *Endocrinology, 153*(1), 316-328. doi: 10.1210/en.2011-1260**

**Gawlik, V., Schmidt, S., Scheepers, A., Wennemuth, G., Augustin, R., Aumuller, G., . . . Schurmann, A. (2008). Targeted disruption of Slc2a8 (GLUT8) reduces motility and mitochondrial potential of spermatozoa. *Mol Membr Biol, 25*(3), 224-235. doi: 10.1080/09687680701855405**

**Goodman, R. L., Coolen, L. M., Anderson, G. M., Hardy, S. L., Valent, M., Connors, J. M., . . . Lehman, M. N. (2004). Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep. *Endocrinology, 145*(6), 2959-2967. doi: 10.1210/en.2003-1305**

**Hill, J. W., Elias, C. F., Fukuda, M., Williams, K. W., Berglund, E. D., Holland, W. L., . . . Elmquist, J. K. (2010). Direct insulin and leptin action on pro-**

**opiomelanocortin neurons is required for normal glucose homeostasis and fertility. *Cell Metab, 11*(4), 286-297. doi: 10.1016/j.cmet.2010.03.002**

**Hill, J. W., Elmquist, J. K., & Elias, C. F. (2008). Hypothalamic pathways linking energy balance and reproduction. *Am J Physiol Endocrinol Metab, 294*(5), E827-832. doi: 10.1152/ajpendo.00670.2007**

**Jope, T., Lammert, A., Kratzsch, J., Paasch, U., & Glander, H. J. (2003). Leptin and leptin receptor in human seminal plasma and in human spermatozoa. *Int J Androl, 26*(6), 335-341.**

**Lehman, M. N., Coolen, L. M., & Goodman, R. L. (2010). Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology, 151*(8), 3479-3489. doi: 10.1210/en.2010-0022**

**Leroux, L., Desbois, P., Lamotte, L., Duvillie, B., Cordonnier, N., Jackerott, M., . . . Joshi, R. L. (2001). Compensatory responses in mice carrying a null mutation for Ins1 or Ins2. *Diabetes, 50 Suppl 1*, S150-153.**

**Lopez-Alvarenga, J. C., Zarinan, T., Olivares, A., Gonzalez-Barranco, J., Veldhuis, J. D., & Ulloa-Aguirre, A. (2002). Poorly controlled type I diabetes mellitus in young men selectively suppresses luteinizing hormone secretory burst mass. *J Clin Endocrinol Metab, 87*(12), 5507-5515.**

**Marks, J. L., Porte, D., Jr., Stahl, W. L., & Baskin, D. G. (1990). Localization of insulin receptor mRNA in rat brain by in situ hybridization. *Endocrinology, 127*(6), 3234-3236.**

**Membrez, M., Hummler, E., Beermann, F., Haefliger, J. A., Savioz, R., Pedrazzini, T., & Thorens, B. (2006). GLUT8 is dispensable for embryonic development but influences hippocampal neurogenesis and heart function. *Mol Cell Biol, 26*(11), 4268-4276. doi: 10.1128/MCB.00081-06**

**Navarro, V. M., & Tena-Sempere, M. (2012). Neuroendocrine control by kisspeptins: role in metabolic regulation of fertility. *Nat Rev Endocrinol, 8*(1), 40-53. doi: 10.1038/nrendo.2011.147**

**Plum, L., Belgardt, B. F., & Bruning, J. C. (2006). Central insulin action in energy and glucose homeostasis. *J Clin Invest, 116*(7), 1761-1766. doi: 10.1172/JCI29063**

**Rodriguez-Martinez, H. (2007). Role of the oviduct in sperm capacitation. *Theriogenology, 68 Suppl 1*, S138-146. doi: 10.1016/j.theriogenology.2007.03.018**

**Ruiz-Pino, F., Navarro, V. M., Bentsen, A. H., Garcia-Galiano, D., Sanchez-Garrido, M. A., Ciofi, P., . . . Tena-Sempere, M. (2012). Neurokinin B and the control of the gonadotropic axis in the rat: developmental changes, sexual dimorphism, and regulation by gonadal steroids. *Endocrinology, 153*(10), 4818-4829. doi: 10.1210/en.2012-1287**

**Schmidt, S., Joost, H. G., & Schurmann, A. (2009). GLUT8, the enigmatic intracellular hexose transporter. *Am J Physiol Endocrinol Metab, 296*(4), E614-618. doi: 10.1152/ajpendo.91019.2008**

**Schoeller, E. L., Albanna, G., Frolova, A. I., & Moley, K. H. (2012). Insulin rescues impaired spermatogenesis via the hypothalamic-pituitary-gonadal axis in Akita diabetic mice and restores male fertility. *Diabetes, 61*(7), 1869-1878. doi: 10.2337/db11-1527**

**Silveira, L. G., Tusset, C., & Latronico, A. C. (2010). Impact of mutations in kisspeptin and neurokinin B signaling pathways on human reproduction. *Brain Res, 1364*, 72-80. doi: 10.1016/j.brainres.2010.08.087**

**Skinner, Michael K., & Griswold, Michael D. (2005). *Sertoli cell biology*. Amsterdam ; Boston: Elsevier Academic Press.**

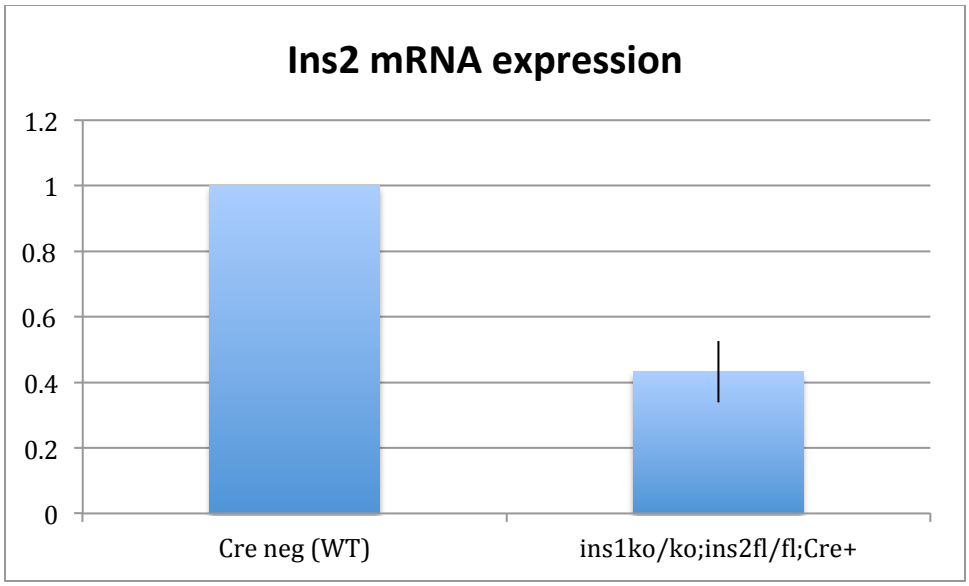
**Stuart, C. A., Howell, M. E., Zhang, Y., & Yin, D. (2009). Insulin-stimulated translocation of glucose transporter (GLUT) 12 parallels that of GLUT4 in normal muscle. *J Clin Endocrinol Metab, 94*(9), 3535-3542. doi: 10.1210/jc.2009-0162**

**Tena-Sempere, M., Manna, P. R., Zhang, F. P., Pinilla, L., Gonzalez, L. C., Dieguez, C., . . . Aguilar, E. (2001). Molecular mechanisms of leptin action in adult rat testis: potential targets for leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger ribonucleic acid expression. *J Endocrinol, 170*(2), 413-423.**

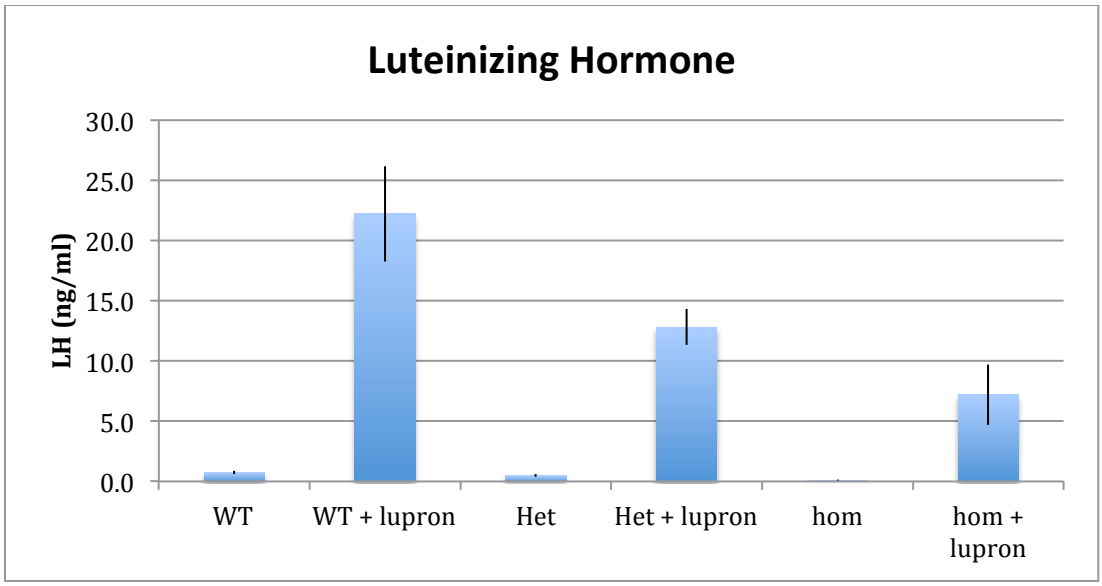
**Tena-Sempere, M., Pinilla, L., Gonzalez, L. C., Dieguez, C., Casanueva, F. F., & Aguilar, E. (1999). Leptin inhibits testosterone secretion from adult rat testis in vitro. *J Endocrinol, 161*(2), 211-218.**

**Topaloglu, A. K., Reimann, F., Guclu, M., Yalin, A. S., Kotan, L. D., Porter, K. M., . . . Semple, R. K. (2009). TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nat Genet, 41*(3), 354-358. doi: 10.1038/ng.306**

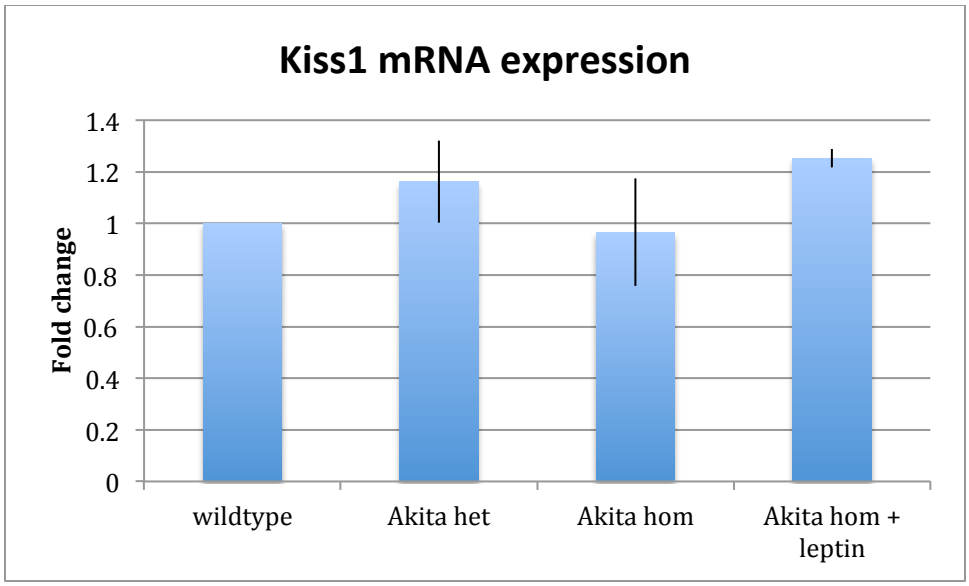
**Zamorano, P. L., Mahesh, V. B., De Sevilla, L. M., Chorich, L. P., Bhat, G. K., & Brann, D. W. (1997). Expression and localization of the leptin receptor in endocrine and neuroendocrine tissues of the rat. *Neuroendocrinology, 65*(3), 223-228.**




**Supplemental Figure 5-1:** Ins2 mRNA expression in tissue specific insulin knockout mice. Mice homozygous for *ins1* complete knockout, homozygous for a floxed allele of *ins2*, and positive for Amh-Cre, a Sertoli cells specific promoter, expressed approximately 40% the transcript levels of *ins2* compared to control mice lacking Cre recombinase activity.

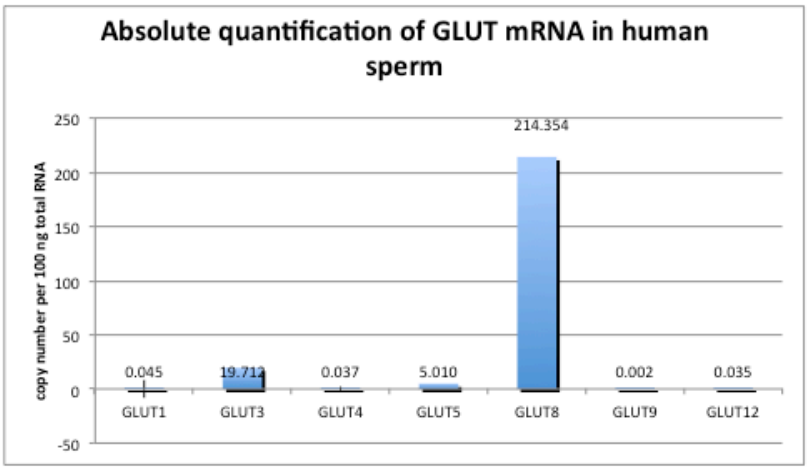



**Supplemental Figure 5-2:** Basal levels of luteinizing hormone are decreased in Akita homozygous mice. Akita heterozygotes and homozygotes have a diminished response to the GnRH agonist, Lupron.

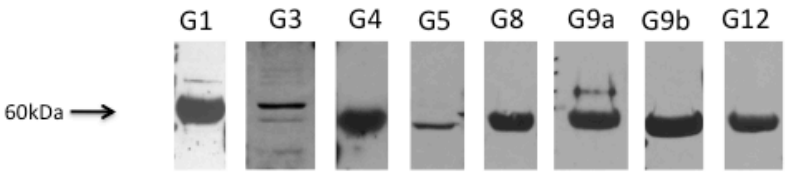



**Supplemental Figure 5-3:** Kisspeptin levels remain unchanged in Akita homozygous mice and Akita homozygous mice treated with leptin.

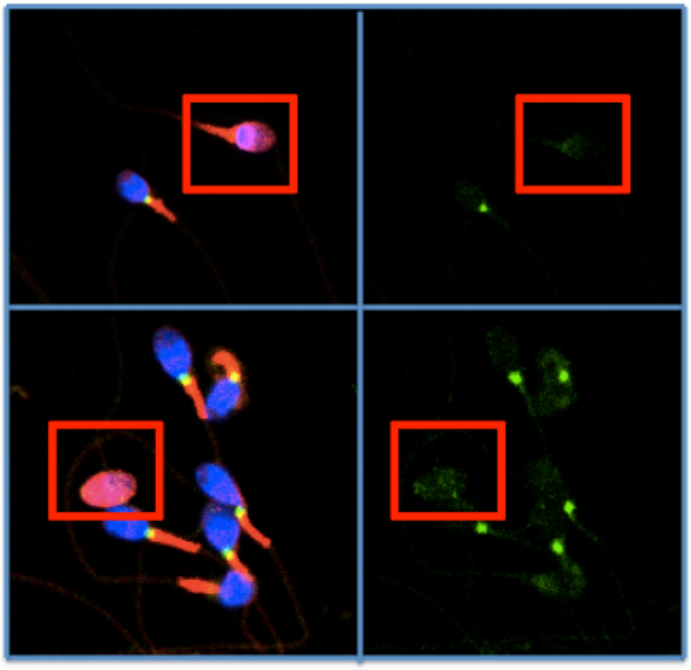
a)



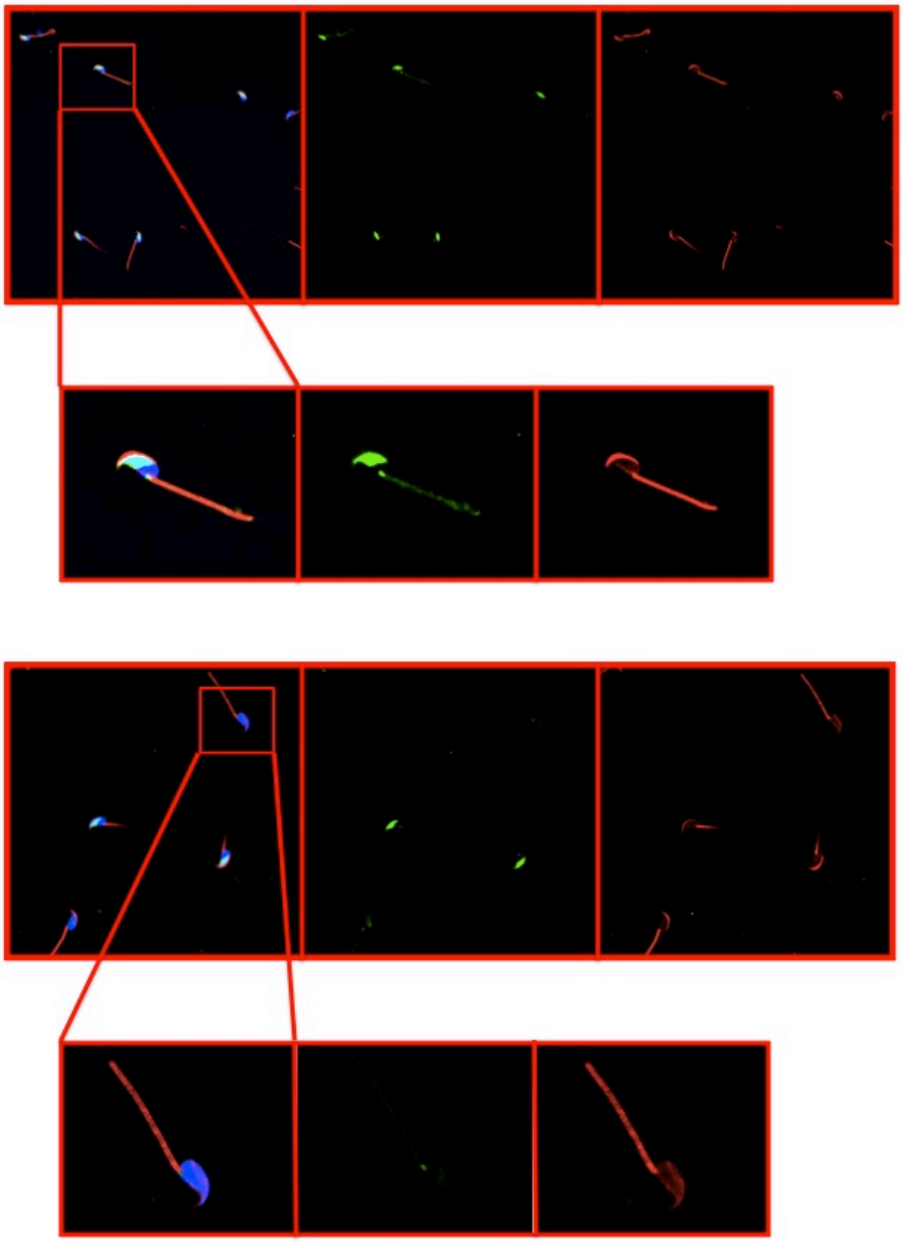

b)



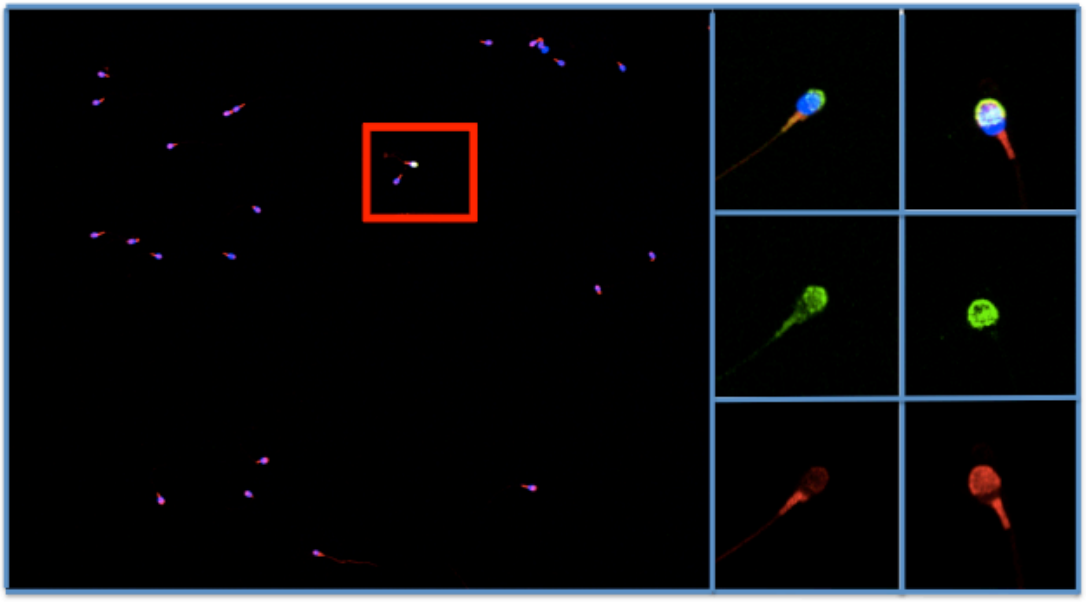

**Supplemental Figure 5-4:** a) qRT-PCR of the glucose transporters expressed in human sperm cells. GLUT8 shows the highest expression levels followed by GLUT3 and then GLUT5. B) Protein expression of the glucose transporters expressed by human sperm cells.



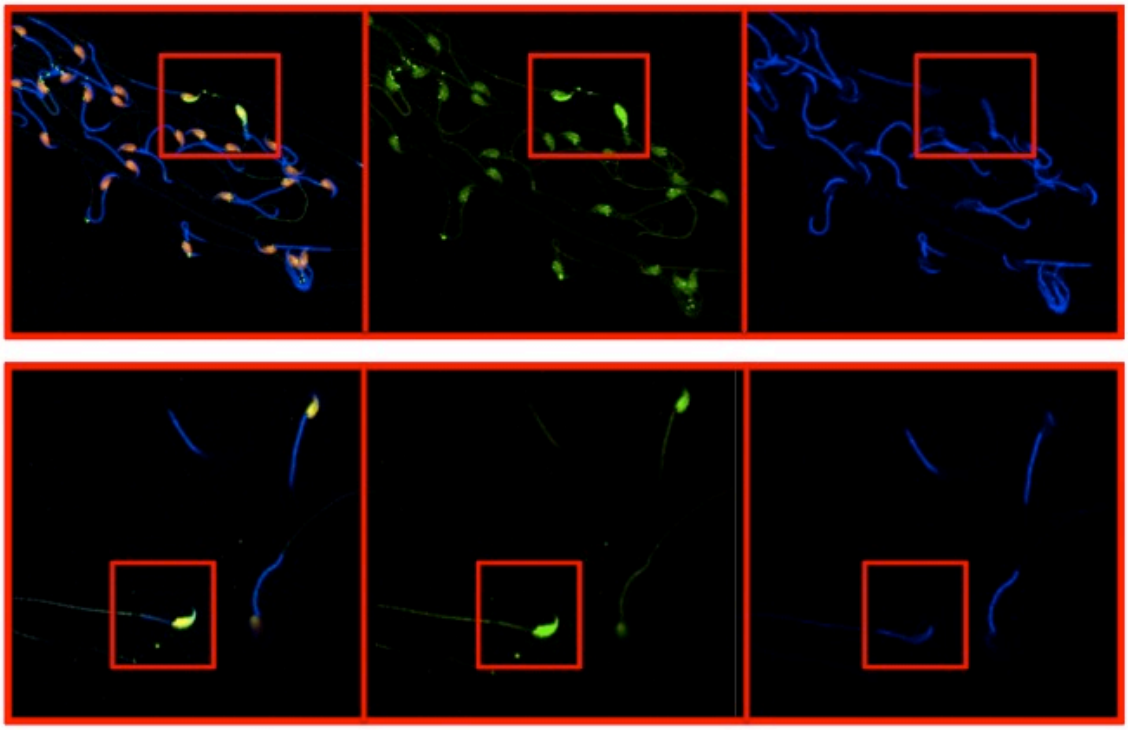
**Supplemental Figure 5-5:** GLUT8 localization in human sperm. GLUT8 localizes to the connecting piece of human spermatozoa. The signal is not present in sperm cells that have not undergone the acrosome reaction. Green = GLUT1; red = PNA-lectin; blue=nuclei



**Supplemental Figure 5-6:** GLUT8 localization in mouse sperm. GLUT8 localizes to the acrosome and the connecting piece of human spermatozoa, with fainter expression on the midpiece. Acrosomal GLUT8 expression disappears after acrosome reaction. Green = GLUT1; red = PNA-lectin; blue=nuclei



**Supplemental Figure 5-7:** GLUT12 in human sperm. GLUT12 localizes strongly to the acrosome of human sperm cells, though only a small percentage of sperm cells stain positively. The presence of GLUT12 does not correlate to the presence of an acrosome. Green: GLUT12, Red: PNA-lectin, Blue: nuclei



**Supplemental Figure 5-8:** GLUT12 in mouse sperm. GLUT12 localizes strongly to the acrosome of mouse sperm cells, though only a small percentage of sperm cells stain positively. The presence of GLUT12 does not correlate to the presence of an acrosome. Green: GLUT12, Blue: PNA-lectin

## Curriculum Vitae:

**Erica Schoeller**

5387 Pershing Avenue, Saint Louis, MO 63112
Mobile: (608) 332-9993 Email: elschoeller@gmail.com

### EDUCATION

**Washington University in Saint Louis, Saint Louis, MO**
2007-Present
*Ph.D. candidate in Molecular Genetics and Genomics*
*Expected graduation: April 2013*
**Dissertation Title:** Type 1 diabetes and male fertility

**University of Wisconsin-Madison**, Madison, WI 2002-2007
*Bachelor of Science in Genetics*

### TEACHING AND WORKING EXPERIENCE

**Graduate Teaching Assistant** 2008
Department of Biology
Washington University in Saint Louis
- Introductory Biology 2960: leading discussion groups, office hours and exam grading

**Research Assistant** 2003-2007
Department of Food Science
Laboratory of Dr. Steve Ingham
- Researched the effects of temperature and pH on food pathogen growth and perseverance

**Undergraduate Research Assistant** 2002-2003
Department of Food Science
Laboratory of Dr. James Steele
- Identification of protective bacterial cultures to improve food safety

**Undergraduate Research Assistant** Summer 2003
Department of Biology
Laboratory of Dr. Susan Golden
- Circadian pathways in cyanobacteria

### SPECIAL COURSES

**Frontiers in Reproduction**
Marine Biological Laboratory, Woods Hole, MA
- Six-week comprehensive laboratory and lecture course in reproductive biology May-June 2011
- Learned multiple techniques in reproductive biology, including in vitro fertilization, ICSI, organ culture, and laser capture microdissection

## AWARDS

- Lalor Foundation Merit Award, Society for the Study of Reproduction 2009
- Best Oral Presentation Award at Washington University Annual Genetics Retreat, 2010

## PUBLICATIONS

- Insulin Rescues Impaired Spermatogenesis via the Hypothalamic-Pituitary-Gonadal Axis in Akita Diabetic Mice and Restores Male Fertility.
  Schoeller EL, Albanna G, Frolova AI, Moley KH.
  Diabetes. 2012 Jul;61(7):1869-78. Epub 2012 Apr 20

- The effects of type 1 diabetes on the hypothalamic, pituitary and testes axis.
  Schoeller EL, Schon S, Moley KH.
  Cell Tissue Res. 2012 Sep;349(3):839-47. Epub 2012 Apr 15

- Mitochondrial dysfunction and apoptosis in cumulus cells of type I diabetic mice.
  Wang Q, Frolova AI, Purcell S, Adastra K, Schoeller E, Chi MM, Schedl T, Moley KH.
  PLoS One. 2010 Dec 28;5(12):e15901.

- GATA4 regulates Sertoli cell function and fertility in adult male mice.
  Kyrönlahti A, Euler R, Bielinska M, Schoeller EL, Moley KH, Toppari J, Heikinheimo M, Wilson DB.
  Mol Cell Endocrinol. 2011 Feb 10;333(1):85-95. Epub 2010 Dec 21.

- Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring.
  Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH.
  Endocrinology. 2010 Aug;151(8):4039-46. Epub 2010 Jun 23.

- Maternal diabetes causes mitochondrial dysfunction and meiotic defects in murine oocytes.
  Wang Q, Ratchford AM, Chi MM, Schoeller E, Frolova A, Schedl T, Moley KH.
  Mol Endocrinol. 2009 Oct;23(10):1603-12. Epub 2009 Jul 2.

- Predicting pathogen growth during short-term temperature abuse of raw sausage.
  Ingham SC, Ingham BH, Borneman D, Jaussaud E, Schoeller EL, Hoftiezer N, Schwartzburg L, Burnham GM, Norback JP.
  J Food Prot. 2009 Jan;72(1):75-84.

- Pathogen reduction in unpasteurized apple cider: adding cranberry juice to enhance the lethality of warm hold and freeze-thaw steps.
  Ingham SC, Schoeller EL, Engel RA.
  J Food Prot. 2006 Feb;69(2):293-8.

- Fate of Staphylococcus aureus on vacuum-packaged ready-to-eat meat products stored at 21 degrees C.
  Ingham SC, Engel RA, Fanslau MA, Schoeller EL, Searls G, Buege DR, Zhu J.
  J Food Prot. 2005 Sep;68(9):1911-5.

## PRESENTATIONS

• **Erica Schoeller, Kelle H. Moley.** Leptin Rescues Spermatogenesis in Akita Diabetic Mice. Society for the Study of Reproduction. State College, PA (August 2012). Poster Presentation.

• **Erica Schoeller, Samantha Schon, Sahar, Stephens, Kelle H. Moley**. Glucose Transporters in Human Sperm. Society for Gynecologic Investigation in San Diego, CA (March 2012). Platform presentation.

• **Erica Schoeller, Kelle H. Moley.** The Functional Significance of Insulin in Murine Testes and Sperm. Frontiers in Reproduction Annual Symposium in Woods Hole, MA (June 2011). Platform presentation.

• **Erica Schoeller, Kelle H. Moley.** Insulin Expression in the Testes of Diabetic Mice. Society for the Study of Reproduction (July 2010). Poster Presentation.

• **Erica Schoeller, Kelle H. Moley**. Insulin Expression in Testes and Sperm of High Fat Fed Mice. Society for the Study of Reproduction (July 2009). Platform Presentation.

• **Erica Schoeller, Kelle H. Moley.** The Significance of Insulin in Murine Testes and Sperm. CRS Annual Minisymposium in Reproductive Science at Northwestern University. (October 2010). Poster Presentation.

• **Erica Schoeller, Kelle H. Moley.** Insulin Expression in Murine Testes and Sperm. Annual Diabetes Day at Washington University in Saint Louis. (November 2011). Poster Presentation.

## EXTRACURRICULAR ACTIVITIES

- **Co-Head of Genetics and Genomics Teaching Team for the Young Scientist Program (2012-present)**
- **Peer Mentor Liaison for the Student Advisory Committee (2009-2010)**
- **Student Advisory Committee Member (2007-2010)**
- **Graduate Student Senate Representative (2009-2011)**