5-24-2012

# The Importance of Glucose Transport in Reproductive Events

Katie Adastra
*Washington University in St. Louis*

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Developmental, Regenerative, and Stem Cell Biology

Dissertation Examination Committee:
Kelle Moley, Chair
Paul Hruz
Kristen Kroll
Jeffrey Miner
Indira Mysorekar
Mark Sands

# THE IMPORTANCE OF GLUCOSE TRANSPORT IN REPRODUCTIVE EVENTS

by

Katie Lynn Adastra

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

May 2012

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

The Importance of Glucose Transport in Reproductive Events

by

Katie Lynn Adastra

Doctor of Philosophy in Biology and Biomedical Sciences

Developmental, Regenerative, and Stem Cell Biology

Washington University in St. Louis, 2012

Professor Kelle H. Moley, Chairperson

Successful pregnancy outcome is contingent on a number of factors, the earliest of which include the events occurring during early pregnancy. This time period from fertilization to implantation requires drastic changes in both the embryo and the uterus, including cell proliferation and differentiation. Alterations in pre-implantation milieu may lead to adverse effects during any of these stages. This emphasizes the need to understand these early processes and the potential deleterious conditions that may be contributing to adverse pregnancy outcome including fetal malformations and demise.

In the second chapter, we provide evidence for a differential response of elevated activation of autophagy in embryos and oocytes exposed to a hyperglycemic environment, which is accompanied by an increase in apoptosis. While this environment

signaled the activation of these pathways, a hyperglycemic environment during the pre-implantation stage has previously been shown to also alter expression levels of facilitated glucose transporters (SLC2As). One SLC2A in particular, SLC2A8, has been implicated as an important glucose transporter during early pregnancy events, however, a detailed study of this transporter during this time period has been lacking in the literature. Using a *Slc2a8* deficient mouse model, the data presented in third chapter is the first to demonstrate that a lack of *Slc2a8* leads to reproductive defects most likely due to abnormal decidualization and impaired implantation. This phenotype leads to decreased litter size, and smaller pups at weaning that continue to display an abnormally small growth phenotype into adulthood. Furthermore, in chapter 4 we detail the novel finding of SLC2A8 co-localization with autophagosomes during autophagy activation in uterine stromal cells. In conclusion, the findings in this dissertation support the hypothesis that alterations of the pre-implantation milieu during early pregnancy events can result in aberrations in processes directly impacting fetal outcome and the success of a pregnancy.

**Acknowledgements**

I am grateful to have completed my graduate work at Washington University. My time at Wash U was wonderful, as intelligent and generous people have surrounded me. People generous with time, knowledge and friendship. I appreciate all the interactions I was lucky enough to have, thank you to all.

Of these interactions, the time I spent with my mentor Kelle Moley has been the most influential. Kelle is a brilliant scientist, a strong woman and a devoted mother. I truly appreciate the immeasurable role she played in developing who I am today. Kelle imparted knowledge, core values, and useful coping skills onto me, allowing me to grow into the accomplished scientist, brilliant woman and loving mother I am today. To Kelle I say, thank you for being an exemplary role model.

Kelle has also taught me to surround myself with people I trust and value. The best example of this person is my lab mom, Maggie Chi. Maggie, you have taught me patience, generosity, creativity and dedication. While I haven't always been the best student, you have been an incredible teacher.

My wonderful lab mates have also played an incredibly supportive role in my graduate career. I knew that I could rely on any one of them for advice on experimental design, analysis or even my personal life. A special thanks to my graduate school colleagues: Lauren Aerni-Flessner, Toni Frolova, and Erica Schoeller. Ladies, I couldn't have done it without you. Thank you Erica Louden for being a great friend and a positive influence in my life.

My family has also played an incredibly supportive role. I am lucky to have you all near me as I completed this part of my life. I am especially grateful for my mother. Mom, you have always shown so much trust in my abilities to succeed. You are the most generous and selfless person I know. I am not sure it can ever be said enough, thank you.

To my husband Bart, this work is dedicated to you, as my success is intertwined with yours. You provided comfort, patience and encouragement when I needed it. I found strength in your total confidence in me. Thank you for being you.

For my children, remember to 'reach for the stars', *sic itur ad astra.*

**Table of Contents**

**List of Figures**

Chapter One



Chapter Two



Chapter Three

Chapter Four

# INTRODUCTION

## Introduction

### *1.1 Early Pregnancy Events*

Crucial to the perpetuation of all species is the ability to reproduce. Pregnancy encompasses the important events from conception to birth. Full-term pregnancy is 40 weeks in a human, however this time is significantly shorter in mice, 19-20 days, which along with other factors makes the mouse a good model organism to study pregnancy events[1]. Years of compounded studies highlight the importance of early embryonic development in pregnancy outcome and reproductive success. These data emphasize the need to understand these early processes and the potential deleterious environments that may be contributing to fetal malformations and demise. This section of the chapter will introduce the important events occurring during early pregnancy in mice, specifically the time period encompassing fertilization through implantation.

#### *1.1.1 Pre-implantation Events*

For the purpose of this manuscript, the pre-implantation time period of embryogenesis refers to the events that occur prior to the blastocyst invasion of the uterus. During this time the embryo will develop from a single cell into a multi-cellular structure with two distinct cell lineages. Concurrent with embryo cleavage and the beginning of differentiation, the embryo will travel through the oviduct, as a result of peristaltic movement, to eventually reach the uterus where implantation will occur. This whole process, from fertilization to blastocyst implantation lasts 4-5 days in the mouse, and 5 days in the human[1, 2].

While the pre-implantation period is considered to begin after fertilization, the contribution of the gametes should not be overlooked. Evidence in the literature suggests the quality of the egg and sperm has a direct impact on the development of the embryo, implantation, fetal outcome and even adult disease[3, 4]. An overview of the female gametes, oocytes will be discussed, followed by a review of the male gamete development, sperm.

Female mice are born with a finite number of oocytes; however, well over half of these will degrade during the first few weeks following birth[5, 6]. The oocytes that remain will be arrested in the diplotene stage of the first meiotic prophase until they are sexually mature, which is characterized by an increase in size and the competency to respond to hormonal stimulation[1]. Upon stimulation by follicle stimulating hormone (FSH), competent oocytes, making up only a few of the total population will respond by preparing for ovulation. Ovulation is signaled by a surge of luteinizing hormone (LH). The pituitary secretes both FSH and LH, and this cycle is repeated every 4 to 5 days in the mature mouse[1, 7]. For experimental purposes, this process can be artificially induced and amplified in the mice by exogenous hormone (over)stimulation, making the mouse a valuable tool in the investigation of early pregnancy processes.

Analogous to oogenesis, the process of spermatogenesis is reliant on pituitary gonadotrophins[8]. Unlike oogenesis, spermatogenesis is reliant on a self-renewing population of stem cells in the testes, taking around 35 days to complete the process from stem cell to spermatozoa[9]. Once copulation has occurred and the sperm are released into the female reproductive tract, the sperm will enter the oocyte, after several well-characterized events and the oocyte will be fertilized in the distal oviduct.

Several factors can affect the quality of the gametes, including genetic, environmental and metabolic factors. Of importance to this manuscript, diabetic conditions, including hyperglycemia, have been shown to lead to a poorer quality oocyte and sperm. In the oocyte this is associated with reduced levels of ATP and necessary metabolites, resulting in poor reproductive outcomes[10, 11].

After fertilization, a series of cleavage events occur developing a single cell into over sixty cells with two differentiated cell lineages, these events are outlined in **Figure 1.1**. As the embryo travels from the oviduct to the uterus, it will continue to increase in cell number through cleavage events but remain the same size. While mouse and human pre-implantation developments are similar, the following events will refer to specific time points within mouse development. During these very early stages of development following fertilization, the embryo relies on maternal mRNA and protein that was synthesized during the period of oogenesis[12]. However, at the two-cell stage (12-36 hours post-fertilization, hpf), the zygotic transcription is activated and the maternal constituents are degraded over the subsequent developmental stages[13]. Another cleavage event will take place resulting in 4-cells around 48 hpf and 8-cells shortly thereafter, around 60 hpf[1].

While the cells of the embryo remain equipotent to this point, the cells of the embryo begin to compact and polarize. As the embryo develops into the morula stage (~70 hpf), it begins to differentiate into two distinct cell lineages, the inner cell mass (ICM) and the trophectoderm (TE). Of these lineages that form, the inner cell mass, represents the cells that will go on to develop into the embryo. The trophectoderm will surround the inner cell mass making up the periphery of the embryo and shield the ICM

from direct contact with the external environment. These cells, referred to as trophoblasts, will go on to develop into the placenta later in pregnancy[1].

During these events leading to blastocyst development (~84-96 hpf); the embryo has relied on the metabolism of both lactate and pyruvate as main substrates for energy[14, 15]. However, concurrent with the hatching of the blastocyst in preparation for implantation, the embryo acquires the ability to perform glycolysis and relies on glucose as its main substrate[14, 16, 17]. An upregulation of glucose transporters is observed during this time, which will be described in detail later in this chapter.

As mentioned previously, the overall quality of the embryo can be impacted by genetic, metabolic and even environmental conditions. Of interest to this study, the literature has detailed the negative impact that obesity and hyperglycemia has on gamete and embryo quality, leading to a poor reproductive outcome[18, 19]. These metabolic conditions will alter gene expression leading to changes in fetal outcome[20, 21]. Overall, the events occurring during pre-implantation can be critical to the success of the pregnancy and overall fetal outcome.

### *1.1.2 Implantation and Decidualization*

The success of a pregnancy is also highly dependent on the subsequent processes of implantation and decidualization. Implantation refers to the attachment and succeeding invasion of the embryo into the uterine wall. The attachment of the embryo to the uterine wall, occurring 4.5 days after fertilization (E4.5) in the mouse, will signal the process of decidualization to begin[22]. This process includes differentiation and remodeling of the stromal cells of the uterus in order to become receptive to the implanting embryo[23].

Both steps are highly dependent on complex signaling cascades regulated by the steroidal hormones, estrogen and progesterone[24]. These signals will bring about changes in the cell morphology and the biochemical properties of these cells, which will provide an environment that facilitates implantation. Defects in these hormonal signaling cascades leads to an impairment in the decidualization process and oftentimes manifests as infertility[25, 26].

Besides hormonal regulation, ongoing research suggests a critical role of glucose metabolism during decidualization[27]. This role for glucose utilization is supported by the upregulation of several glucose transporters in accordance with this event; with facilitative glucose transporter 1 exhibiting the highest response[28]. The importance of glucose transporters during these events will also be discussed later in this chapter.

Activation of several genes central to decidualization occurs in both the mouse and the human. These genes, although distinct between species, represent common markers of the decidualization process. Increased amounts of bone morphogenetic protein 2 (Bmp2), prolactin-related protein (Prp) and cyclooxygenase 2 (Cox2) are observed in the mouse, while changes in prolactin (Prl), and insulin-like growth factor binding protein (Igfbp1) are evident in humans[29-33].

In summation, events occurring during early pregnancy have great impact on the outcome of the pregnancy. Evidence in the literature suggests that the quality of the gametes has a direct impact on embryo quality. Furthermore, the processes of implantation and decidualization are highly regulated and the success of the pregnancy depends greatly on the completion of these processes. The importance of these processes

to pregnancy outcome highlights the need to continue to fully understand the mechanisms underlying them.

## *1.2 Autophagy*

### *1.2.1 Autophagy Overview*

Autophagy is a catabolic process by which long-lived proteins and damaged organelles are sequestered for degradation via the lysosome. Three types of autophagic degradation have been described in the literature (1) microautophagy, (2) macroautophagy and (3) chaperone-mediated autophagy. Microautophagy occurs as an invagination of the lysosomal membrane. This is unlike the process of macroautophagy, which involves the sequestration of damaged organelles or long-lived proteins in double membrane structures, autophagosomes, which then fuse with lysosomes. And lastly, chaperone-mediated autophagy involves translocation of unfolded, soluble proteins across the lysosomal membranes[34-36]. For the purposes of this manuscript, the word autophagy will be used to refer to the process of macroautophagy.

It is evident in the literature that autophagy is a critical pathway in a number of biological processes, such as cell growth, cellular homeostasis, and development[36, 37]. Moreover, there is a clearly established role of autophagy as a protective process in response to environmental stress. These stressors can include nutrient deprivation, oxidative stress, and infection[37]. Signaling pathways activated in these conditions closely regulate induction of autophagy. In particular, the TOR pathway, which is activated during nutrient starvation and the RAS/camp/PKA pathway, which plays a role in glucose sensing, are important in autophagy induction[38, 39]. Other signaling

pathways, such as AMPK signaling also play a role[40]. Interestingly, while autophagy is initially activated for protective purposes, excess of autophagy can actually be toxic and result in cell death[41]. Furthermore, the process of autophagy may be altered in disease states, which can result in protective effects or even harmful effects[35]. These findings suggest that the process of autophagy must be highly regulated in normal states to maintain a threshold of autophagic activity.

The process of autophagy involves a number of key players in a series of defined steps. The steps in the autophagic pathway that this manuscript will focus on are vesicle formation and elongation, fusion, and the breakdown and recycling of the autolysosome contents. These steps are detailed in **Figure 1.2**.

The origin of the autophagosomal membrane is a highly debated topic in the current literature. A number hypotheses have been considered, however two remain to be debated. One is that the origin of the membrane is formed from the membrane of an intracellular organelle, namely the endoplasmic reticulum, the other hypothesis is that the membrane is synthesized *de novo* in the cytosol[42-44]**.** While both represent attractive hypotheses, sufficient data defining the source of the membrane has yet to be elucidated. It is known, however, that the induction of autophagy occurs via activation of the Atg1 complex (ULK1 and ULK2, in mammals). This complex acts to recruit other Atg proteins to what is known as the Phagophore Activation Site on the autophagosomal membrane[45-47]. The key players that aid in vesicle formation and elongation have been identified as Atg12/Atg8 complex (Atg12/LC3 in mammals), which is an ubiquitin-like protein system, and the Atg16L complex, which aids in both elongation and closure of the autophagosome[48, 49]. These complexes continue to elongate the vesicle until it

has fully engulfed the contents to be degraded. The outer membrane of autophagosome will then fuse with a lysosome and the inner membrane is subsequently degraded forming the autolysosome[50]. The long-lived proteins are degraded by lysosomal hydrolases and the amino acids are returned to the cytosol for use by the cell. Although this process of recycling is not well characterized, it is believed that permeases are responsible for, at the least, amino acid recycling[50]. An interesting question that remains unanswered is: how do the carbohydrates and other macromolecules that remain get recycled back to the cytosol?

### *1.2.2 Autophagy during Early Pregnancy Events*

In recent years the importance of autophagy in normal biological processes, such as development, is becoming increasingly evident. This section of the manuscript will focus on the role of autophagy in early pregnancy events.

As mentioned previously, in both humans and mice, females are born with a finite number of oocytes. A large number of oocytes are destroyed, however this attrition does not correspond with high levels of apoptosis in mice; instead it is hypothesized that autophagy contributes to this process, because an increase in autophagic markers are observed during this process[6].

While autophagy is not observed in unfertilized oocytes in sexually mature female mice, the published data suggests that autophagy is activated 4 hours after fertilization of the oocyte[51]. However, this expression wanes shortly after and then is reactivated at the two-cell stage[51]. The role for autophagy during this early time period of development is unknown, but there is speculation that autophagy may be activated in order to degrade

maternal proteins once the zygotic genome is activated (E2.0). This hypothesis is supported by a developmental arrest between the 4-cell and 8-cell stage in an autophagy deficient model[52]. This indicates that proper autophagic degradation within early embryos is essential for progression of pre-implantation development.

Recently published data suggests a role for autophagy during embryoid cavitation[53]. This group reported that loss of either *Beclin-1* (required for the initiation of autophagosome formation) or *Atg5* (a critical component to the formation of autophagosomes), both essential autophagic genes, leads to failure of cavitation due to the persistence of cellular corpses in murine embryonic stem cells. These cells fail to display signals to indicate removal. This dysregulation is also associated with low levels of ATP[53]. Overall, the lethality associated with removal of this pathway, indicates the critical roles that this pathway plays in embryo development, many of which may not be elucidated yet.

In humans the timing of implantation is important for the success of the pregnancy, and implantation occurring more than 8 days after ovulation correlates to an increase in pregnancy loss in humans[54]. It has been suggested that autophagy may also play a role in this critical process. Lee et al. reported that at the time of implantation in the mouse (E4.5) there is an increase of autophagosomes formed in the trophoblast cells of the blastocyst[55]. This differential response of autophagy during this critical time, mainly an upregulation in the placental cell lineage, indicates a potential role for autophagy in the implantation of the blastocyst.

In conclusion, many questions still remain about the role autophagy plays during early pregnancy events. However, it is known that this process is occurring in both gametes and pre-implantation embryos and is upregulated during implantation in the trophectoderm of the blastocyst. Furthermore, using knockout mouse technology, it has been determined that autophagy is necessary for pre-implantation development beyond the 8-cell stage. These data highlight the need for further studies investigating autophagy during early pregnancy events.

### *1.3 Glucose Transporters*

#### *1.3.1 Overview of Glucose Transporters*

Glucose transport across membranes can be accomplished by either sodium-coupled glucose transporters (SGLTs) or facilitative glucose transporters, known as the SLC2A family. The SLC2A family (with members commonly referred to as GLUTs), is the family of solute carriers known to transport various hexose molecules across membranes. This family is comprised of 14 members[56]. All 14 known SLC2As have shared characteristics including 12 transmembrane spanning helices and an exofacial N-linked glycosylation site, however, each SLC2A family member is unique in regards to tissue localization, kinetics and substrate specificity[56].

The 14 members of the SLC2A family are divided into three classes, Class I, II and III. Class I is comprised of SLC2As 1-4 and 14. Often referred to as the classical transporters, this class of glucose transporters was the first to be described in the literature and has since been the most well studied of the family. Typically, these transporters are either localized to the cell surface, such as SLC2A1, or shuttled to the cell surface from an

intracellular compartment in response to signal, such as insulin or high glucose, as in the case of SLC2A4, to aid in whole body glucose homeostasis[56].

Class II, or the "odd" transporters, consists of SLC2As 5, 7, 9, and 11. Consistent with Class I SLC2As, these facilitative glucose transporters have their N-linked glycosylation site positioned in the exofacial loop between helices 1 and 2. However, this class of transporters are unique as they are able to transport fructose[57]. While SLC2A5 and SLC2A7 are found primarily in the intestine, SLC2A11 has a much wider distribution pattern. And SLC2A9 is widely recognized as a transporter of uric acid and has been shown to play a key role in urate homeostasis[58].

Lastly, Class III, or the "even" transporters encompasses SLC2As 6, 8, 10, 12 and the proton myoinositol transporter or SLC2A13. Unlike, Class I and II, the N-linked glycosylation site in Class III transporters is not in the first exofacial loop but rather in the fifth loop. Intriguingly, another unique characteristic is the retention of these SLC2As to an intracellular location, which has been attributed to the presence of an internalization signaling motif[59]. While the majority of these glucose transporters have not been well studied, research is ongoing to characterize these SLC2As.

Dysregulation of glucose transporters often results in adverse metabolic conditions, as implied by their function. While manipulation of SLC2A gene expression results in a variety of severity in phenotypes in mice, in humans four known inherited syndromes have been linked to mutations in the genes of SLC2A family members to date[56]. Notably, GLUT-1 deficiency syndrome causes infantile seizures, microcephaly and a delay in development due to a decrease of glucose transport across the brain-blood

barrier[60]. Another condition is Fanconi-Bickel Syndrome, which is caused by mutations in SLC2A2 resulting metabolic dysfunctions along with hepatomegaly and dwarfism[61]. These adverse outcomes reveal a necessity for a clear understanding of the role of each facilitative glucose transporter in physiological events.

### *1.3.2 Glucose Transporter 8*

Glucose Transporter 8 was the first SLC2A identified by database mining and was determined to have 44% sequence similarity to SLC2A5[62]. A member of the Class III SLC2As, SLC2A8 has the shared features of a N-glycosylation site on the fifth extracellular loop and a intracellular localization, which is signaled by a dileucine motif (DEXXXLL), trafficking SLC2A8 to the late endosome/lysosome[59]. In order to determine affinity for glucose, Xenopus oocyte glucose uptake studies are used. In these studies, the *Slc2a8* gene must be manipulated by changing an amino acid in the intracellular localization signal to allow for SLC2A8 to be translocated to the plasma membrane. These studies have suggested that SLC2A8 has a high affinity for glucose, with a Km~2[63]. Additionally, transport of glucose in this system by SLC2A8 can be competed out with the addition of fructose or galactose, suggesting that SLC2A8 may transport these and potentially other hexoses. Additionally, glucose transport by SLC2A8 can be blocked by the addition of cytochalasin B, a known glucose transport inhibitor[63]. Research on this SLC2A is ongoing, particularly concerning physiological function of SLC2A8.

Expression profiling of SLC2A8 has revealed a wide distribution of this transporter with the most abundant expression exhibited in the testes. SLC2A8 is also

expressed in other reproductive tissues such as the ovary and uterus, in addition to various other tissues including the brain, liver, adipose, and heart. It is also present in the blastocyst[63].

Due to the expression of SLC2A8 in reproductive and embryonic tissues, it was speculated to have a role in early pregnancy events. In fact, while SLC2A8 is not known to be present at the cell membrane in any of the investigated cell types, there is data to support its translocation to the plasma membrane in blastocysts in an insulin responsive manner[64]. This data suggests a possible novel role for SLC2A8 in glucose utilization during blastocyst development and reflects the unique environment in embryonic stem cells. Furthermore, knockdown of *Slc2a*8 in the embryo, by anti-sense technology, resulted in an increase of cell death in blastocysts and a decrease in successful pregnancies by E14.5[65]. Again, this supports the hypothesis that SLC2A8 is critical for normal embryonic development.

While data obtained from blastocyst studies predict a requirement of SLC2A8 in normal embryonic development, two published *Slc2a8* deficient models do not exhibit embryonic lethality as expected. This unexpected finding could be attributed to knockout strategy, as both models of *Slc2a8* deficient mouse models were created by unconventional strategies. Membrez et al. created a conditional mouse, which expressed a truncated form of the SLC2A8 protein. This group targeted exon 10, the last exon of the *Slc2a8* gene, by flanking it with loxP sites and crossed lox-positive mice with NesCreI mice, which express Cre recombinase driven by the Nestin promoter. The use of the alternative, NesCreI mice, was supported by literature reporting activation of Nestin in the germ line. The authors acknowledge that this results in a truncated SLC2A8 protein,

which was determined to be non-functional. Studies to determine functionality are were conducted using Xenopus uptake assays, which require a mutation in the intracellular localization for retention to the plasma membrane along with the deletion of exon 10[66]. A second group, Gawlik et al., constructed a targeting site flanking exons 5-7 with loxP sites and introducing this by homologous recombination into embryonic stem cells (ES). The ES cell clones, which had successfully incorporated the targeted allele, were transiently trasnfected with pCre to generate ES clones with a deleted allele, which were used for morula aggregration and the resultant blastocysts were transferred to a pseduopregnancy female mouse[67]. However, the resultant *Slc2a8-/-* mice were only shown to not possess the full length mRNA by quantitative RT-PCR and not by conventional standards of Southern Blot. Therefore, due to the alternative strategies employed and inadequate published verification of non-functional SLC2A8 protein in these *Slc2a8-/-* mice, careful consideration must be taken when reviewed the resultant phenotypes.

While neither group observed embryonic lethality, both groups noted that the heterozygous mating of *Slc2a8* mice resulted in lower than expected percentage of *Slc2a8-/-* and *Slc2a8+/-* pups[66, 67]. Gawlik et al. attributed this phenotype to an observable reduction in sperm motility[67]. Investigation into the reproductive phenotype revealed a decrease in mitochondrial membrane potential leading to decreased ATP levels and a reduced sperm motility[67]. Overall, the authors hypothesized the lower than expected number of homozygous null and heterozygous pups in the heterozygous matings to the possibility of the *Slc2a8-/-* sperm being slower to reach the egg. However, this is

unlikely, as the time necessary in the uterine tract for the sperm to even become competent for fertilization is around an hour[1].

Other notable phenotypic observations includes a mild brain phenotype consisting of increased proliferation of hippocampal cells and an increased P-wave duration in the heart, in the mouse model created by Membrez et al.[66]. And several behavioral alterations, including hyperactivity in the previously mentioned mouse model with reduced sperm motility[68]. All of these phenotypes are not surprising as SLC2A8 is highly expressed in the testes and expressed to a lesser extent in the brain and heart. Of note, neither of these groups reported changes in normal glucose homeostasis or growth at baseline in these models.

Overall, in the field of glucose transport, questions surrounding the function of SLC2A8 still remain unanswered. However, the combination of data obtained by *in vitro* and *in vivo* studies, suggest an importance of SLC2A8 in several processes, including embryonic development, sperm functionality, normal neuronal activities and normal heart function. These findings emphasize the need for a clear understanding of the role of SLC2A8.

### *1.3.3 Glucose Transporters in Early Pregnancy Events*

#### *1.3.3.1 Glucose Transporters in Pre-implantation Events*

As the gametes and the embryo journey from the oviduct to the uterus, and progress from a single cell into a blastocyst, they are subjected to a variety of environments. This requires the gametes and the embryo to be able to adapt to these

differing surroundings. One way in which they accomplish this is by utilizing of a variety of glucose transporters during these processes.

As previously mentioned, the oocyte and early embryo exhibit a low level of glucose consumption, as pyruvate is the main energy source. However, of the facilitative glucose transporters that have been investigated, several are expressed in the oocyte of the mouse, including SLC2A1, 7, 9 and 13[69-71]. Additionally, conditions that lead to abnormal glucose transporter expression, such as hyperglycemia and hyperinsulemnia, have suggested a need for normal glucose transporter expression in oocyte progression and function. This is evident from studies using oocytes obtained from mouse models of Type I diabetes. These oocytes are smaller and exhibit a delayed maturation[72]. Additionally exposure to this aberrant metabolic milieu for merely 24 hours after fertilization will lead to detrimental fetal outcomes. This is evident in a study conducted by Moley et al., in which fertilized oocytes from diabetic female mice were transferred into pseudo-pregnant control mice. The resultant fetuses showed an increased incidence of malformations and growth retardation when compared to fetuses obtained from transferring control oocytes into pseudo-pregnant control females[73]. This data along with previous studies which report a decrease in glucose transporter expression during diabetic conditions in the blastocyst, suggests that normal glucose transport is critical to normal development at very early stages of pregnancy and that alterations in this function leads to an increase in poor fetal outcome[20].

Previously, a role for SLC2A8 in energy metabolism of the sperm was addressed. While the function of glucose transporters in sperm remains elusive, we are able to understand a need for normal glucose transporter expression in the male reproductive

tissue by examining situations in which this expression is disrupted[74]. For example, in a mouse model of Type I diabetes, the mice exhibit a decrease in sperm concentration, motility and fertilization[74]. Additionally, in human studies, fertilization rates and progression to blastocyst development are decreased. Both of these studies reflect the importance of glucose homeostasis in normal sperm development and highlight the need for ongoing research in this field[74].

Briefly alluded to previously, facilitative glucose transporters have been shown to be important in embryogenesis in the mouse model. Several SLC2As are expressed at various stages of pre-implantation development and alteration of the expression of these SLC2As, either by genetic means or metabolically, results in deleterious effects on the embryo. This section will detail the current field of SLC2As in pre-implantation embryogenesis.

Of the facilitative glucose transporters that have been studied, several have been identified in the mouse in various stage of pre-implantation development, including SLC2As 1-4, 8, 9 and 12. Using mouse models of glucose transporter knockdown or knockout, we are able to investigate the role of these glucose transporters in reproduction.

SLC2A1 is expressed throughout pre-implantation embryogenesis[75]. However, SLC2A1 expression remains cytoplasmic until the morula stage when the embryo is polarized, at this time GLUT1 is localized to basolateral membrane of the trophoblast and the plasma membrane of the ICM, presumably to transport glucose into the ICM**(Figure 1.3)**[75, 76]. Deletion of SLC2A1 does not result in embryonic lethality, however the *Slc2a1-/-* mice exhibit lethality around E13[77]. Survival of the fetuses to this later stage

in development indicates SLC2A1 is not required for pre-implantation embryonic development to occur or that possibly another SLC2A is able to compensate for the loss. SLC2A2 is also found basolaterally in the trophoblast cells of the mouse. This transporter represents an inefficient transporter in the low glucose environment due to its high Km value (~20mM)[78]. Therefore, it may represent either no function or a compensatory mechanism for SLC2A1 loss. Furthermore, conventional global knockout of *Slc2a2* in mice does not produce embryonic lethality, however, the mice develop symptoms of Type II diabetes and die shortly after birth, indicating a role in whole body glucose homeostasis[79].

SLC2A3 is found in both human and mouse starting during compaction, around the 8-cell stage[80]. SLC2A3 is a high capacity transporter and is often found in tissues which require an abundance of glucose. Contrary to SLC2A1 and SLC2A2, SLC2A3 is found on the apical membrane, indicating a possible role in delivering glucose from the maternal environment[80]. Deletion of *Slc2a3* by gene trap results in demise of *Slc2a3-/-* fetuses by E12.5. Another model of *Slc2a3* deficiency by conventional knockout of *Slc2a3*, results in an increase in apoptosis in the trophectoderm and overall demise of the embryo by E9.5. In this *Slc2a3-/-* mouse model, diffuse SLC2A1 expression was observed, as opposed to the normal expression pattern at the basolateral membrane in trophoblast cells[81]. The presence of another facilitative glucose transporter, SLC2A4 in pre-implantation embryos remains controversial in the literature, and deficiency of this gene in mice does not present with defects in reproduction[82, 83].

As previously discussed in the last section, SLC2A8 expression is localized to the apical plasma membrane in the trophoblast cells in response to insulin and knockdown of

SLC2A8 by anti-sense technology led to an increase in apoptosis[64]. Transfer of these treated embryos to pseudo-pregnant females resulted in decreased pregnancy rate and a high rate of resorptions[65]. However, surprisingly, knockout of *Slc2a8* in the mouse did not result in embryonic lethality in two published models. However, as mentioned previously, this may be a result of the unconventional strategies employed in the creation of the mouse. SLC2A9 has also been investigated in the mouse. It is expressed during the one-to-two cell stage and again later in the blastocyst intracellularly. By blocking *Slc2a9* expression by antisense technology and transferring the embryos into pseudo-pregnant females, Carayannopoulos et al. were able to show the importance of SLC2A9 in embryogenesis, as rates of pregnancy were decreased[84]. Lastly, SLC2A12 is seen very early in development, with expression apparent in the oocyte and two-cell embryo however this expression lessens around E3[85]. Further studies are warranted to fully understand the role these facilitative glucose transporters represent in pre-implantation embryonic development.

#### *1.3.3.2 Glucose Transporters in Implantation and Decidualization*

Uterine decidualization is a remarkable process that requires the differentiation of a population of uterine cells, the stromal cells. Several studies have indicated the importance of glucose metabolism during decidualization[28, 86-88]. Recently, Frolova et al. reported that culture conditions of low glucose resulted in impaired decidualization in both mouse and human *in vitro* endometrial stromal cell cultures[28]. Furthermore, the increase of facilitative glucose transporter expression during this critical time window for decidualization indicates a requirement of glucose utilization to complete the decidualization process[28, 87, 88].

Of the glucose transporters that have been investigated, several SLC2As are expressed in the uterus during decidualization in both mice and humans, including SLC2A1, 3, 6, 8, 10, and 12[28]. Expression of SLC2A2, 4, and 5 are either not expressed (SLC2A2) or expressed at almost undetectable levels (SLC2A4 and 5). While several SLC2As are present during decidualization, only a few are shown to increase concurrent with the decidualization process. In mice SLC2A1, 6 and 8 exhibit significant increase in mRNA copy number during this process *in vitro*. In human *in vitro* endometrial stromal cell culture, SLC2A1, 8, 10 and 12 exhibit increased mRNA copy number upon decidualization[28].

In both mice and human, SLC2A1 represents the most abundant facilitative glucose transporter with the highest fold increase of the SLC2As during decidualization[28, 87]. Frolova et al. demonstrated the importance of this SLC2A during decidualization by using lentivirus shRNA technology to knockdown *Slc2a1* in human endometrial stromal cells. This deficiency in SLC2A1 led to a significant decrease in decidualization marker expression, suggesting a major role for SLC2A1 in the decidualization process. Furthermore, compensation for loss of SLC2A1 was not evident based on expression of the other SLC2As, indicating a specific role for SLC2A1 in this process[28]. Overall, this indicates a unique and critical role for SLC2A1 in decidualization.

The only other SLC2A to exhibit an increase in mRNA copy number upon decidualization in both mice and humans is SLC2A8[28]. Unlike, SLC2A1 which is localized specifically to the basolateral surface to presumably transport glucose into the stromal cells, SLC2A8 is found in high- and low- density intracellular membrane fractions

using cellular fractionation of mouse endometrial stromal cells[89]. This localization is concurrent with reported intracellular localization of SLC2A8 in other cell types[59, 90]. These distinct localization patterns suggest differing roles for these SLC2As in the uterus and warrant further studies to elucidate the role of SLC2A8 during decidualization.

In conclusion, the decidualization process requires an abundance of energy to undergo the functional and morphological changes involved in preparation of the uterus for the implanting blastocyst. One important energy source for this transformation is glucose. The need for glucose during this process is evident by the increased abundance of SLC2As during decidualization. In particular, SLC2A1 has been identified as the most abundant SLC2A, and expression of this transporter is critical for proper uterine decidualization. However, further studies are warranted to determine the role of several other SLC2As that are expressed during this time, in particular SLC2A8.

In summation, events occurring during the pre-implantation period are critical to fetal outcome and the success of a pregnancy. Aberrations in this process can arise from alterations of glucose transport, both by genetic and metabolic means. These potentially detrimental outcomes reinforce the need for a clear understanding of the role of glucose transport in early pregnancy events.

### *1.4 References*


1. Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R., *Manipulating the Mouse Embryo: A Laboratory Manual* Third ed. 2003, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
2. Fritz, M.A. and L. Speroff, *Clinical gynecologic endocrinology and infertility*. 8th ed, Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins. x, 1439 p.
3. Krisher, R.L., *The effect of oocyte quality on development.* J Anim Sci, 2004. **82 E-Suppl**: p. E14-23.
4. Sirard, M.A., et al., *Contribution of the oocyte to embryo quality.* Theriogenology, 2006. **65**(1): p. 126-36.
5. Faddy, M.J., R.G. Gosden, and R.G. Edwards, *Ovarian follicle dynamics in mice: a comparative study of three inbred strains and an F1 hybrid.* J Endocrinol, 1983. **96**(1): p. 23-33.
6. Rodrigues, P., et al., *Multiple mechanisms of germ cell loss in the perinatal mouse ovary.* Reproduction, 2009. **137**(4): p. 709-20.
7. Richards, J.S., et al., *Ovarian follicular development: from physiology to molecular biology.* Recent Prog Horm Res, 1987. **43**: p. 231-76.
8. Pareek, T.K., et al., *Insights into male germ cell apoptosis due to depletion of gonadotropins caused by GnRH antagonists.* Apoptosis, 2007. **12**(6): p. 1085-100.
9. Eddy, E.M., *Male germ cell gene expression.* Recent Prog Horm Res, 2002. **57**: p. 103-28.

10. Wang, Q. and K.H. Moley, *Maternal diabetes and oocyte quality.* Mitochondrion. **10**(5): p. 403-10.

11. Ratchford, A.M., et al., *Maternal diabetes adversely affects AMP-activated protein kinase activity and cellular metabolism in murine oocytes.* Am J Physiol Endocrinol Metab, 2007. **293**(5): p. E1198-206.

12. Stitzel, M.L. and G. Seydoux, *Regulation of the oocyte-to-zygote transition.* Science, 2007. **316**(5823): p. 407-8.

13. Piko, L. and K.B. Clegg, *Quantitative changes in total RNA, total poly(A), and ribosomes in early mouse embryos.* Dev Biol, 1982. **89**(2): p. 362-78.

14. Leese, H.J. and A.M. Barton, *Pyruvate and glucose uptake by mouse ova and preimplantation embryos.* J Reprod Fertil, 1984. **72**(1): p. 9-13.

15. Harris, S.E., et al., *Pyruvate and oxygen consumption throughout the growth and development of murine oocytes.* Mol Reprod Dev, 2009. **76**(3): p. 231-8.

16. Gardner, D.K. and H.J. Leese, *Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos.* Hum Reprod, 1986. **1**(1): p. 25-7.

17. Gardner, D.K., T.B. Pool, and M. Lane, *Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation, and viability.* Semin Reprod Med, 2000. **18**(2): p. 205-18.

18. Yang, J., et al., *Fetal and neonatal outcomes of diabetic pregnancies.* Obstet Gynecol, 2006. **108**(3 Pt 1): p. 644-50.

19. Moley, K.H., et al., *Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways.* Nat Med, 1998. **4**(12): p. 1421-4.

20. Moley, K.H., M.M. Chi, and M.M. Mueckler, *Maternal hyperglycemia alters glucose transport and utilization in mouse preimplantation embryos.* Am J Physiol, 1998. **275**(1 Pt 1): p. E38-47.

21. Chi, M.M., et al., *Decreased glucose transporter expression triggers BAX-dependent apoptosis in the murine blastocyst.* J Biol Chem, 2000. **275**(51): p. 40252-7.

22. Paria, B.C., Y.M. Huet-Hudson, and S.K. Dey, *Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus.* Proc Natl Acad Sci U S A, 1993. **90**(21): p. 10159-62.

23. Ramathal, C.Y., et al., *Endometrial decidualization: of mice and men.* Semin Reprod Med. **28**(1): p. 17-26.

24. Dey, S.K., et al., *Molecular cues to implantation.* Endocr Rev, 2004. **25**(3): p. 341-73.

25. Lubahn, D.B., et al., *Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 11162-6.

26. Lydon, J.P., et al., *Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities.* Genes Dev, 1995. **9**(18): p. 2266-78.

27. Frolova, A.I. and K.H. Moley, *Glucose transporters in the uterus: an analysis of tissue distribution and proposed physiological roles.* Reproduction. **142**(2): p. 211-20.

28. Frolova, A.I. and K.H. Moley, *Quantitative analysis of glucose transporter mRNAs in endometrial stromal cells reveals critical role of GLUT1 in uterine receptivity.* Endocrinology. **152**(5): p. 2123-8.

29. Bell, S.C., et al., *Regulation of insulin-like growth factor-binding protein-1 synthesis and secretion by progestin and relaxin in long term cultures of human endometrial stromal cells.* J Clin Endocrinol Metab, 1991. **72**(5): p. 1014-24.

30. Giudice, L.C., et al., *Insulin-like growth factor binding proteins in human endometrium: steroid-dependent messenger ribonucleic acid expression and protein synthesis.* J Clin Endocrinol Metab, 1991. **72**(4): p. 779-87.

31. Ying, Y. and G.Q. Zhao, *Detection of multiple bone morphogenetic protein messenger ribonucleic acids and their signal transducer, Smad1, during mouse decidualization.* Biol Reprod, 2000. **63**(6): p. 1781-6.

32. Lim, H., et al., *Hoxa-10 regulates uterine stromal cell responsiveness to progesterone during implantation and decidualization in the mouse.* Mol Endocrinol, 1999. **13**(6): p. 1005-17.

33. Gu, Y., et al., *Expression of decidual prolactin-related protein in the rat decidua.* Endocrinology, 1994. **135**(4): p. 1422-7.

34. Massey, A., R. Kiffin, and A.M. Cuervo, *Pathophysiology of chaperone-mediated autophagy.* Int J Biochem Cell Biol, 2004. **36**(12): p. 2420-34.

35. Martinet, W., et al., *Autophagy in disease: a double-edged sword with therapeutic potential.* Clin Sci (Lond), 2009. **116**(9): p. 697-712.

36. Levine, B. and D.J. Klionsky, *Development by self-digestion: molecular mechanisms and biological functions of autophagy.* Dev Cell, 2004. **6**(4): p. 463-77.

37. Yang, Z. and D.J. Klionsky, *Mammalian autophagy: core molecular machinery and signaling regulation.* Curr Opin Cell Biol. **22**(2): p. 124-31.

38. Kamada, Y., et al., *Tor-mediated induction of autophagy via an Apg1 protein kinase complex.* J Cell Biol, 2000. **150**(6): p. 1507-13.

39. Mavrakis, M., et al., *Depletion of type IA regulatory subunit (RIalpha) of protein kinase A (PKA) in mammalian cells and tissues activates mTOR and causes autophagic deficiency.* Hum Mol Genet, 2006. **15**(19): p. 2962-71.

40. Liang, J., et al., *The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis.* Nat Cell Biol, 2007. **9**(2): p. 218-24.

41. Akazawa, H., et al., *Diphtheria toxin-induced autophagic cardiomyocyte death plays a pathogenic role in mouse model of heart failure.* J Biol Chem, 2004. **279**(39): p. 41095-103.

42. Kovacs, A.L., et al., *Sequestration revisited: integrating traditional electron microscopy, de novo assembly and new results.* Autophagy, 2007. **3**(6): p. 655-62.

43. Hayashi-Nishino, M., et al., *A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation.* Nat Cell Biol, 2009. **11**(12): p. 1433-7.

44. Yla-Anttila, P., et al., *3D tomography reveals connections between the phagophore and endoplasmic reticulum.* Autophagy, 2009. **5**(8): p. 1180-5.

45. Hara, T., et al., *FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells.* J Cell Biol, 2008. **181**(3): p. 497-510.

46. Jung, C.H., et al., *ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery.* Mol Biol Cell, 2009. **20**(7): p. 1992-2003.

47. Mizushima, N., *The role of the Atg1/ULK1 complex in autophagy regulation.* Curr Opin Cell Biol. **22**(2): p. 132-9.

48. Geng, J. and D.J. Klionsky, *The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series.* EMBO Rep, 2008. **9**(9): p. 859-64.

49. Fujita, N., et al., *The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy.* Mol Biol Cell, 2008. **19**(5): p. 2092-100.

50. Chen, Y. and D.J. Klionsky, *The regulation of autophagy - unanswered questions.* J Cell Sci. **124**(Pt 2): p. 161-70.

51. Tsukamoto, S., et al., *Autophagy is essential for preimplantation development of mouse embryos.* Science, 2008. **321**(5885): p. 117-20.

52. Tsukamoto, S., A. Kuma, and N. Mizushima, *The role of autophagy during the oocyte-to-embryo transition.* Autophagy, 2008. **4**(8): p. 1076-8.

53. Qu, X., et al., *Autophagy gene-dependent clearance of apoptotic cells during embryonic development.* Cell, 2007. **128**(5): p. 931-46.

54. Wilcox, A.J., D.D. Baird, and C.R. Weinberg, *Time of implantation of the conceptus and loss of pregnancy.* N Engl J Med, 1999. **340**(23): p. 1796-9.

55. Lee, J.E., et al., *Autophagy regulates embryonic survival during delayed implantation.* Endocrinology. **152**(5): p. 2067-75.

56. Augustin, R., *The protein family of glucose transport facilitators: It's not only about glucose after all.* IUBMB Life. **62**(5): p. 315-33.

57. Manolescu, A.R., et al., *Facilitated hexose transporters: new perspectives on form and function.* Physiology (Bethesda), 2007. **22**: p. 234-40.

58. Doblado, M.A. and K.H. Moley, *Facilitative glucose transporter 9 (GLUT9), a unique hexose and urate transporter.* Am J Physiol Endocrinol Metab, 2009.

59. Augustin, R., J. Riley, and K.H. Moley, *GLUT8 contains a [DE]XXXL[LI] sorting motif and localizes to a late endosomal/lysosomal compartment.* Traffic, 2005. **6**(12): p. 1196-212.

60. Seidner, G., et al., *GLUT-1 deficiency syndrome caused by haploinsufficiency of the blood-brain barrier hexose carrier.* Nat Genet, 1998. **18**(2): p. 188-91.

61. Santer, R., B. Steinmann, and J. Schaub, *Fanconi-Bickel syndrome--a congenital defect of facilitative glucose transport.* Curr Mol Med, 2002. **2**(2): p. 213-27.

62. Phay, J.E., H.B. Hussain, and J.F. Moley, *Strategy for identification of novel glucose transporter family members by using internet-based genomic databases.* Surgery, 2000. **128**(6): p. 946-51.

63. Ibberson, M., M. Uldry, and B. Thorens, *GLUTX1, a novel mammalian glucose transporter expressed in the central nervous system and insulin-sensitive tissues.* J Biol Chem, 2000. **275**(7): p. 4607-12.

64. Carayannopoulos, M.O., et al., *GLUT8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst.* Proc Natl Acad Sci U S A, 2000. **97**(13): p. 7313-8.

65. Pinto, A.B., et al., *Glucose transporter 8 expression and translocation are critical for murine blastocyst survival.* Biol Reprod, 2002. **66**(6): p. 1729-33.

66. Membrez, M., et al., *GLUT8 is dispensable for embryonic development but influences hippocampal neurogenesis and heart function.* Mol Cell Biol, 2006. **26**(11): p. 4268-76.

67. Gawlik, V., et al., *Targeted disruption of Slc2a8 (GLUT8) reduces motility and mitochondrial potential of spermatozoa.* Mol Membr Biol, 2008. **25**(3): p. 224-35.

68. Schmidt, S., et al., *Deletion of glucose transporter GLUT8 in mice increases locomotor activity.* Behav Genet, 2008. **38**(4): p. 396-406.

69. Morita, Y., et al., *Expression and possible function of glucose transporter protein GLUT1 during preimplantation mouse development from oocytes to blastocysts.* Biochem Biophys Res Commun, 1992. **188**(1): p. 8-15.

70. Lee, J.H., Y.W. Kim, and S.G. Chang, *Glucose transporter-1 expression in urothelial papilloma of the bladder.* Urol Int, 2005. **74**(3): p. 268-71.

71. Dan-Goor, M., et al., *Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos.* Hum Reprod, 1997. **12**(11): p. 2508-10.

72. Colton, S.A., G.M. Pieper, and S.M. Downs, *Altered meiotic regulation in oocytes from diabetic mice.* Biol Reprod, 2002. **67**(1): p. 220-31.

73. Wyman, A., et al., *One-cell zygote transfer from diabetic to nondiabetic mouse results in congenital malformations and growth retardation in offspring.* Endocrinology, 2008. **149**(2): p. 466-9.

74. Kim, S.T. and K.H. Moley, *Paternal effect on embryo quality in diabetic mice is related to poor sperm quality and associated with decreased glucose transporter expression.* Reproduction, 2008. **136**(3): p. 313-22.

75. Aghayan, M., et al., *Developmental expression and cellular localization of glucose transporter molecules during mouse preimplantation development.* Development, 1992. **115**(1): p. 305-12.

76. Pantaleon, M., et al., *An unusual subcellular localization of GLUT1 and link with metabolism in oocytes and preimplantation mouse embryos.* Biol Reprod, 2001. **64**(4): p. 1247-54.

77. Wang, D., et al., *A mouse model for Glut-1 haploinsufficiency.* Hum Mol Genet, 2006. **15**(7): p. 1169-79.

78. Pantaleon, M. and P.L. Kaye, *Glucose transporters in preimplantation development.* Rev Reprod, 1998. **3**(2): p. 77-81.

79. Guillam, M.T., et al., *Early diabetes and abnormal postnatal pancreatic islet development in mice lacking Glut-2.* Nat Genet, 1997. **17**(3): p. 327-30.

80. Pantaleon, M., et al., *Glucose transporter GLUT3: ontogeny, targeting, and role in the mouse blastocyst.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3795-800.

81. Ganguly, A., et al., *Glucose transporter isoform-3 mutations cause early pregnancy loss and fetal growth restriction.* Am J Physiol Endocrinol Metab, 2007. **292**(5): p. E1241-55.

82. Riley, J.K. and K.H. Moley, *Glucose utilization and the PI3-K pathway: mechanisms for cell survival in preimplantation embryos.* Reproduction, 2006. **131**(5): p. 823-35.

83. Katz, E.B., et al., *Cardiac and adipose tissue abnormalities but not diabetes in mice deficient in GLUT4.* Nature, 1995. **377**(6545): p. 151-5.

84. Carayannopoulos, M.O., et al., *GLUT9 is differentially expressed and targeted in the preimplantation embryo.* Endocrinology, 2004. **145**(3): p. 1435-43.

85. Zhou, Y., P.L. Kaye, and M. Pantaleon, *Identification of the facilitative glucose transporter 12 gene Glut12 in mouse preimplantation embryos.* Gene Expr Patterns, 2004. **4**(6): p. 621-31.

86. von Wolff, M., et al., *Glucose transporter proteins (GLUT) in human endometrium: expression, regulation, and function throughout the menstrual cycle and in early pregnancy.* J Clin Endocrinol Metab, 2003. **88**(8): p. 3885-92.

87. Frolova, A., et al., *Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells.* Endocrinology, 2009. **150**(3): p. 1512-20.

88. Kim, S.T. and K.H. Moley, *Regulation of facilitative glucose transporters and AKT/MAPK/PRKAA signaling via estradiol and progesterone in the mouse uterine epithelium.* Biol Reprod, 2009. **81**(1): p. 188-98.

89. Flessner, L.B. and K.H. Moley, *Similar [DE]XXXL[LI] motifs differentially target GLUT8 and GLUT12 in Chinese hamster ovary cells.* Traffic, 2009. **10**(3): p. 324-33.

90. Schmidt, S., H.G. Joost, and A. Schurmann, *GLUT8, the enigmatic intracellular hexose transporter.* Am J Physiol Endocrinol Metab, 2009. **296**(4): p. E614-8.

### *1.5 Figure Legends*

**Figure 1.1. Pre-implantation stages of development in the mouse.** Upon fertilization of the oocyte in the oviduct, the developing embryo travels toward the uterus while undergoing several cell cleavages. After entering the uterine cavity, at about 4.5dpc, the blastocyst will hatch and begin implanting in the uterine wall.

**Figure 1.2. A schematic of autophagy in mammals.** Several autophagic proteins are recruited to the phagosome activation site (PAS) during autophagy initiation including the ULK1/2 complex. The Atg12-Atg5-Atg16L complex along with LC3-II aids in phagopore elongation to form the enclosed autophagosome. The autophagsome then fuses with the lysosome, and lysosomal enzymes degrade the internal membrane and autolysosomal contents. Recycling of the amino acids to the cytosol is performed by the lysosomal permeases.

**Figure 1.3. Glucose transporter localization in murine blastocysts.** SLC2A1 is localized to the basolateral membranes of the trophophectoderm in the murine blastocyst, while SLC2A3 is localized on the apical membranes. The localization of SLC2A8 is unique in blastocysts as it is translocated to the plasma membrane of the trophoblast cells in response to insulin.

**Figure 1.4. Glucose transporter localization in endometrial stromal cells.** SLC2A1 is the most abundant facilitative glucose transporter present in the endometrial stromal cells (ESC) of the uterus, followed by SLC2A3 in the mouse. These SLC2As are localized to the plasma membrane of ESCs. SLC2A4 a well-characterized insulin-responsive SLC2A, is reported at almost undetectable levels. Localization of SLC2A8 is unknown, however it has been localized intracellularly to either the endoplasmic reticulum or lysosome in other tissues.

**Figure 1.1**

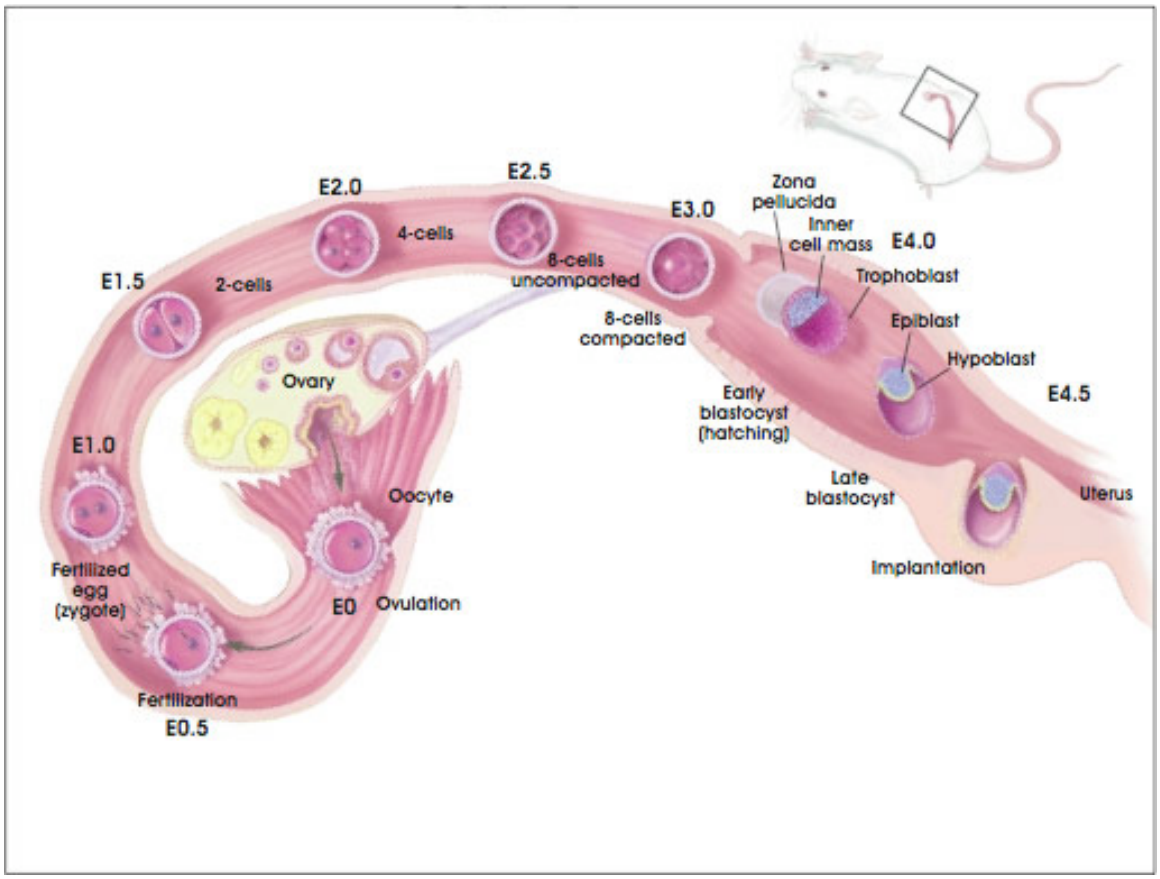
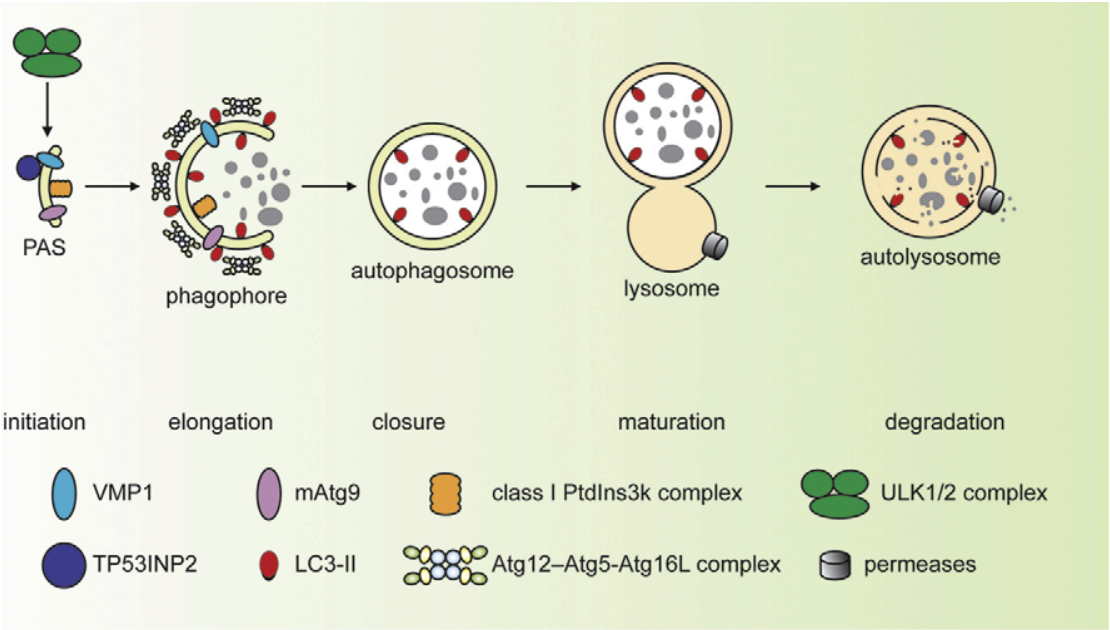


**Figure 1.2**




Adapted from: Current Opinion in Cell Biology 2010, 22:124–13. [37]

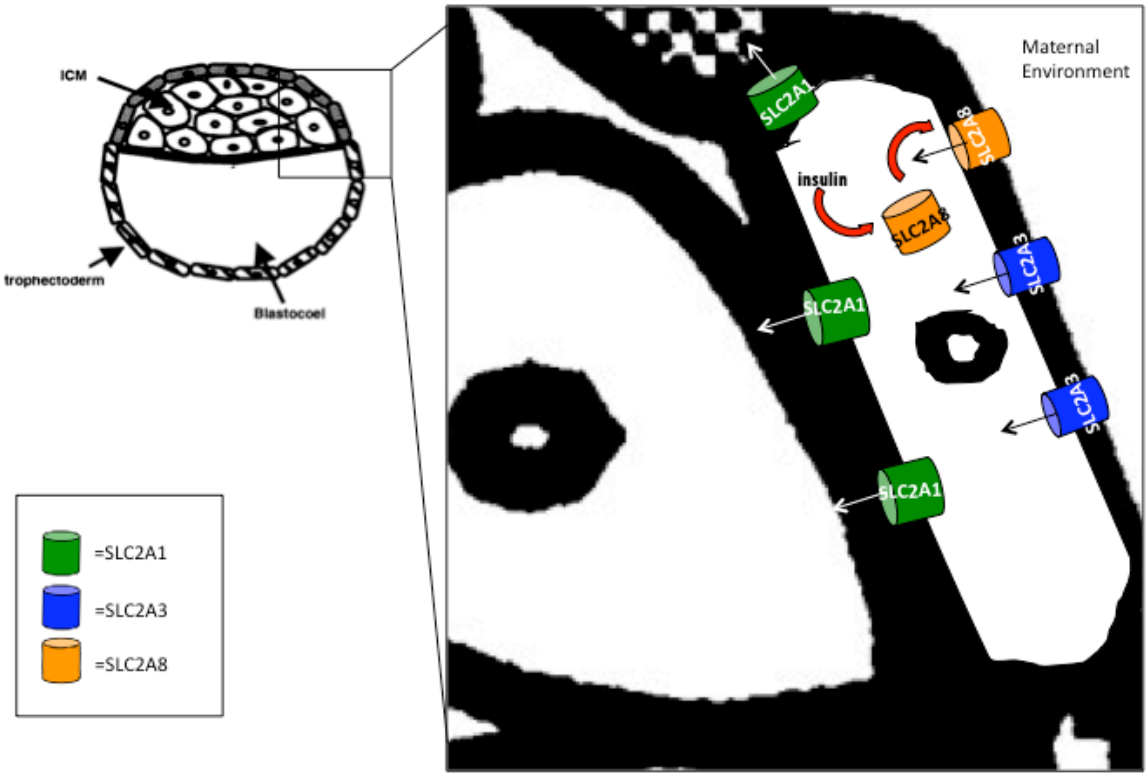
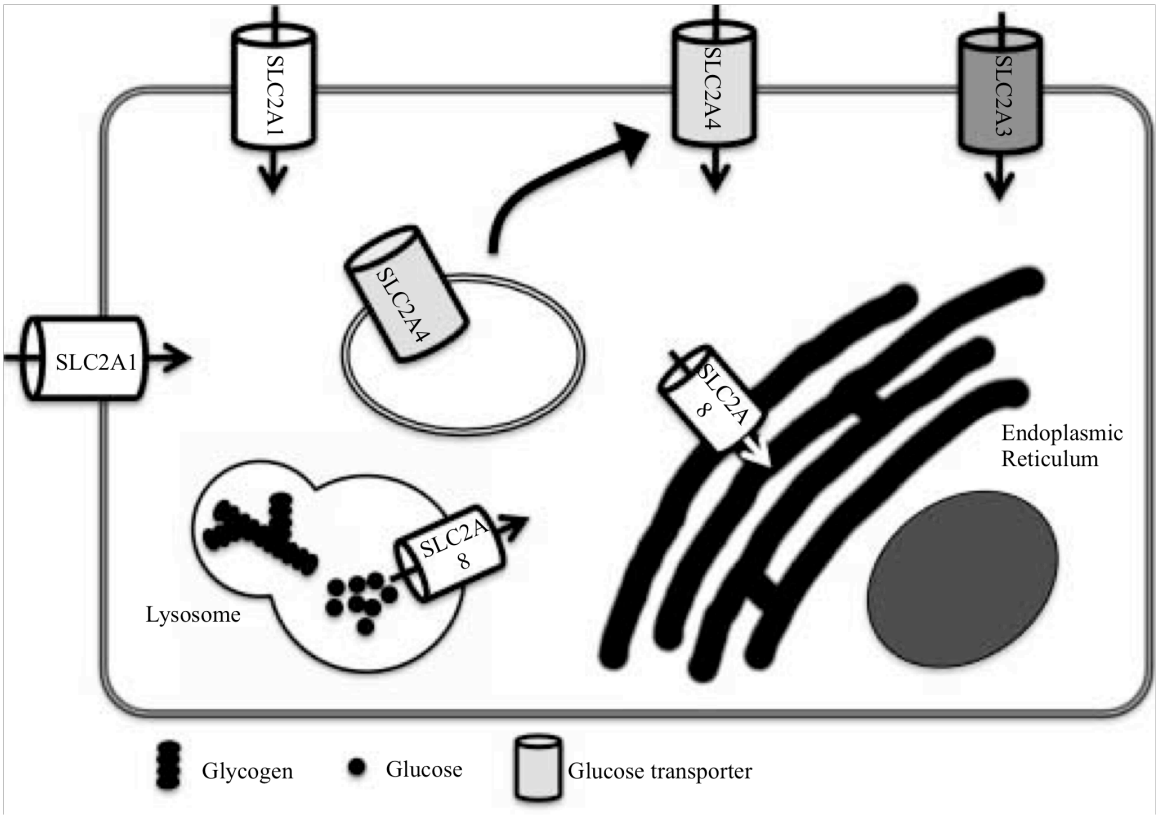

ICM
trophectoderm
Blastocoel
Maternal
Environment
SLC2A1
SLC2A8
insulin
SLC2A8
SLC2A3
SLC2A1
SLC2A3
SLC2A1
=SLC2A1
=SLC2A3
=SLC2A8

**Figure 1.3**

**Figure 1.4**




Adapted from: Reproduction (2011) 142: 211-220. [27]

# CHAPTER 2

## A Differential Autophagic Response to Hyperglycemia in the Developing Murine Embryo

**Adastra KL, Chi MM, Riley JK, Moley KH.**

**Published in Reproduction 141(5):607-15, 2011.**

**Abstract**

Autophagy is critical to the process of development as mouse models have shown that lack of autophagy leads to developmental arrest during the pre-implantation stage of embryogenesis. The process of autophagy is regulated through signaling pathways, which respond to the cellular environment. Therefore, any alteration in environment may lead to the dysregulation of the autophagic process potentially resulting in cell death. Using both *in vitro* and *in vivo* models to study autophagy in the pre-implantation murine embryo, we observed that the cells responds to environmental stressors (i.e. hyperglycemic environment) by increasing activation of autophagy in a differential pattern within the embryo. This upregulation is accompanied by an increase in apoptosis, which appears to plateau at high concentrations of glucose. Activation of the autophagic pathway was further confirmed by an increase in GAPDH activity in both *in vivo* and *in vitro* hyperglycemic models, which has been linked to autophagy through activation of the *Atg12* gene. Furthermore, this increase in autophagy in response to a hyperglycemic environment was observed as early as the oocyte stage. In conclusion, in this paper we provided evidence for a differential response of elevated activation of autophagy in embryos and oocytes exposed to a hyperglycemic environment.

## 2.1 Introduction

Autophagy is a programmed method of protein degradation and recycling of the necessary cellular building blocks of glucose, amino acids and fatty acids. This process has been shown to be critical to cell survival during periods of nutrient and specifically glucose deprivation, as well as during development and differentiation (Aki *et al.* 2003, Mizushima *et al.* 2004, Scott *et al.* 2004, Singh *et al.* 2009). During the developmental progression from oocyte to cleavage stage embryo, maternal proteins and RNAs are degraded and quickly replaced by newly synthesized embryonic counterparts. Mizushima *et al.* recently elucidated a physiological role of autophagy to aid in this process (Tsukamoto *et al.* 2008a, Tsukamoto *et al.* 2008b). By creating an oocyte-specific *Atg5* (autophagy-related 5, a critical component to the formation of autophagosomes) knockout mouse, the authors determined that fertilization of *Atg5 null* oocytes with *Atg5 null* sperm resulted in developmental arrest between the 4-cell and 8-cell stage. Fertilization of the *Atg5 null* oocyte with wild-type sperm, however, resulted in normal embryo development. They also demonstrated that protein-recycling rates were abnormal in the embryos entirely devoid of ATG5, and concluded that proper autophagic degradation within early embryos is essential for pre-implantation development. Results suggesting a role for autophagy during embryoid cavitation were also recently published (Qu *et al.* 2007). This group reported that loss of either *Beclin-1* (required for the initiation of autophagosome formation) or *Atg5*, both essential autophagic genes, leads to failure of cavitation due to the persistence of cellular corpses in murine embryonic stem cells(ES). These cells fail to display signals to indicate removal. This dysregulation

is also associated with low levels of ATP. Overall, the lethality associated with removal of this pathway, indicates the critical roles that this pathway plays in embryo development, many of which may not be elucidated yet.

The activation of autophagy has been characterized as a survival mechanism in the prevention of cell death (Boya *et al.* 2005). This role for autophagy has been reviewed elsewhere (Maiuri *et al.* 2007, Thorburn 2008). Briefly, the interplay between survival and autophagy is demonstrated by studies showing that lack of Beclin 1 expression, leads to excessive apoptotic cell death with embryo lethality at a peri-implantation stage (Yue *et al.* 2003). While the role for autophagy as a pro-survival mechanism is well accepted, the idea that excess autophagy with elevated levels of autophagosomes leads to cell death is still in debate. However, it is accepted that excessive autophagy leads to cell death during ischemia (Akazawa *et al.* 2004). Therefore, there may be certain circumstances in which this interrelationship exists. And while the mechanisms may not be entirely clear, recent studies suggest that oxidative stress plays a role in this switch from survival to death (Sakaida *et al.* 1990, Grune *et al.* 2003, Rodriguez-Enriquez *et al.* 2004, Kiffin *et al.* 2006).

In diabetes models it has been postulated that, autophagy is activated in certain tissues as a mechanism of protection against cellular damage resulting from oxidative stress. This activation is evident in the pancreatic beta cells of diabetic animal models and humans (Fujitani *et al.* 2009, Masini *et al.* 2009). Other tissues that exhibit an increased level of autophagy in diabetic models include the muscle,

neuronal tissue, and liver (Amherdt *et al.* 1974, Towns *et al.* 2005). However, a decrease in autophagy mediated protein turnover is observed in the renal cortex of the diabetic kidney (Sooparb *et al.* 2004). Therefore, it appears that tissues may regulate autophagy differently in response to a diabetic environment, and thus, investigation of this pathway is important as a potential therapeutic target for problems associated with diabetes.

The uterine milieu plays an important role in the development and implantation of an embryo. Previously, we have determined that a maternal diabetic state in mice results in an increase in number of fetal resorptions and an increase in congenital malformations of the fetus. These deleterious effects resulted with only a 96-hour exposure to the diabetic environment, as blastocyst stage embryos transferred from diabetic mothers into non-diabetic control mothers also displayed these phenotypes (Wyman *et al.* 2008). In addition, we have shown that the hyperglycemic environment of the uterus, by treatment with high glucose leads to an increase in apoptosis in the embryo (Moley *et al.* 1998a). Therefore, we wanted to investigate the autophagic pathway in a diabetic milieu.

In this paper we present evidence that the pre-implantation embryo alters autophagy in response to the external environment. The embryos respond to stressors including a known activator of autophagy and a hyperglycemic environment by increasing the activation of autophagy, identifying autophagy as a pathway for survival in the blastocyst. Furthermore, the embryonic response is differentially activated in what appears to be specific cell niches within the embryos.

## 2.2 Material and Methods

### *2.2.1 Oocyte and embryo retrieval*

To collect ovulated oocytes, control and diabetic B6SJL mice (Jackson Laboratory, Bar Harbor, Maine) received an injection of 10 IU human Chorionic Gonadotropin (hCG) 2 days after PMSG priming (PSMG 2000I.U., National Hormone & Peptide Program, Torrance, California; hCG, Sigma, St. Louis, Missouri). Oocytes were recovered from oviductal ampullae 13.5 h post-hCG, and cumulus cells were removed by incubating briefly in 1 mg/mL hyaluronidase. For embryo retrieval, the mice received the hormonal stimulation mentioned above and then were mated overnight with males of proven fertility. Mating was confirmed by the presence of a vaginal plug. Embryos for the *in vivo* blastocyst study (**Figure 2.3A**) were obtained by flushing the uterine horns 96 h post-hCG. For the *in vitro* hyperglycemia and autophagy activation studies (**Figures 2.1A-E, 2.2A-J and 2.3B**), embryos were recovered from control mice 76 h post-hCG and mating, and were cultured for 30 h in KSOM/0.25%BSA(v/v) (KSOM, Millipore Specialty Media, Massachusetts) and the indicated culture conditions at 37°C in 5% $CO_2$.

### *2.2.2 Immunofluorescence staining*

Embryos were collected and fixed in 3% Paraformaldehyde in PBS/2%BSA(v/v) for 20 minutes at room temperature. The embryos were permeabilized in 0.5% TritonX-100 in PBS/2%BSA for 30 minutes. To block, the embryos were placed in 5% normal goat serum in PBS/2%BSA for one hour. Then the embryos were incubated in the primary antibody (1:250) in PBS/2%BSA overnight at 4°C,

(LC3:Novus Biologicals, Littleton, Colorado; Beclin-1, Cell Signaling Technology, Massachusetts). The embryos were washed three times in PBS/2%BSA and then incubated in secondary antibody (Alexa Fluor goat-anti-rabbit IgG 488, Molecular Probes, Eugene, Oregon) for 45 minutes at room temperature. The embryos were rinsed three times again and placed in To-pro®-3-iodide (Molecular Probes, Eugene, Oregon) (1:500) in PBS for 15 minutes. After three more rinses in PBS, the embryos were mounted on slides using Vectashield (Vecta, Burlingame, California) and visualize by confocal microscopy. All experimental groups (i.e. **Figure 2.1A-C or D and E; Figure 2.2**) were conducted on the same day in order to use the same prepared secondary antibody preparation. Relative fluorescence was quantified by a blinded observer as described previously (Jungheim *et al.* 2010).

*2.2.3 Cell death assay*

Apoptosis was detected using the In Situ Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany). The embryos were fixed and permeabilized as indicated in the immunofluorescence staining method. A positive control was obtained by treating embryos with DNase (0.5μL/mL in PBS) for 20 minutes at 37°C. Then the embryos were incubated in the reaction mix, as per manufacturer's specifications for 1 hour at 37°C in the dark. All subsequent reactions were also carried out in the dark. After three rinses in PBS/2%BSA, the embryos were counterstained with To-pro®-3-iodide as mentioned before, rinsed and then mounted on slides. The slides were viewed on a confocal microscope.

*2.2.4 GAPDH activity assay*

This assay has been previously described for individual cell activity measurements (Passonneau 1993). Individual mouse blastocysts were extracted in 20mM Phosphate buffer (pH 7.4), 0.02% BSA, 0.5mM EDTA (pH 7.0), 5mM B-Me, 0.25% glycerol and 0.5% Triton X-100(v/v) at room temperature for 120 min under oil and stored at -75°C. A 0.1µL aliquot was added to 1µL GAPDH reagent containing 50mM Imidazole HCL (pH 7.0), 0.05%BSA, 1mM EDTA (pH 7.0), 1mM B-Me, 1mM $Na_2HA_SO_4$, 100µM NAD, and 100µM Glyceraldehyde 3-phosphate at room temperature for 1 hour. NADH standards in GAPDH reagent were added in this step. The reaction was stopped by the addition of 1µL of 0.12N NaOH and heated to 80°C for 25 min. To amplify, a 0.5µL aliquot was taken out and added to 10µL NAD cycling reagent at room temperature overnight under oil for 15,000 fold amplification, as performed previously by our group (Chi *et al.* 2002). The reaction was stopped by the addition of 1µL 1N NaOH and heated at 80°C for 25 min. To read the samples, a 10µL aliquot was taken out and added to 1mL Malate indicator reagent, as performed previously to obtain the NADH final reading in an A-1 Filter Fluorometer (Farrand Optical Components and Instruments, Valhalla, New York) (Chi *et al.* 2002). All calculations were based on the internal NADH standards.

*2.2.5 Generation of diabetic mice*

To generate an *in vivo* diabetic model, 3-week old female B6SJLF1 mice received a single injection of streptozotocin at a dose of 190 mg/kg. Four days after injection, a tail-blood sample was measured for glucose concentrations via a Contour TS One

Touch Glucometer (Bayer, Mishawaka, Indiana). Glucose levels greater than 300 mg/dl, were considered diabetic. Age-matched controls injected with PBS were used.

*2.2.6 Transmission electron microscopy*

For ultrastructural analysis of autophagy, 100 oocytes of each group were processed for transmission electron microscopy as described previously in the Molecular Microbiology Imaging Facility at Washington University in St. Louis (Gualtieri *et al.* 2009). The presence of autophagosomes was determined from electron micrographs at 10,000X magnification. To quantify the autophagosomes, the number of autophagosomes and the area in 10 random sections of each group were recorded using ImageJ (National Institutes of Health, Bethesda, Maryland). The following criteria for identifying autophagosomes were used: vesicles with a double membrane, between 0.3 and 2 um, with clearly recognizable cytoplasmic contents and are not multilamellar bodies.

*2.2.7 Statistical analysis*

All experiments were completed in triplicate with at least 10 embryos or oocytes per group in each experiment. The images in the figures are representative of the group of embryos in each group. For the GAPDH assay, a student's t-test was performed. Significance was defined as $p<0.05$.

## 2.3 Results

### *2.3.1 Embryonic autophagy is influenced by environment*

Our lab and others have observed a basal level of autophagic activation during all stages of pre-implantation embryonic development in mice (Cooper A *et al.* 2008, Tsukamoto *et al.* 2008a, Tsukamoto *et al.* 2008b). However, an autophagic response due to environmental stress or a known inhibitor has yet to be studied during this crucial developmental time period. It has been well established that mTOR is an upstream repressor of autophagy. Therefore, the mTOR inhibitor rapamycin is widely used to activate autophagy within *in vitro* systems. In order to investigate the embryonic response in the presence of an autophagic activator, we exposed morula stage embryos (76 h post-hCG) to culture media containing either 1μM or 5μM rapamycin for 30 hours and observed the levels of Beclin-1 and LC-3 expression by immunofluorescence compared to embryos treated with DMSO (vehicle control) (**Figure 2.1, A-C**). We observed autophagic activation as an increase in the levels of Beclin-1 protein expression in the treated groups. This activation was confirmed by LC3 protein expression, a major component of the autophagosomal membrane (**Figure 2.1, D-E**). While the increase in autophagic response was expected, there was an unusual variable patterning of response between the single cells within the blastocyst. Beclin-1 protein expression was limited to the trophectoderm of the blastocyst, with the ICM seemingly unaffected (**Figure 2.1C**). In addition, we examined the expression of LC3, a well know marker for autophagy, as it is localized to the autophagosomal membrane throughout the

bulk of the autophagic process. The expression of LC3 was also detected in a sporadic pattern throughout the embryo, however, this pattern was different than that of Beclin-1 (**Figure 2.1E**). This pattern may represent a differential autophagic response in certain cell niches.

Additionally, a cell death assay was used to determine whether the treatment affected cell quality. We found no significant difference in cell death, the percentage of apoptotic cells, between the rapamycin treated and control groups; however there was a difference in total cell number (**Supplemental Figure 2.1A and B**). This difference may be due to either a decrease in proliferation or non-apoptotic cell death.

*2.3.2 The amount of glucose in the environment is critical to baseline autophagy levels*

Due to the importance of autophagy in energy homeostasis in other tissues and the alterations of this pathway in diabetic models, we investigated autophagy in the developing embryo exposed to different glucose levels *in vitro*.

As an embryo transitions from the morula to blastocyst stage, the amount of environmental glucose is critical as the embryos switches from using pyruvate and lactate as energy to glucose (Brown & Whittingham 1991, Leese 1991). In previous papers, we have determined that the hyperglycemic conditions present in the milieu of diabetic mothers leads to a decrease in glucose transporter expression, deceased intracellular glucose and an increase in apoptosis in murine blastocysts (Moley *et al.* 1998a, Moley *et al.* 1998b). Additionally, when embryos were exposed to a hyperglycemic environment for 72 hours and then transplanted to a control pseudo-

pregnant foster mouse, an increased number of resorptions and malformations in the fetuses were observed (Wyman *et al.* 2008). Therefore, the maternal environment and in particular the amount of glucose present is critical to fetal outcome.

To investigate whether the amount of glucose present in the environment alters the basal level of autophagy present in embryos, we exposed the developing embryos to varying concentrations of glucose *in vitro* during morula to blastocyst transition for 30 hours. We have previously determined that a level of 52mM D-glucose closely mimics the phenotypic effects we observe in the embryos exposed to an *in vivo* diabetic environment, with similar apoptosis, glucose transporter downregulation and decreased intracellular glucose (Moley *et al.* 1998a). Following this exposure to high glucose, we then attempted to detect LC3 by immunofluorescence, as a marker for autophagosomes. At more physiological levels (5mM glucose) a minimal level autophagy is observed. However, the levels of LC3 appear to increase as the embryos are cultured in higher concentrations of glucose (**Figure 2.2A-E**). We anticipate that the drop in intracellular glucose triggers an autophagic response in a rescue attempt to recycle cellular substrates and generate alternative energy substrates.

To investigate the interchange between autophagy and apoptosis in the developing blastocyst, we repeated the experiment and used a cell death detection assay to determine the level of cell death occurring in each of the conditions. Previously, we have published results indicating an increase in apoptosis in

embryos exposed to a hyperglycemic environment *in vivo* or *in vitro* (Moley *et al.* 1998a). While we observed a similar result, the levels of apoptosis appear to plateau above 20mM glucose concentrations (**Figure 2.2F-J**).

Therefore, we conclude that higher levels of cell stress induced by a diabetic milieu result in a dose-dependent increase of autophagy, as measured by the autophagosomal marker LC3. Interestingly, the embryos appear to display an internal rheostat that controls the level of apoptosis that occurs. We speculate that this persistence of the cells within the blastocyst that do not undergo apoptosis but display a compensatory increase in autophagy may result in significant changes in these specific cells thus permanently altering the lineages derived from this cell of the blastocyst. These cell specific lineages changes could be responsible for the occurrence of malformations and/or miscarriages of these fetuses.

*2.3.3 Changes in GAPDH activity reflect autophagic activation and inhibition*

While it is well established that increased GAPDH activity triggers flux through the glycolytic pathway in general and triose metabolism specifically, a recent study has demonstrated a previously unknown role for GAPDH in signaling the activation of autophagy (Colell *et al.* 2007). By overexpressing GAPDH the investigators were able to protect the cells from caspase-independent cell death and even promote cell survival. Additionally, they showed that increased GAPDH activity leads to an elevation in intracellular ATP as well as upregulation of Atg12, an autophagic protein involved in formation of the autophagosome. We chose GAPDH activity as a metabolic marker of the autophagic response in these embryos.

To test whether GAPDH activity could be used as a marker of autophagy in blastocysts, we measured the activity using microanalytic enzymatic assays (Passonneau 1993) in individual blastocysts exposed to either DMSO as vehicle control or 5mM rapamycin for 30 hours to activate autophagy (**Suppl Figure 2.2A**). Activity increased significantly over vehicle. Conversely, blastocysts were exposed to control media versus media with added 4mM or 8mM 3-methyladenine (3-MA), a known inhibitor of autophagy for 30 hours. Activity of GAPDH was significantly decreased in a dose dependent manner by 3-MA (**Suppl Figure 2.2B**). After this confirmation that GAPDH activity could serve as an accurate measure of autophagic activation in the embryos, blastocysts were collected from both *in vivo* and *in vitro* diabetic conditions and GAPDH activity was measured in each group (**Figure 2.3A & B**). Our results indicate that these diabetic conditions result in an increase in GAPDH activity and we believe this increase signifies an upregulation autophagy in an attempt to protect the cell from damage as our other results suggest.

*2.3.4 Autophagosomes are more abundant in oocytes of diabetic mice*

Previously, our lab has published results indicating that *in vivo* maternal diabetes leads to an increase of apoptosis in the ovarian follicles of mice (Chang *et al.* 2005). Recently, our lab has also concluded that the mitochondria of diabetic oocytes display an abnormal morphology and distribution leading to a decrease in the metabolites. Furthermore, these oocytes display meiotic spindles defects and chromatin misalignment (Wang *et al.* 2009). Using GFP-LC3 mice, Mizushima *et al.* observed a basal level of autophagy occurring at the unfertilized oocyte stage,

however, much lower than that of the fertilized oocyte (Tsukamoto *et al.* 2008a). Therefore, we hypothesize; the metabolic milieu of diabetes may lead to a dysregulation of autophagy in the oocytes of these mice.

By using transmission electron microscopy we were able to obtain a more accurate estimation of the prevalence of autophagy during this stage (**Figure 2.4**). Our results indicate the presence of a basal level autophagosomes in control oocytes. The numbers of autophagosomes per area increase in oocytes obtained from diabetic mice. This increase suggests an increased activation of the autophagic pathway.

## 2.4 Discussion

While it has been determined that genetic removal of key players in the autophagic pathway leads to developmental arrest and embryonic malformations (Zheng *et al.* 2006, Fimia *et al.* 2007, Cecconi *et al.* 2008), the dysregulation of this pathway in response to embryonic environmental stressors has yet to be elucidated. This study suggests that an autophagic response to the environmental stress of a hyperglycemic milieu occurs during the oocyte and blastocyst stages of development and that this event is heterogeneously triggered at the blastocyst stage with some cells undergoing cell death and others surviving, but significantly and perhaps permanently altered by the autophagic process. We speculate that these remaining cells may alter their fate, thus changing the originally assigned cell lineages and possibly having developmental consequences.

By exposing the embryos to an autophagic activator, rapamycin, we were able to observe an increase in autophagic proteins, Beclin-1 and LC3 as indicators of an increased activation of autophagy. Interestingly, the embryos display a heterogeneous autophagic response to culture conditions. This differential display of activation of a molecular pathway in embryos has been detailed before, however, this was in regards to apoptosis (Pampfer 2000). In those studies, it was suggested that the inner cell mass (ICM), which will develop into the embryo, and the trophectoderm (TE), which develops into the extraembryonic tissues including the placenta, consist of separate "micro-environments." This internal separation establishes differential gene expression, different levels of ion and glucose transport and a varying response to cytotoxic agents (Pampfer 2000). Furthermore, cells within each of these compartments display a disparity. It has been reported, using cell lines derived from either the ICM or the trophectoderm, that the trophectoderm is more viable in a hyperglycemic environment, whereas, the ICM is more susceptible to hyperglycemic environments, leading to an increase incidence in apoptosis (Pampfer 2000). This is recapitulated *in vivo* as the number of cells in the ICM of embryos exposed to maternal hyperglycemia is reduced compared to controls (Lea *et al.* 1996, Pampfer *et al.* 1997).

In this study we show a different pattern of expression than apoptosis. Although, the expression of Beclin-1 appears exclusively in the trophectoderm, the autophagic activation by LC3 is seen in random individual cells of the blastocyst. Murine embryos inherit a pool of maternal transcripts that are progressively degraded and replaced by the products of embryonic transcription. This embryonic

genomic activation is triggered when the blastomeres still exhibit developmental plasticity and can change their cell fate (Zernicka-Goetz *et al.* 2009). It is possible that this differential autophagic response in single cells in the embryo during pre-implantation development affects the subsequent lineage allocation in that cell and its resulting daughter cells. We postulate that this early change in lineage patterning may result not only in the growth abnormalities of infants and placentas, but also in malformations commonly seen in fetuses from diabetic mothers.

While the effect of a maternal hyperglycemic environment on embryo development has been long studied, much is still left unanswered. Our lab has previously published data establishing a result of increased apoptosis in murine blastocysts exposed to a hyperglycemic diabetic environment, *in vivo* or *in vitro* (Moley *et al.* 1998a). Furthermore, when transferred into pseudo-pregnant females, these exposed blastocysts display an increase rate of resorptions and malformations (Wyman *et al.* 2008). Surprisingly, one-cell zygotes when transferred from diabetic to non-diabetic mice also demonstrated a significantly higher rate of malformations and growth retardation, suggesting an even earlier period of vulnerability of the zygote to hyperglycemia. More recent studies by our lab have confirmed this hypothesis. Recently, we determined that GV and MII oocytes from diabetic mice have biochemical and meiotic abnormalities which could predispose them to developmental problems post fertilization and perhaps implantation (Wang *et al.,* 2009). In this study we demonstrate a significantly higher number of autophagosomes in GV stage oocytes by electron microscopy. Other groups have shown that the autophagic protein LC3 is not detected until after fertilization

(Tsukamoto *et al.* 2008a), however, we show a significant difference in activation in the unfertilized oocyte from diabetic mice. This raises the possibility that maternal proteins at this very early stage may be prematurely degraded by autophagy, resulting in oocytes deficient in protein and perhaps predisposed to increased autophagy and other abnormalities, as we see in the blastocysts from diabetic mice. Future studies will be designed to test this hypothesis.

Recent studies have implicated GAPDH as a possible mediator and biochemical indicator of autophagic activation. Although the mechanism is not entirely elucidated, those studies demonstrated that overexpressing GAPDH in cells induced to undergo autophagy, prevented this process. The conclusions were that GAPDH activity not only triggered metabolism of trioses with in the glycolytic pathway but also, directly or indirectly, induced an increase in the transcription of Atg12, a key autophagic protein. Our study demonstrated a significant increase in GAPDH activity within the whole blastocyst in response to maternal diabetes or in vitro high glucose. In addition, we confirmed that changes in this enzyme activity are predictive of either activation of autophagy by rapamycin or inhibition of autophagy by 3-methyladenine.

In conclusion, we show in this paper that the levels of autophagy are altered during oocyte and pre-implantation development in response to environmental stressors **(Figure 2.5).** With the addition of the autophagy activator rapamycin, levels of autophagy are increased in a cell specific manner. Additionally, embryos cultured in a hyperglycemic environment, mimicking maternal diabetes result in the

elevation of the autophagic pathway. This elevation is also seen as an increase in GAPDH activity, which has recently been suggested as an activator of the autophagic pathway. Furthermore, it appears that the autophagic pathway may be altered as early as the oocytes as a result of maternal diabetes. This early alteration in autophagy may result in changes in quality of the embryo after fertilization. However, further studies will need to be conducted to validate this hypothesis. Overall, our data suggests that the hyperglycemic environment leads to an increased activation of autophagy during the oocyte growth phase and pre-implantation development resulting in a differential response by the individual cells within the embryo.

## 2.5 Acknowledgements

The authors would like to acknowledge Wandy Beatty in the Molecular Microbiology Imaging Facility at Washington University in St. Louis for the transmission electron microscopy imaging and The Bakewell Neuroimaging Laboratory for the use of the confocal microscopy for imaging.

## 2.6 References

**Akazawa H, Komazaki S, Shimomura H, Terasaki F, Zou Y, Takano H, Nagai T & Komuro I** 2004 Diphtheria toxin-induced autophagic cardiomyocyte death plays a pathogenic role in mouse model of heart failure. *J Biol Chem* **279** 41095-41103.

**Aki T, Yamaguchi K, Fujimiya T & Mizukami Y** 2003 Phosphoinositide 3-kinase accelerates autophagic cell death during glucose deprivation in the rat cardiomyocyte-derived cell line H9c2. *Oncogene* **22** 8529-8535.

**Amherdt M, Harris V, Renold AE, Orci L & Unger RH** 1974 Hepatic autography in uncontrolled experimental diabetes and its relationships to insulin and glucagon. *J Clin Invest* **54** 188-193.

**Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Metivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P & Kroemer G** 2005 Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol* **25** 1025-1040.

**Brown JJ & Whittingham DG** 1991 The roles of pyruvate, lactate and glucose during preimplantation development of embryos from F1 hybrid mice in vitro. *Development* **112** 99-105.

**Cecconi F, Piacentini M & Fimia GM** 2008 The involvement of cell death and survival in neural tube defects: a distinct role for apoptosis and autophagy? *Cell Death Differ* **15** 1170-1177.

**Chang AS, Dale AN & Moley KH** 2005 Maternal diabetes adversely affects preovulatory oocyte maturation, development, and granulosa cell apoptosis. *Endocrinology* **146** 2445-2453.

**Chi MM, Hoehn A & Moley KH** 2002 Metabolic changes in the glucose-induced apoptotic blastocyst suggest alterations in mitochondrial physiology. *Am J Physiol Endocrinol Metab* **283** E226-232.

**Colell A, Ricci JE, Tait S, Milasta S, Maurer U, Bouchier-Hayes L, Fitzgerald P, Guio-Carrion A, Waterhouse NJ, Li CW, Mari B, Barbry P, Newmeyer DD, Beere HM & Green DR** 2007 GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. *Cell* **129** 983-997.

**Cooper A SR, Boehle K, Riley J, Moley K. 2008.** Autophagy is a physiologic process regulated by glucose availability in the murine preimplantation blastocyst. In *Reproductive Sciences (Supplement) 1*, pp. 73A.

**Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P, Gruss P, Piacentini M, Chowdhury K & Cecconi F** 2007 Ambra1 regulates autophagy and development of the nervous system. *Nature* **447** 1121-1125.

**Fujitani Y, Kawamori R & Watada H** 2009 The role of autophagy in pancreatic beta-cell and diabetes. *Autophagy* **5** 280-282.

**Grune T, Merker K, Sandig G & Davies KJ** 2003 Selective degradation of oxidatively modified protein substrates by the proteasome. *Biochem Biophys Res Commun* **305** 709-718.

**Gualtieri R, Iaccarino M, Mollo V, Prisco M, Iaccarino S & Talevi R** 2009 Slow cooling of human oocytes: ultrastructural injuries and apoptotic status. *Fertil Steril* **91** 1023-1034.

**Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE & Moley KH** 2010 Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. *Endocrinology* **151** 4039-4046.

**Kiffin R, Bandyopadhyay U & Cuervo AM** 2006 Oxidative stress and autophagy. *Antioxid Redox Signal* **8** 152-162.

**Lea RG, McCracken JE, McIntyre SS, Smith W & Baird JD** 1996 Disturbed development of the preimplantation embryo in the insulin-dependent diabetic BB/E rat. *Diabetes* **45** 1463-1470.

**Leese HJ** 1991 Metabolism of the preimplantation mammalian embryo. *Oxf Rev Reprod Biol* **13** 35-72.

**Maiuri MC, Zalckvar E, Kimchi A & Kroemer G** 2007 Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* **8** 741-752.

**Masini M, Bugliani M, Lupi R, del Guerra S, Boggi U, Filipponi F, Marselli L, Masiello P & Marchetti P** 2009 Autophagy in human type 2 diabetes pancreatic beta cells. *Diabetologia* **52** 1083-1086.

**Mizushima N, Yamamoto A, Matsui M, Yoshimori T & Ohsumi Y** 2004 In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell* **15** 1101-1111.

**Moley KH, Chi MM, Knudson CM, Korsmeyer SJ & Mueckler MM** 1998a Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. *Nat Med* **4** 1421-1424.

**Moley KH, Chi MM & Mueckler MM** 1998b Maternal hyperglycemia alters glucose transport and utilization in mouse preimplantation embryos. *Am J Physiol* **275** E38-47.

**Pampfer S** 2000 Apoptosis in rodent peri-implantation embryos: differential susceptibility of inner cell mass and trophectoderm cell lineages--a review. *Placenta* **21 Suppl A** S3-10.

**Pampfer S, Vanderheyden I, McCracken JE, Vesela J & De Hertogh R** 1997 Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumor necrosis factor-alpha in vitro. *Development* **124** 4827-4836.

**Passonneau JL, OH.** 1993 *Enzymatic Analysis*. Totowa, NJ: Humana Press.

**Qu X, Zou Z, Sun Q, Luby-Phelps K, Cheng P, Hogan RN, Gilpin C & Levine B** 2007 Autophagy gene-dependent clearance of apoptotic cells during embryonic development. *Cell* **128** 931-946.

**Rodriguez-Enriquez S, He L & Lemasters JJ** 2004 Role of mitochondrial permeability transition pores in mitochondrial autophagy. *Int J Biochem Cell Biol* **36** 2463-2472.

**Sakaida I, Kyle ME & Farber JL** 1990 Autophagic degradation of protein generates a pool of ferric iron required for the killing of cultured hepatocytes by an oxidative stress. *Mol Pharmacol* **37** 435-442.

**Scott RC, Schuldiner O & Neufeld TP** 2004 Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev Cell* **7** 167-178.

**Singh R, Xiang Y, Wang Y, Baikati K, Cuervo AM, Luu YK, Tang Y, Pessin JE, Schwartz GJ & Czaja MJ** 2009 Autophagy regulates adipose mass and differentiation in mice. *J Clin Invest* **119** 3329-3339.

**Sooparb S, Price SR, Shaoguang J & Franch HA** 2004 Suppression of chaperone-mediated autophagy in the renal cortex during acute diabetes mellitus. *Kidney Int* **65** 2135-2144.

**Thorburn A** 2008 Apoptosis and autophagy: regulatory connections between two supposedly different processes. *Apoptosis* **13** 1-9.

**Towns R, Kabeya Y, Yoshimori T, Guo C, Shangguan Y, Hong S, Kaplan M, Klionsky DJ & Wiley JW** 2005 Sera from patients with type 2 diabetes and neuropathy induce autophagy and colocalization with mitochondria in SY5Y cells. *Autophagy* **1** 163-170.

**Tsukamoto S, Kuma A & Mizushima N** 2008a The role of autophagy during the oocyte-to-embryo transition. *Autophagy* **4** 1076-1078.

**Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A & Mizushima N** 2008b Autophagy is essential for preimplantation development of mouse embryos. *Science* **321** 117-120.

**Wang Q, Ratchford AM, Chi MM, Schoeller E, Frolova A, Schedl T & Moley KH** 2009 Maternal diabetes causes mitochondrial dysfunction and meiotic defects in murine oocytes. *Mol Endocrinol* **23** 1603-1612.

**Wyman A, Pinto AB, Sheridan R & Moley KH** 2008 One-cell zygote transfer from diabetic to nondiabetic mouse results in congenital malformations and growth retardation in offspring. *Endocrinology* **149** 466-469.

**Yue Z, Jin S, Yang C, Levine AJ & Heintz N** 2003 Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A* **100** 15077-15082.

**Zernicka-Goetz M, Morris SA & Bruce AW** 2009 Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. *Nat Rev Genet* **10** 467-477.

**Zheng B, Tang T, Tang N, Kudlicka K, Ohtsubo K, Ma P, Marth JD, Farquhar MG & Lehtonen E** 2006 Essential role of RGS-PX1/sorting nexin 13 in mouse development and regulation of endocytosis dynamics. *Proc Natl Acad Sci U S A* **103** 16776-16781.

## 2.7 Figure Legends

**Figure 2.1. Beclin-1 and LC3 expression in rapamycin treated embryos.** Ten mouse control blastocysts were incubated for 30 hours in each group: DMSO control (A), 1μM (B), or 5μM (C) rapamycin. This experiment was repeated 3 times. Beclin-1 expression is increased in a variable pattern, with the most expression observed in the trophectoderm cells. LC3 expression in mouse control blastocysts incubated in DMSO (D, n=10) or 5μM rapamycin (E, n=10). This experiment was conducted 3 times.

**Figure 2.2. Autophagic and apoptotic responses to varying levels of excess glucose.** LC3 expression in embryos. Negative control (A), physiologic 5mM (B), 20mM (C), 35mM (D) and 52mM (E) glucose. Cell death detection assay for apoptosis. Positive control of DNase treated embryos (F), physiologic 5mM (G), 20mM (H), 35mM (I), 52mM (J) glucose. Each group consisted of at least 30 embryos and the experiment was repeated 3 times.

**Figure 2.3. GAPDH activity and autophagy are increased in embryos exposed to diabetic environments both *in vivo* and *in vitro*.** GAPDH activity (*in vivo*): embryos from control versus streptozocin-induced diabetic mothers (A) *p-value<0.0001. GAPDH activity (*in vitro*): embryos exposed to 2.78mM versus 52mM D-glucose (B), *p-value<0.0005 (n= at least 50 for each group).

**Figure 2.4. A representative autophagosome in a control (left) and diabetic (right) oocyte.** There is a 43% increase in number of autophagosomes in the diabetic oocyte per area (sample size=100, 10 sections from each group quantitated).

**Figure 2.5. Cartoon of level of autophagy in control vs. diabetic oocytes and embryos.** Whereas, no autophagy was detected in MII oocytes from control mice, we determined increased autophagosomes and decreased ATP in MII oocytes from diabetic mice. In addition, although some autophagic proteins have been detected in control blastocysts, diabetic blastocysts demonstrated increased LC3 and Beclin-1 protein as well as decreased ATP. We speculate that increased autophagy at the oocyte stage may prematurely degrade maternal proteins leading to developmental arrest and increased autophagy at the blastocyst stage may lead to abnormal degradation of embryonic proteins essential for proper development.

**Supplemental Figure 2.1**. **Total nuclei number and percent apoptotic nuclei of blastocysts cultured in rapamycin (5mM) for 30 hours vs. control.** (A) Although the number of apoptotic cells was not significantly different between the groups, the total number of nuclei was greater in the control group (n=29) vs. the rapamycin treated group (n=24) ($p<0.04$), (B) the percentage of apoptotic cells

(TUNEL positive cells/total nuclei) was not significantly different between the two groups.

**Supplemental Figure 2.2. GAPDH activity and autophagy are increased and decreased in embryos exposed to rapamycin and 3-methyladenosine (3-MA), respectively.** (A) Blastocysts were incubated for 30 hours in either DMSO vehicle control (n=15) vs. 5μM rapamycin (n=15). GAPDH activity was significantly higher in rapamycin treated blastocysts ($p<0.05$). (B) Blastocysts were incubated for 30 hours in control media (n=15), 4mM 3-MA (n=15) or 8mM 3-MA (n=14). GAPDH activity was negatively affected in a dose-dependent fashion. (*$p<0.001$; **$p<0.001$).

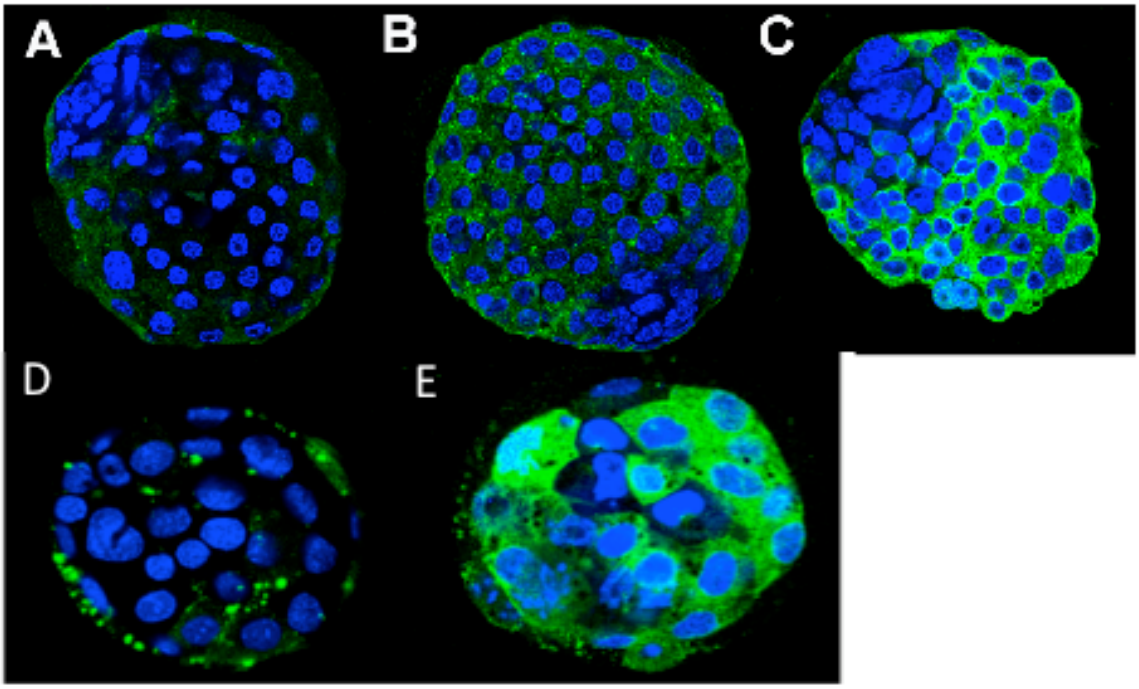



Figure 2.1

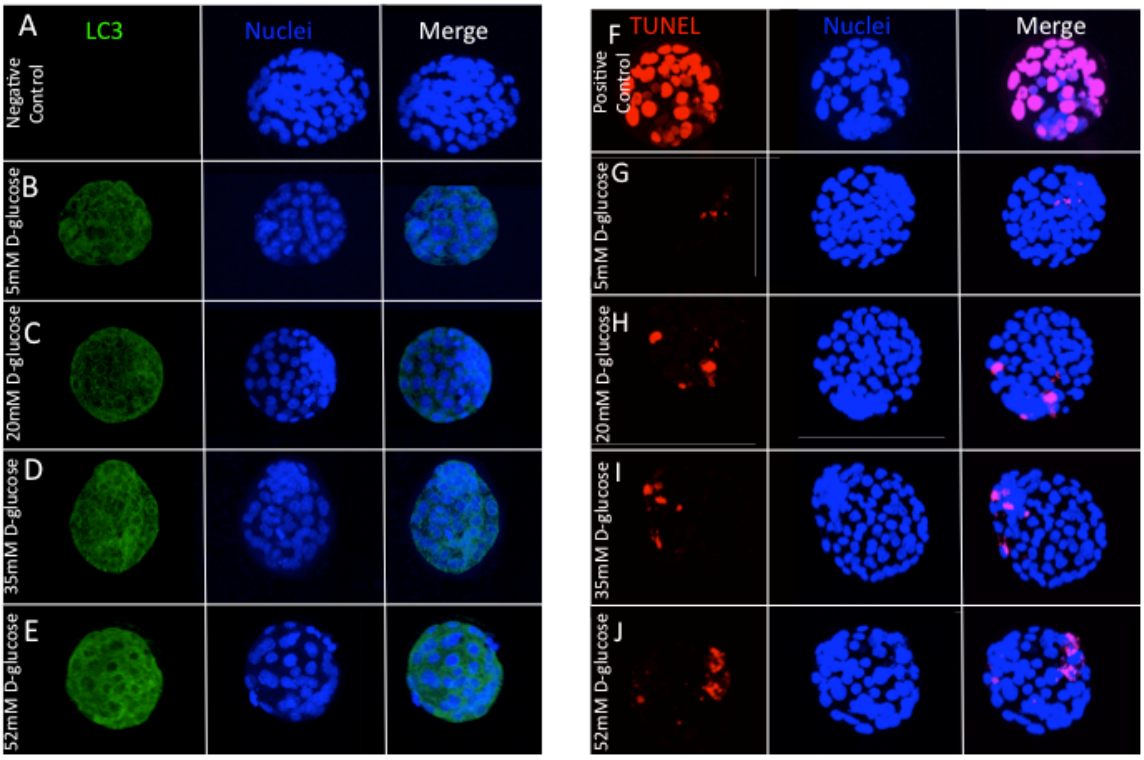

A
LC3
Nuclei
Merge
Negative Control
B
5mM D-glucose
C
20mM D-glucose
D
35mM D-glucose
E
52mM D-glucose
F
TUNEL
Nuclei
Merge
Positive Control
G
5mM D-glucose
H
20mM D-glucose
I
35mM D-glucose
J
52mM D-glucose

Figure 2.2

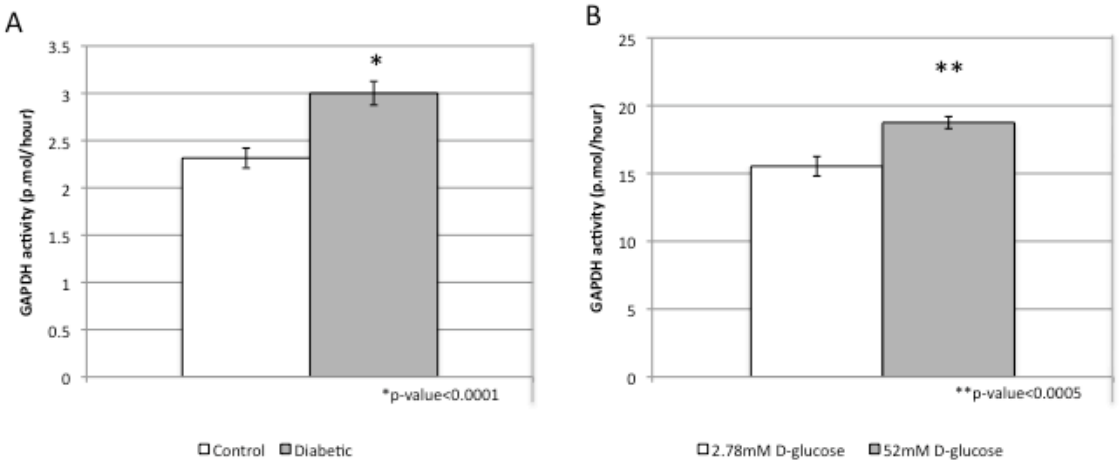

A
3.5
3
2.5
2
1.5
1
0.5
0
GAPDH activity (p.mol/hour)
*
*p-value<0.0001
Control
Diabetic
B
25
20
15
10
5
0
GAPDH activity (p.mol/hour)
**
**p-value<0.0005
2.78mM D-glucose
52mM D-glucose

Figure 2.3

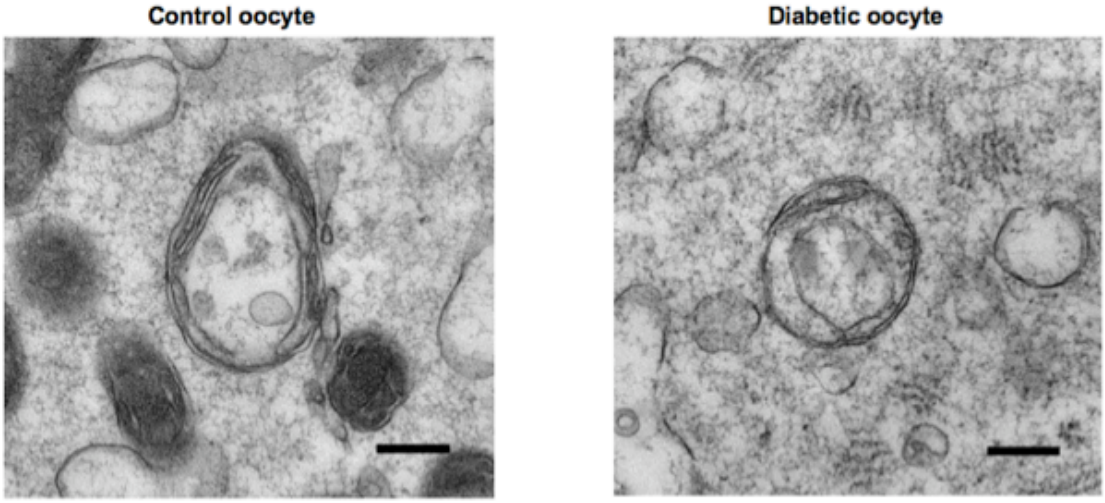

Control oocyte
Diabetic oocyte
Number of Autophagosomes per area
Control oocyte: 1.60/1000µm²
Diabetic oocyte: 2.82/1000µm²

Figure 2.4

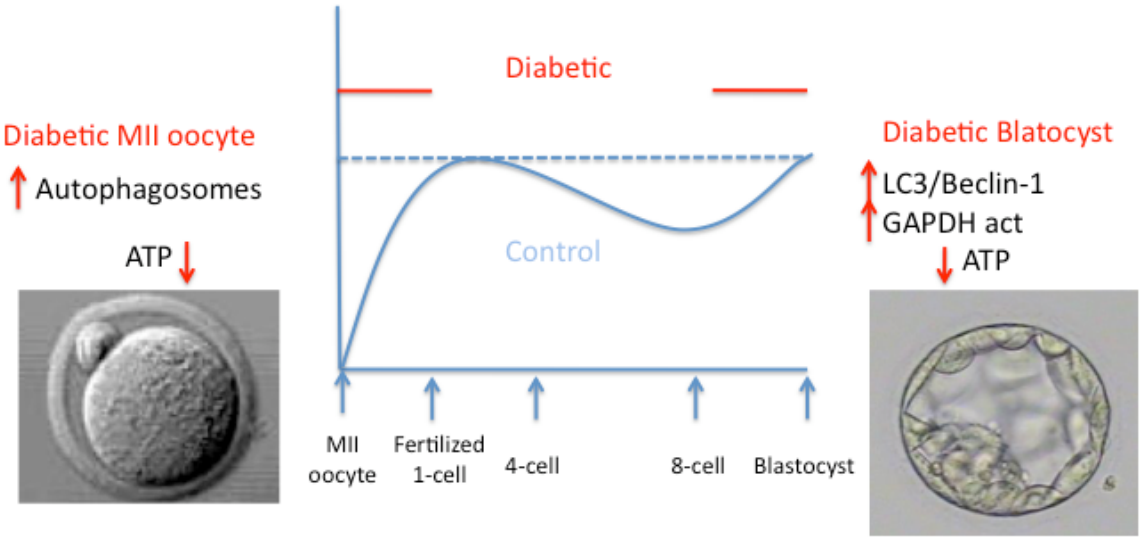
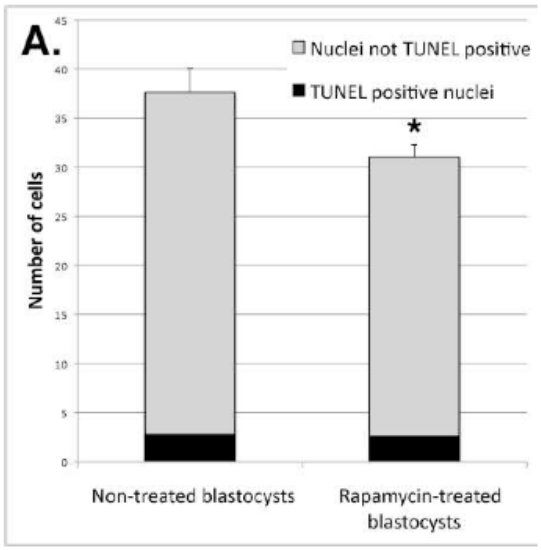
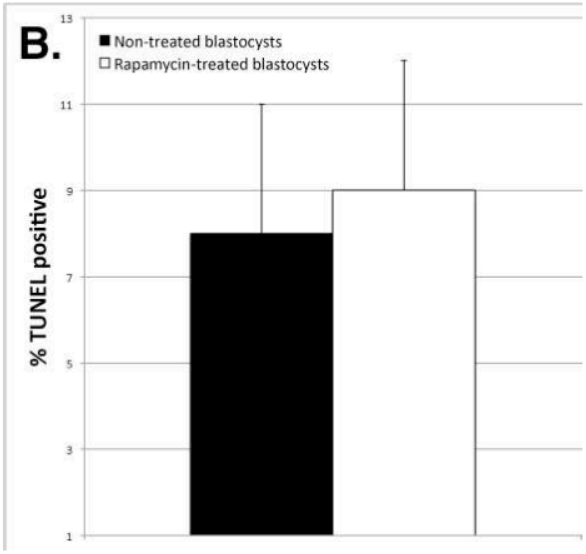

Diabetic MII oocyte
Autophagosomes
ATP
Diabetic
Control
MII oocyte
Fertilized 1-cell
4-cell
8-cell
Blastocyst
Diabetic Blatocyst
LC3/Beclin-1
GAPDH act
ATP

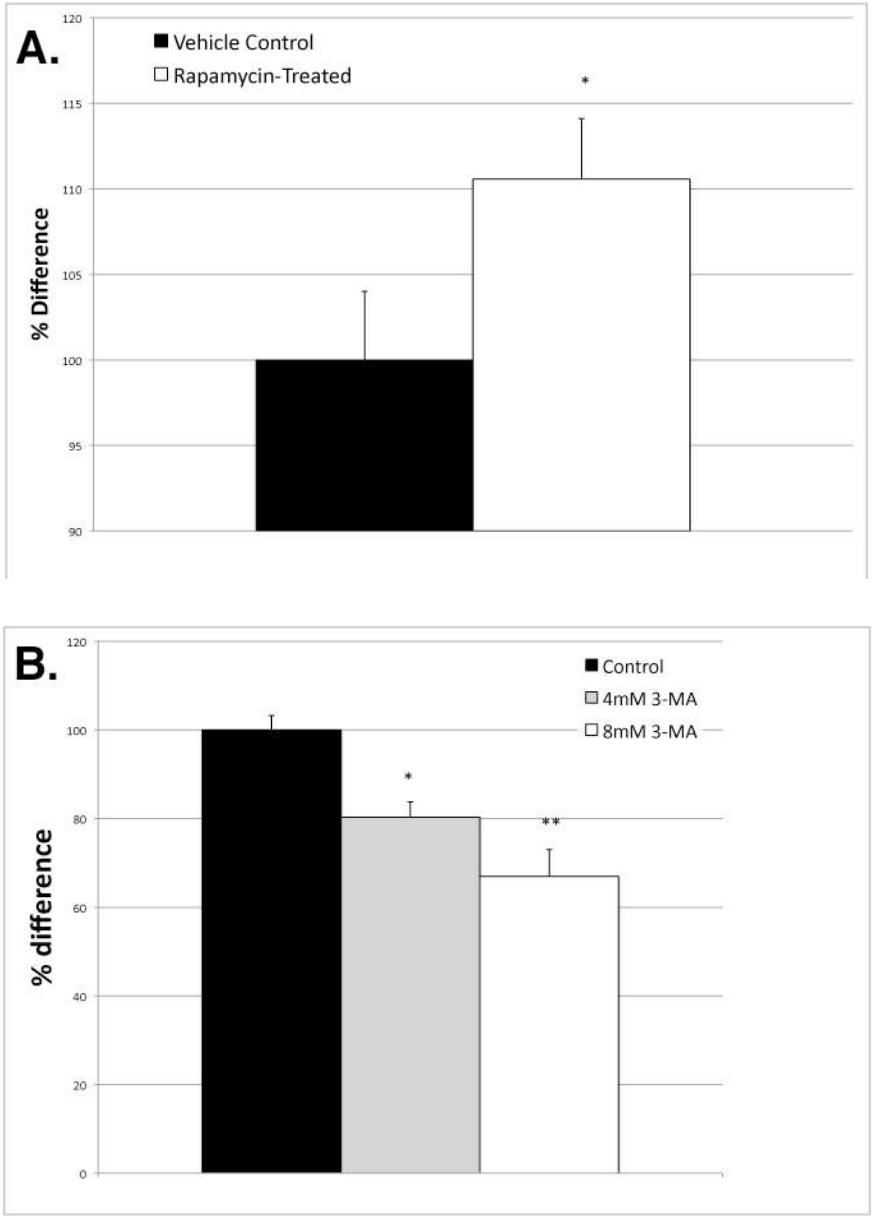
Figure 2.5




Supplemental Figure 2.1

GAPDH activity




Supplemental Figure 2.2

# CHAPTER 3

## *Slc2a8* Deficiency in Mice Results in Reproductive and Growth Impairments

Adastra K, Frolova A, Chi MM, Cusumano D, Bade M, Carayannopoulos M, Moley K

**Abstract**

SLC2A8, also known as GLUT8, is a facilitative glucose transporter expressed in the testis, brain, liver, heart, uterus, ovary and fat. In this study we examined the effect of *Slc2a8* deficiency on mouse gamete, preimplantation embryo and implantation phenotype, as well as postnatal growth and physiology. For this model, the transcriptional start site and exons 1-4 were targeted and a lack of protein expression was confirmed by western immunoblot. Both sperm cells and oocytes demonstrated abnormal metabolism and ATP production, respectively. The most significant difference between wild-type and null mice was the process of embryo implantation. A lack of SLC2A8 expression resulted in impaired decidualization, a critical step in the differentiation of endometrial stromal cells (ESC), necessary for implantation. SLC2A8 expression in both mouse and human ESCs increases dramatically in response to hormonal changes occurring during the process of implantation. This study is the first to demonstrate that a lack of *Slc2a8* leads to reproductive defects most likely due to abnormal decidualization and impaired implantation. This phenotype leads to decreased litter size, and smaller pups at weaning that continue to display an abnormally small growth phenotype into adulthood. By MRI they displayed decreased body fat and interestingly, they are resistant to a diet high in fat and carbohydrates.

## 3.1 Introduction

Glucose Transporter 8 (SLC2A8) is a member of the Class III of facilitative glucose transporters (SLC2As). All 14 known SLC2As have shared characteristics including 12 transmembrane spanning helices and an exofacial N-linked glycosylation site[1], however, SLC2As are unique in tissue localization, kinetics and substrate specificity. More specifically, SLC2A8 has a high affinity for glucose (Km~2mM), which can be competed with fructose, galactose and cytochalasin B, indicating that it may transport other hexoses[2].

SLC2A8 is expressed at high levels in the testes and is also found in brain, liver, heart, uterus, ovary and fat[2, 3]. Furthermore, SLC2A8 is localized entirely intracellular, except in blastocysts where it has been shown to translocate to the plasma membrane in response to insulin stimulation[3, 4]. This intracellular localization is signaled by a dileucine motif (DEXXXLL), which traffics SLC2A8 to the late endosome/lysosome[5]. While research on this SLC2A is ongoing, a known function for the SLC2A8 remains elusive.

Our lab has previously indicated a role for SLC2A8 in pre-implantation embryo survival in mice. Knockdown of *Slc2a*8 in the embryo, by anti-sense technology, resulted in an increase of apoptosis in blastocysts and a decrease in successful pregnancies by E14.5[6]. This data, along with data indicating that SLC2A8 is translocated to the membrane in response to insulin[3], suggests that SLC2A8 plays an important role in embryo development, possibly by facilitation of glucose utilization.

Previously published *Slc2a8* deficient models, however, do not exhibit embryonic lethality, but do display mild reproductive and behavioral phenotypes. Membrez et al. reported a mild brain phenotype consisting of increased proliferation of hippocampal cells and an increased P-wave duration in the heart[7]. Another group described a phenotype in which deletion of *Slc2a8* resulted in reduced sperm motility and ATP and behavioral alterations, in particular hyperactivity[8, 9]. These phenotypes are not surprising as SLC2A8 is highly expressed in the testes and also in the brain and heart. Both groups noted that the heterozygous mating of *Slc2a8* mice resulted in lower than expected percentage of *Slc2a8-/-* and *Slc2a8+/-* pups, potentially resulting from the decreased motility observed in the sperm. Neither of these groups reported changes in normal glucose homeostasis or growth at baseline in these models.

In this paper we detail a distinct phenotype resulting from the creation of a *Slc2a8* null mouse using conventional knockout system, deleting the first four exons including the transcription start site globally. These mice exhibit a more severe reproductive phenotype than previous models, as litters born to *Slc2a8-/-* mating pairs are smaller in number than the wild-type controls (*Slc2a8+/+*). A role for SLC2A8 in the metabolism of both female and male gametes is uncovered as the sperm motility is decreased (which is attributed to decreased ATP levels previously[8]), and ATP levels in the oocyte are significantly decreased as well. Additionally, *Slc2a8-/-* females do not undergo complete decidualization of the uterus upon *in vivo* artificial stimulation, suggesting a role for SLC2A8 in the event of implantation and possibly placentation. Furthermore, in our model, SLC2A8 is required for normal growth, as *Slc2a8-/-* mice are smaller in size than their *Slc2a8+/+* counterparts. Finally, although SLC2A8 does not seem to be required

for normal glucose homeostasis under basal conditions, the lack of SLC2A8 appears to protect the mice from the metabolic conditions induced by high fat feeding.

## 3.2 Material and Methods

### *3.2.1 Generation of Slc2a8 Targeting Construct*

A P1 genomic clone generated from the 129 mouse strain was screened for the presence of *Slc2a8* by a commercial screening service (Genome Systems, St. Louis, MO). Genomic DNA was prepared following the manufacturer's protocol and used as the template for generation of the *Slc2a8* targeting construct. A 2.9kb BglI fragment was used for the 5' arm (*forward* 5'-CACAAGGCCACATTGTCAGCCCA-3', *reverse* 5'-GCCGCGCTCGCCCGAGCGGC-3') and a 2.5kb PCR generated fragment for the 3' arm (*forward* 5'-GGGGGTTCCAGTGCAAGGAAGGGTG-3' and *reverse* 5'-TCGTGGTACCGGTCATTC-3') (**Figure 3.1A**). The targeting vector (pLNTK) contains PGK-*neo*$^r$ and HSV-TK genes for positive and negative selection, respectively.

### *3.2.2 Generation of Recombinant Slc2a8 ES Clones and Southern Blot Analysis*

RW4 ES cells (Washington University ES Core) were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) in standard ES medium. ES cells were electroporated with 25 μg of linearized *Slc2a8* targeting vector and cultured on MEFs resistant to G418. After 24 hr, the medium was supplemented with 400 μg/ml G418 (GIBCO BRL), and resistant ES clones were isolated after 6 days of selection. Individual clones were expanded, and genomic DNA was prepared and analyzed by southern blot

analysis following established methods[10]. Briefly, genomic DNA was digested with EcoRV, resolved on 0.8% agarose gels, and transferred to nitrocellulose filters. Filters were hybridized with a 5' external $^{32}$P-labeled probe as indicated (**Figure 3.1A**).

*3.2.3 Western Blot*

Protein was extracted from whole testes digest. The extracted protein was then run on 10% SDS-Page gel, transferred to nitrocellulose membrane, then blocked in 5% milk-TBST, primary antibody specific to the C-terminal of SLC2A8 (created by KHM) was used at 1:1000 in 5% milk-TBS-T overnight at 4ºC, goat anti-rabbit secondary was used at 1:10,000 in 1% milk/TBS-T for 1 hour, washed and developed by ECL (Amersham). The membrane was stripped and re-probed for actin (primary 1:10,000, secondary goat anti-mouse 1:10,000) as a loading control.

*3.2.4 Computer Assisted Sperm Analysis (CASA)*

The cauda epididymis was diced, and the sperm were allowed to disperse into the HTF embryo max (Millipore) for 10 min at 37ºC. Motility parameters were measured by CASA system (Hamilton-Thorne Research, Beverly, MA, USA).

*3.2.5 Metabolic Analyses*

Mice were primed with 10 IU pregnant mare serum gonadotropin (PMSG) by ip injection, and 48 h later cumulus enclosed oocytes were obtained by manual rupturing of antral ovarian follicles. The ATP [11] and Hadh2 [12] microanalytic assays were described elsewhere. The assays are linked to NADPH. The NADPH by-product is then

enzymatically amplified in a cycling reaction, and a byproduct of the amplification step is measured in a fluorometric assay.

*3.2.6 Artificial Decidualization*

To generate deciduomas, 7- to 8-wk-old female mice were used and treated as described previously[13]. Briefly, mice were ovariectomized and allowed 2 wk for recovery. They were then injected with 100 ng 17β-estradiol (E2) (Sigma) for 3 d, rested 2 d, and then injected with 10 ng of E2/1 μg of Progesterone (P4) (Sigma) for 3 d. The steroids were diluted in sunflower seed oil (Sigma) and injections were all sc, conducted at 1000–1030 h, with the total volume being 50 μl. Immediately after the last of the above sensitization injections, the right uterine horn received an intraluminal injection of 100 μl of sunflower seed oil. Mice continued to receive 1 mg of P4 sc for up to 4 d. On the day after the last injection the mice were killed, and uterine horns dissected out and weighed.

*3.2.7 Glucose Tolerance and Insulin Tolerance Tests*

Both of these methods were described elsewhere[14]. Briefly, for the glucose tolerance test (GTT), mice (n=10 per group) were fasted 16 h prior to an oral gavage of 30% D-glucose (2 mg/g body wt; Sigma). Blood glucose was measured at 30 min intervals for 120 min via the tail vein blood on a contour TS glucometer. For the insulin tolerance test (ITT), mice (n=10 per group) were fasted 4 h prior to an intraperitoneal injection of bovine insulin (0.75 mU/g body wt; Sigma). Blood glucose was measured at 15 min intervals for 75 min via the tail vein. For the ITT, the percentage decreases in blood glucose from the 0 min time point was calculated.

*3.2.8 High Fat Diet*

4-week old mice were fed a diet ad libitum consisting of either 58% fat (AIN-76A, Test Diet) or 4% fat standard control diet (5053, PicoLab) for a total of 12 weeks. Mice were allowed free access to water and maintain on a 12h light/dark cycle. Mice were weighed weekly.

*3.2.9 Statistical Analysis*

All experiments were completed in triplicate. Data was analyzed using the student's t-test, and significance was reached at $p<0.05$.

## 3.3 Results

### *3.3.1 Generation of SLC2A8 deficient mice*

Targeted disruption of the *Slc2a8* gene was accomplished via a *Slc2a8*-targeting construct designed to replace the first 4 exons, including the transcriptional start site and 200bp of 5' upstream sequence, of the *Slc2a8* gene with a neomycin resistance cassette (**Figure 3.1A**). Genomic DNA isolated from a P1 clone screened for the presence of the *Slc2a8* gene was used as the template for the construct. The *Slc2a8* gene spans ~9kb and consists of 10 exons[15]. Successful targeting was achieved by electroporation of ES stem cells with the linearized *Slc2a8* targeting construct and subsequent culture in the presence of G418. A total of 750 resistant clones were screened by Southern blot and 1 recombinant ES clones identified. A recombinant ES clone was microinjected into blastocysts of C57BL/6 mice. Chimeric mice were screened by Southern blot analysis

and founders were intercrossed. Germline transmission of F1 offspring was confirmed by Southern blot analysis (**Figure 3.1B**).

We also confirmed deletion of *Slc2a8* by reverse-transcriptase PCR in testes, heart and liver tissues, which are all known to express *Slc2a8*. While *Slc2a8* was detectable in these tissues in the wild-type animals, no expression was detectable in the testes, heart and liver of *Slc2a8-/-* mice.

### *3.3.2 Impaired protein expression in Slc2a8 deficient mice*

To confirm that the deletion of the *Slc2a8* gene resulted in loss of protein expression, we performed western blot and immunofluorescence analysis of testes. SLC2A8 is most abundantly expressed in the testes, in particular the spermatids, spermatozoa and the leydig cells[16]. As illustrated in **Figure 3.2A**, SLC2A8 is present, as expected, in the *Slc2a8+/+* mice but is completely absent in testes from *Slc2a8-/-* mice. Furthermore, immunofluorescence staining of testes also confirmed the absence of SLC2A8 protein in the *Slc2a8-/-* mice (**Figure 3.2B**). These results confirm that the *Slc2a8-/-* mice are devoid of SLC2A8 protein.

### *3.3.3 Slc2a8-/- mice mating result in smaller litter sizes*

Other mouse models deficient in *Slc2a8* display a reproductive phenotype with less than expected percentage of heterozygous and homozygous null mice in Mendelian crosses[7, 8]. One group determined that the sperm in their *Slc2a8-/-* mice was less motile than *Slc2a8+/+* as a result of decreased ATP and mitochondria membrane potential in the sperm[8]. While the matings from their *Slc2a8-/-* mice produced smaller litters, they reported that this phenomenon was neither significant nor investigated. Our

*Slc2a8-/-* mice exhibit a more severe reproductive phenotype as litters that result from *Slc2a8-/-* mating are significantly smaller in number (~4 pups/litter) than litters from *Slc2a8+/+* x *Slc2a8+/+* mating (~6 pups/litter) (**Table 3-I**). To determine whether this phenotype was due to a male or female reproductive effect, we mated *Slc2a8-/-* males or females with their *Slc2a8+/+* counterpart. Interestingly, the matings resulted in a decreased number of pups similar to the numbers we observed in *Slc2a8-/-* x *Slc2a8-/-* matings. This suggests that these mice have a complex reproductive phenotype that may stem from an overarching phenotype. We will address these phenotypes individually in this paper.

### *3.3.4 Slc2a8-/- males have normal testes histology but exhibit reduced sperm motility*

SLC2A8 is most abundantly expressed in the testes in both mice and humans. While the function of SLC2A8 is unknown, it has been previously suggested that SLC2A8 is important for energy metabolism in the sperm[16]. Using hematoxylin and eosin (H&E) staining we examined the morphology of testes obtained from *Slc2a8-/-* compared to *Slc2a8+/+* mice. The overall morphology is normal and all cell types are present (**Figure 3.3A**). In previously reported *Slc2a8* deficient mice, no changes in morphology were observed but the mice did exhibit a reduction in motility by Computer Assisted Sperm Analysis (CASA)[8]. This reduction in motility is consistent with data that SLC2A8 is localized mainly to the acrosome region, which is responsible for supplying the sperm with energy[16-18]. Therefore, we repeated this assay with the *Slc2a8-/-* mice we generated (**Figure 3.3B**). The mice did exhibit a reduction in motility with a correlating low number of rapid sperm and an increased number of slow and static sperm; however, this data did not reach significance. While there may be a trend towards

a decrease in motility, this phenotype appears to be a minor factor contributing to the reduction in litter size observed.

### *3.3.5 Slc2a8-/- females have normal ovarian histology but an abnormal metabolic profile*

We also examined the histology of the ovary, as the litter numbers obtained from our breeding crosses indicated a female reproductive phenotype as well. SLC2A8 is expressed in the tissues of the female reproductive tract, including the ovaries (and oocytes) and the uterus[19, 20]. The histology of ovaries from *Slc2a8-/-* mice was comparable to the *Slc2a8+/+* mice ovaries and all cell types were present (**Figure 3.3C**). Additionally, we sought to determine if the oocytes exhibited any metabolic disturbances as a result of being devoid of SLC2A8. Previous data from our lab and others have reported that glucose metabolism is critical to the process of meiosis in the oocyte[11, 21, 22]. A dysregulation of glucose metabolism leading to a decrease in ATP production results in oxidative stress and overall poorer outcomes in oocyte development [23, 24]. Using a microanalytic technique in which we can measure the metabolites of a single oocyte, we determined that the ATP content in *Slc2a8-/-* oocytes is significantly decreased compared to *Slc2a8+/+* oocytes (~25% less, **Figure 3.3D**). These data are similar to reported metabolic abnormalities in oocytes obtained from diabetic mice and older human patients undergoing IVF[11, 25].

To further profile these metabolic abnormalities we examined an important enzyme in fatty acid metabolism, hydroxyacyl-CoA dehydrogenase or Hadh2 (formerly BOAC). Our results indicate a decrease in Hadh2 activity in oocytes obtained from

*Slc2a8-/-* compared to wild-type (**Figure 3.3E**). In humans, a decrease in this enzyme indicates a poorer quality oocyte[25]. Our lab has also published results indicating a decrease in this enzyme in oocytes from diabetic mice along with a decrease in ATP due to a decrease in AMPK. This metabolic profile results in a poorer quality oocyte[11]. Furthermore, it has been hypothesized that Hadh2 is critical to blastocyst development as the acyl-CoA dehydrogenase null mouse is embryonic lethal at the morula-to-blastocyst transition[26].

Therefore, we conclude, while overall morphology of the ovary is normal, *Slc2a8-/-* oocytes exhibit abnormalities in metabolic pathways potentially leading to a poorer quality oocyte and contributing to the reduced litter size seen among the *Slc2a8-/-* mice.

### *3.3.6 In vivo uterine decidualization is affected in Slc2a8-/- mice*

SLC2A8 is expressed in the uterus of both mice and humans. It is expressed in the whole uterus including the stroma, the decidua and both the luminal and glandular epithelium[20]. While the function of SLC2A8 in the uterus is unknown, expression of *Slc2a8* is dramatically increased upon embryo implantation and decidualization, indicating that it may play an important role in this process[19]. Therefore, we were interested in whether the *Slc2a8-/-* mice exhibited any disturbance in the uterine decidualization process. Using a rodent specific *in vivo* artificial decidualization technique in which we ovariectomize the mice and supplement hormonal stimuli by subcutaneous injection of 17ß-estradiol and progesterone[13], we were able to decidualize the uterus *in vivo* in the absence of an embryo allowing for examination the uterus without the interference of the embryo or ovarian effects.

The *Slc2a8-/-* mice had observably smaller decidualized horns compared to the *Slc2a8+/+* mice (**Figure 3.4A**). Moreover, the mass of the decidualized horn compared to the internal control was significantly smaller when compared to the *Slc2a8+/+* counterparts (~35% less, **Figure 3.4B**). This indicated that there is a disruption in the completion of the decidualization process. This disruption may lead to the smaller litter numbers obtained in the breeding crosses due to abnormal implantation.

### *3.3.7 Slc2a8-/- mice weigh significantly less than Slc2a8+/+ mice*

While other groups have reported no overall change in whole glucose homeostasis in mice deficient in the *Slc2a8-/-* gene, our *Slc2a8-/-* mice are observably smaller than their *Slc2a8+/+* counterparts. The graph in **Figure 3.5A** details weights of the mice starting at 3 weeks of age until 8 weeks of age. The *Slc2a8-/-* mice start out significantly smaller than the *Slc2a8+/+* mice at weaning and continue to be smaller despite maturation into adulthood.

To determine whether the mice exhibit this small size phenotype starting at birth, we weighed the pups at postnatal day 1. The pup sizes were not significantly different between the two groups (**Figure 3.5B**) suggesting that this phenotype manifests later in life. Next, we examined the daily food intake of these mice to determine if a difference in food consumption was responsible for the smaller size phenotype. The *Slc2a8-/-* mice consumed a similar amount of food (in grams) to the *Slc2a8+/+* over several weeks (**Figure 3.5C**). Finally, we investigated the body composition and fat storage and distribution by gonadal fat pad weight and body fat percentage using Magnetic Resonance Imaging (MRI). The gonadal fat pad was smaller (not shown) and

the body fat percentage by MRI was similarly less, however neither of these numbers reaches significance (**Figure 3.6A**).

Our results indicate that lack of SLC2A8 protein during development is resulting in a smaller body size starting with differences observed at weaning. These differences result in a decrease in body fat, although not significant, and are not attributed to food intake.

### *3.3.8 Slc2a8-/- mice do not exhibit a change in whole body glucose homeostasis*

In order to test whether a dysregulation of whole body glucose homeostasis is contributing to the smaller size phenotype observed in the *Slc2a8-/-* mice, we completed both glucose tolerance tests and insulin tolerance tests. The literature suggests that SLC2A8 is not critical in maintaining whole body glucose homeostasis at the basal state. Both tests exhibited clearance results similar to *Slc2a8+/+* mice (**Figures 3.6B & C**). Therefore, our data also suggests that *Slc2a8-/-* does not play a role in whole body glucose homeostasis under basal conditions.

### *3.3.9 Slc2a8-/- mice are resistant to High Fat Diet*

While SLC2A8 does not appear to be important in overall whole body glucose homeostasis during basal conditions, we noticed a distinct phenotype when the mice are fed a diet high in fat and carbohydrates (58%) compared to control diet (4%). We fed the mice the diet for a total of 12 weeks and then completed a regimen of assays to determine the response of the mice. We found that the *Slc2a8-/-* mice did not gain as much weight on the diet as the *Slc2a8+/+* mice (**Figure 3.6D**). This could be attributed to a lack of body fat stores, as the *Slc2a8-/-* exhibited significantly less body fat by MRI (**Figure**

**3.6D**). Moreover, the *Slc2a8-/-* mice fed the high fat diet exhibited improved glucose clearance at 60 minutes during the GTT (**Figure 3.6E**), as well as a normal insulin tolerance test as compared to the *Slc2a8+/+* fed a high fat diet (**Figure 3.6F**). This suggests that the *Slc2a8-/-* mice are somewhat resistant to the effects of a diet high in fats and sugars compared to controls. The mechanism, however, is not clear.

### 3.4 Discussion

SLC2A8 is expressed in insulin-sensitive tissues such as brain and muscle as well as other tissues such as ovary, uterus, liver, and heart and it is most highly expressed in the testes[2, 3]. Interestingly, unlike other transporters which either reside at the plasma membrane (i.e. SLC2A1) or are shuttled there (i.e.SLC2A4), SLC2A8 is found intracellularly, with reported localization in the membranes of late endosomes/lysosomes and of the ER in the brain[5, 27]. One exception to this localization pattern is in the murine blastocyst where SLC2A8 can be triggered to translocate to the plasma membrane in response to insulin[3]. While a few papers have reported a *Slc2a8* deletion model in mice, the function of this unique transporter remains elusive.

The *Slc2a8-/-* mice we generated exhibit a reproductive phenotype resulting in a significant decrease in the number of pups per litter. The *Slc2a8-/-* mating pairs display 30% fewer mice per litter as compared to *Slc2a8+/+* pairs (**Table 3-I**). This phenotype is more severe than previously observed in other *Slc2a8* deficient models. This disparity could be due to the fact that one conditional knockout was made with a *Nestin* cre-knockout model, or due to strain differences or some yet uncovered overarching phenotype in the *Slc2a8* null mice.

In order to investigate the reproductive phenotype, we examined both gonadal and gestational characteristics in the mice. The male mice exhibit overall normal testes morphology, however, the sperm were less motile (**Figure 3.3A & B**). This finding is supported by previously published models, in which deletion of *Slc2a8* resulted in lower ATP in the sperm leading to a decrease in sperm motility[8]. Next, we examined the ovaries of the female mice. Again, overall, the morphology was similar to the wild-type; however, ATP levels were significantly (25%) lower in the *Slc2a8* null oocytes compared to wild-type (**Figure 3.3C & D**). This discrepancy along with a decrease in Hadh2 indicates aberrant energy metabolism in the gonad. Changes in energy metabolism and in particular ATP have been associated with poorer quality gametes, which lead to unfavorable embryonic outcomes[23, 24, 28]. The reason for this difference in the *Slc2a8-/-* oocytes is not clear but may be related to a decrease in glucose uptake in the COC[29].

The process of uterine decidualization is a glucose dependent process, which is mediated mainly by SLC2A1[19, 30]. However, our lab and others have shown a significant increase in SLC2A8 expression during this process[19]. Therefore, to determine if aberrant decidualization is occurring in the *Slc2a8-/-* mice leading to unsuccessful implantation, we examine this process *in vivo*. Using a rodent specific artificial decidualization technique, we report that the *Slc2a8-/-* mice exhibit an incomplete decidualization of the uterus (**Figure 3.4A & B**), as demonstrated by a lack of artificial decidualization in the mice. Disruption of this process could explain the reproductive failures as a function of the poor implantation of embryos leading to a decrease in successful pups per pregnancy.

In conclusion, the *Slc2a8* deficient mice generated by our lab display a more severe reproductive phenotype then previously reported in other models, leading to a smaller litter size. This difference may be due to the fact that the first null model was a *Nestin*-cre and the second mouse still produced a truncated version of the SLC2A8 protein[7-9]. We attribute our reproductive findings to potentially both a gonadal and uterine phenotype. The gametes exhibit abnormal energy metabolism in both previous reported findings and in our study, in which the oocytes have lower ATP levels. Furthermore, we have found a disruption in the uterine decidualization process. While these findings are novel, there may be an overarching phenotype, which is not yet elucidated in the SLC2A8 mice we have generated, such as a hormonal dysregulation.

While previous *Slc2a8* deficient mice models have had no growth disturbances, our *Slc2a8* deficient mice reveal a need for SLC2A8 in normal growth, as the mice are smaller than their wild-type counterparts at 3 weeks of age and into adulthood (in both sexes) (**Figure 3.5A**). However, this growth phenotype is not associated with a dysregulation of overall whole body glucose homeostasis as the ability to clear glucose/insulin is similar to wild-type (**Figure 3.6B & C**). Furthermore, this small size phenotype is not attributed to an increase in food intake as the mice eat similar amounts of food to the wild-type (**Figure 3.5C**).

While SLC2A8 does not appear to be necessary for normal whole body glucose homeostasis, other SLC2As may be compensating for SLC2A8 masking a role for this transporter. Therefore, we were interested in whether stressing the mice would uncover a role for SLC2A8 in this process. We fed the mice a high fat, high carbohydrate diet for 12 weeks and assayed the mice for changes in glucose homeostasis. After 12 weeks on

the diet, the *Slc2a8* deficient mice exhibited significantly less body fat than the wild-type mice (**Figure 3.6D**). And interestingly, *Slc2a8* deficient mice appear to have an improved glucose clearance compared to wild-type as a GTT revealed (**Figure 3.6E**). Additionally, an ITT performed after 12 weeks on the diet showed comparable insulin tolerance to mice on normal chow (**Figure 3.6F**). Further studies to determine the reasons for these differences will need to be completed in the future. It is possible that other SLC2As are upregulated in response to the deletion of *Slc2a8* or that there is a function for SLC2A8 that is yet to be elucidated leading to these findings.

In conclusion, *Slc2a8* deficient mice are smaller at 3 weeks of age but exhibit a normal glucose homeostasis on normal chow despite a smaller growth trajectory as compared to controls that extends into adulthood. However, when the mice are fed a diet consisting of high fat and high carbohydrate, the mice appear resistant to the deleterious effects of this diet, observed in the wild-type mice. Therefore, we conclude that SLC2A8 is important in normal growth. While both the reproductive and growth phenotype are novel and distinct, we believe that there may be an overall disruption of energy metabolism during development, which explains our observations. It is possible that SLC2A8 is present in lysosomal membranes to transport and thus recycle glucose residues from degraded glycosylation sites on proteins targeted for autophagy or lysosomal degradation. This function may be critical in processes which require an abundance of glucose (and SLC2A8 is shown to be increased), such as uterine decidualization and fat differentiation[19, 20, 27, 30, 31], and thus lack of SLC2A8 expression globally accounts for abnormal decidualization and possibly, subsequent placentation and fetal growth; as well as differentiation of adipose cells thus accounting

for elimination of lipid accumulation and fat pad mass in the knockout mice. Further studies, however, will need to be completed to fully understand the role of SLC2A8.

### 3.5 Acknowledgements

The authors would like to acknowledge the NORC for use of the MRI supported by grant number P30DK056341, also The Developmental Biology Histology & Microscopy Core for tissue sectioning.

### 3.6 References


1. **Augustin, R.,** ***The protein family of glucose transport facilitators: It's not only about glucose after all.*** **IUBMB Life. 62(5): p. 315-33.**
2. **Ibberson, M., M. Uldry, and B. Thorens,** ***GLUTX1, a novel mammalian glucose transporter expressed in the central nervous system and insulin-sensitive tissues.*** **J Biol Chem, 2000. 275(7): p. 4607-12.**
3. **Carayannopoulos, M.O., et al.,** ***GLUT8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst.*** **Proc Natl Acad Sci U S A, 2000. 97(13): p. 7313-8.**
4. **Diril, M.K., et al.,** ***Lysosomal localization of GLUT8 in the testis--the EXXXLL motif of GLUT8 is sufficient for its intracellular sorting via AP1- and AP2-mediated interaction.*** **FEBS J, 2009. 276(14): p. 3729-43.**
5. **Augustin, R., J. Riley, and K.H. Moley,** ***GLUT8 contains a [DE]XXXL[LI] sorting motif and localizes to a late endosomal/lysosomal compartment.*** **Traffic, 2005. 6(12): p. 1196-212.**
6. **Pinto, A.B., et al.,** ***Glucose transporter 8 expression and translocation are critical for murine blastocyst survival.*** **Biol Reprod, 2002. 66(6): p. 1729-33.**
7. **Membrez, M., et al.,** ***GLUT8 is dispensable for embryonic development but influences hippocampal neurogenesis and heart function.*** **Mol Cell Biol, 2006. 26(11): p. 4268-76.**

8. **Gawlik, V., et al.,** ***Targeted disruption of Slc2a8 (GLUT8) reduces motility and mitochondrial potential of spermatozoa.*** **Mol Membr Biol, 2008. 25(3): p. 224-35.**

9. **Schmidt, S., et al.,** ***Deletion of glucose transporter GLUT8 in mice increases locomotor activity.*** **Behav Genet, 2008. 38(4): p. 396-406.**

10. **Sambrook, J. and D.W. Russell,** ***Molecular cloning : a laboratory manual.*** **3rd ed. 2001, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.**

11. **Ratchford, A.M., et al.,** ***Maternal diabetes adversely affects AMP-activated protein kinase activity and cellular metabolism in murine oocytes.*** **Am J Physiol Endocrinol Metab, 2007. 293(5): p. E1198-206.**

12. **Lowry, O.H., et al.,** ***Diversity of metabolic patterns in human brain tumors: enzymes of energy metabolism and related metabolites and cofactors.*** **J Neurochem, 1983. 41(4): p. 994-1010.**

13. **Frolova, A.I., K. O'Neill, and K.H. Moley,** ***Dehydroepiandrosterone inhibits glucose flux through the pentose phosphate pathway in human and mouse endometrial stromal cells, preventing decidualization and implantation.*** **Mol Endocrinol. 25(8): p. 1444-55.**

14. **Purcell, S.H., et al.,** ***Improved insulin sensitivity by GLUT12 overexpression in mice.*** **Diabetes. 60(5): p. 1478-82.**

15. **Scheepers, A., et al.,** ***Mouse GLUT8: genomic organization and regulation of expression in 3T3-L1 adipocytes by glucose.*** **Biochem Biophys Res Commun, 2001. 288(4): p. 969-74.**

16. **Kim, S.T. and K.H. Moley, *The expression of GLUT8, GLUT9a, and GLUT9b in the mouse testis and sperm.* Reprod Sci, 2007. 14(5): p. 445-55.**

17. **Gomez, O., et al., *Differential expression of glucose transporter GLUT8 during mouse spermatogenesis.* Reproduction, 2006. 131(1): p. 63-70.**

18. **Schurmann, A., et al., *The glucose transport facilitator GLUT8 is predominantly associated with the acrosomal region of mature spermatozoa.* Cell Tissue Res, 2002. 307(2): p. 237-42.**

19. **Frolova, A.I. and K.H. Moley, *Quantitative analysis of glucose transporter mRNAs in endometrial stromal cells reveals critical role of GLUT1 in uterine receptivity.* Endocrinology. 152(5): p. 2123-8.**

20. **Kim, S.T. and K.H. Moley, *Regulation of facilitative glucose transporters and AKT/MAPK/PRKAA signaling via estradiol and progesterone in the mouse uterine epithelium.* Biol Reprod, 2009. 81(1): p. 188-98.**

21. **Downs, S.M., P.G. Humpherson, and H.J. Leese, *Meiotic induction in cumulus cell-enclosed mouse oocytes: involvement of the pentose phosphate pathway.* Biol Reprod, 1998. 58(4): p. 1084-94.**

22. **Sutton-McDowall, M.L., R.B. Gilchrist, and J.G. Thompson, *The pivotal role of glucose metabolism in determining oocyte developmental competence.* Reproduction. 139(4): p. 685-95.**

23. **Van Blerkom, J., P.W. Davis, and J. Lee, *ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer.* Hum Reprod, 1995. 10(2): p. 415-24.**

24. **Guerin, P., S. El Mouatassim, and Y. Menezo, *Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings.* Hum Reprod Update, 2001. 7(2): p. 175-89.**

25. **Yazigi, R.A., et al., *Enzyme activities and maturation in unstimulated and exogenous gonadotropin-stimulated human oocytes.* Am J Physiol, 1993. 264(4 Pt 1): p. C951-5.**

26. **Berger, P.S. and P.A. Wood, *Disrupted blastocoele formation reveals a critical developmental role for long-chain acyl-CoA dehydrogenase.* Mol Genet Metab, 2004. 82(4): p. 266-72.**

27. **Schmidt, S., H.G. Joost, and A. Schurmann, *GLUT8, the enigmatic intracellular hexose transporter.* Am J Physiol Endocrinol Metab, 2009. 296(4): p. E614-8.**

28. **Menezo, Y. and P. Guerin, *[Gamete and embryo protection against oxidative stress during medically assisted reproduction].* Bull Acad Natl Med, 2005. 189(4): p. 715-26; discussion 726-8.**

29. **Purcell, S., Chi, MM., Lazendorf, S., Moley, K., *Insulin-stimulated glucose uptake occurs in specialized cells within the cumulus oocyte complex* 2011: p. (in submission).**

30. **Frolova, A., et al., *Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells.* Endocrinology, 2009. 150(3): p. 1512-20.**

31. **von Wolff, M., et al., *Glucose transporter proteins (GLUT) in human endometrium: expression, regulation, and function throughout the menstrual cycle and in early pregnancy.* J Clin Endocrinol Metab, 2003. 88(8): p. 3885-92.**

### 3.7 Figure Legends

**Figure 3.1 Targeted Disruption of *Slc2a8*.** **(A)** Schematic representation of the wild type *Slc2a8* allele, the targeting vector used, the *Slc2a8* targeting construct and the targeted *Slc2a8* gene are illustrated. Note the neomycin resistance gene replaces exons 1 -4 of the *Slc2a8* gene. Additionally, the location of the external 5' probe used to in Southern blot analysis of resistant ES clones and Slc2a8 deficient mice is indicated. Abbreviations: Bgl I (B); HindIII (H); BamHI (BHI); PvuI (P). **(B)** Southern blot analysis of EcoRV digested genomic tail DNA from the progeny of *Slc2a8*+/- mice. **(C)** Reverse transcriptase PCR. Lane 1: Positive control, 2: marker, 3-5: *Slc2a8*+/+ testes, heart, liver, 6-8: *Slc2a8*-/- testes, heart, liver.

**Figure 3.2 Impaired protein expression in *Slc2a8*-/- mice.** **(A)** Western blot of SLC2A8 expression in testes protein. SLC2A8 is absent in testes from *Slc2a8*-/- mice. **(B)** Immunofluorescence staining for SLC2A8 in paraffin sectioned testes. SLC2A8 is not expressed in the *Slc2a8*-/- mice.

**Figure 3.3 Male and Female Gonadal Phenotype.** **(A)** No changes in overall testes morphology observed in *Slc2a8*-/- mice. **(B)** *Slc2a8*-/- exhibits reduction in sperm motility approaching significance. **(C)** No changes in overall ovary morphology observed in *Slc2a8*-/- mice. **(D)** ATP is significantly decreased in *Slc2a8*-/- oocytes by ~25%, n=30. **(E)** Hadh2 is significantly decreased in *Slc2a8*-/- oocytes by ~23%, n=20.

**Figure 3.4 SLC2A8 is required for complete uterine decidualization.** **(A)** Representative image of control horn (left) and deciduoma (right) in the *Slc2a8*+/+ (left)

and *Slc2a8-/-* (right) uterus. **(B)** The *Slc2a8-/-* decidualized horn weighed significantly less than the *Slc2a8+/+* decidualized horn compared to their respective control horns.

**Figure 3.5 *Slc2a8-/-* mice exhibit aberrant growth. (A)** *Slc2a8-/-* mice are smaller at 3 weeks of age and into adulthood. Male mice are shown but females exhibit the same pattern. **(B)** Weights of pups at postnatal day 1 are similar to *Slc2a8+/+* mice. **(C)** *Slc2a8-/-* mice consume the same amount of food as *Slc2a8+/+* indicating this is not related to a decrease in calorie consumption.

**Figure 3.6 Overall glucose homeostasis is not affected under normal conditions and maintained during high fat feedings. (A)** *Slc2a8-/-* mice have less body fat by MRI than *Slc2a8+/+* mice (n.s.). **(B & C)** Glucose and insulin tolerance tests are similar to *Slc2a8+/+* mice. **(D)** *Slc2a8-/-* mice do not gain significant body fat on a high fat diet compared to the *Slc2a8+/+* mice. **(E & F)** *Slc2a8-/-* maintains a normal GTT and ITT on a high fat diet.

**Table 3-I *Slc2a8-/-* mice display reduced litter sizes.** Wild-type x wild-type mice have 6.0 pups per litter on average, while mating with a male or female *Slc2a8-/-* results in a reduction in the number of pups per litter.

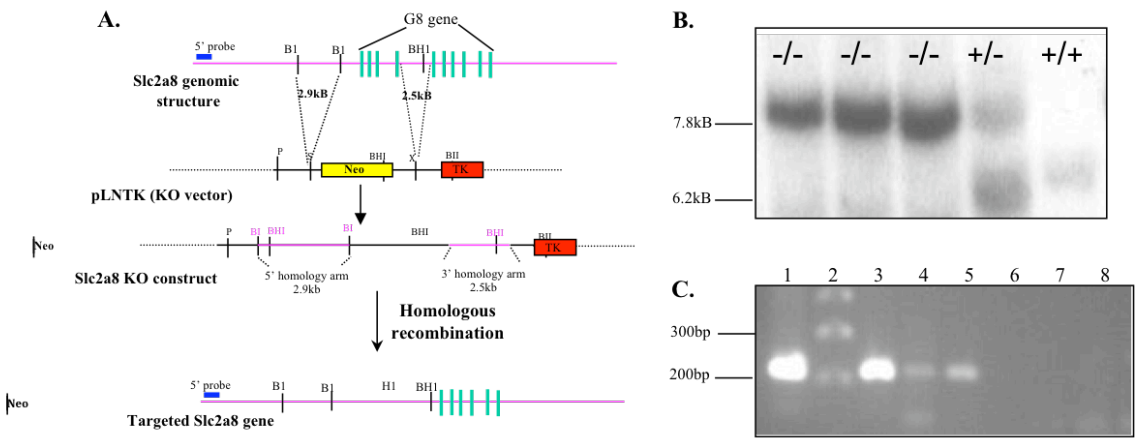
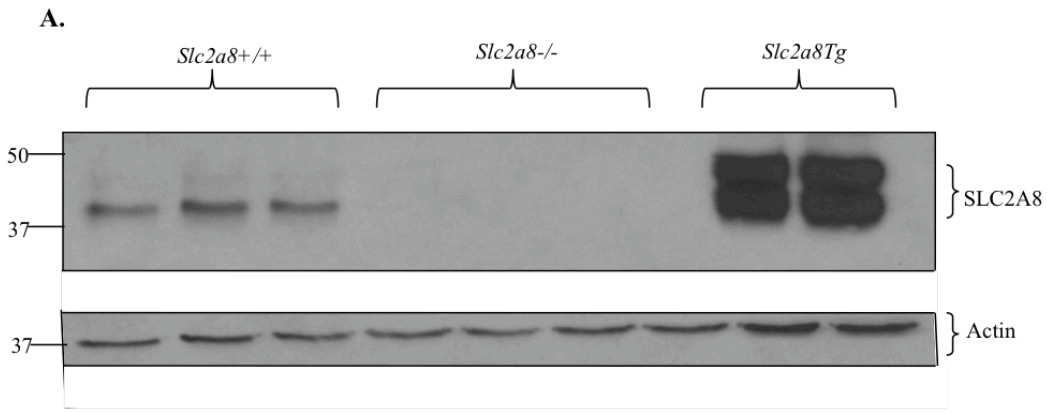

A.
G8 gene
5' probe
B1
B1
BH1
Slc2a8 genomic structure
2.9kB
2.5kB
P
Neo
BHI
BII
TK
pLNTK (KO vector)
Neo
P
BI
BHI
BI
BHI
BII
BII
TK
Slc2a8 KO construct
5' homology arm 2.9kb
3' homology arm 2.5kb
Homologous recombination
Neo
5' probe
B1
B1
H1
BH1
Targeted Slc2a8 gene
B.
-/-
-/-
-/-
+/-
+/+
7.8kB
6.2kB
C.
1
2
3
4
5
6
7
8
300bp
200bp

**Figure 3.1**


A.
Slc2a8+/+
Slc2a8-/-
Slc2a8Tg
50
37
SLC2A8
37
Actin

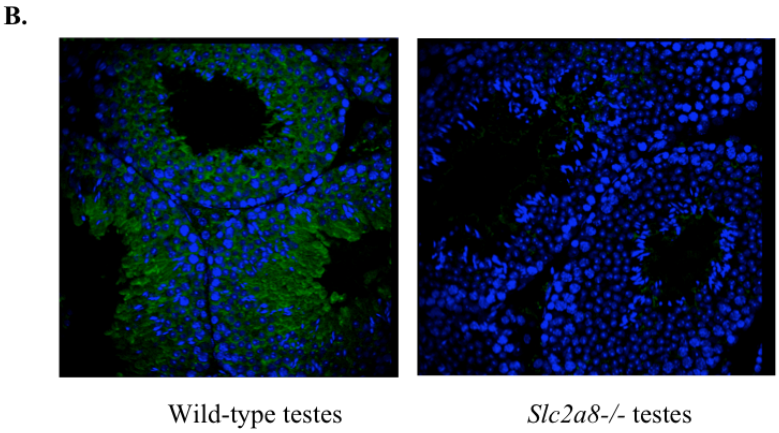

B.
Wild-type testes
Slc2a8-/- testes

**Figure 3.2**

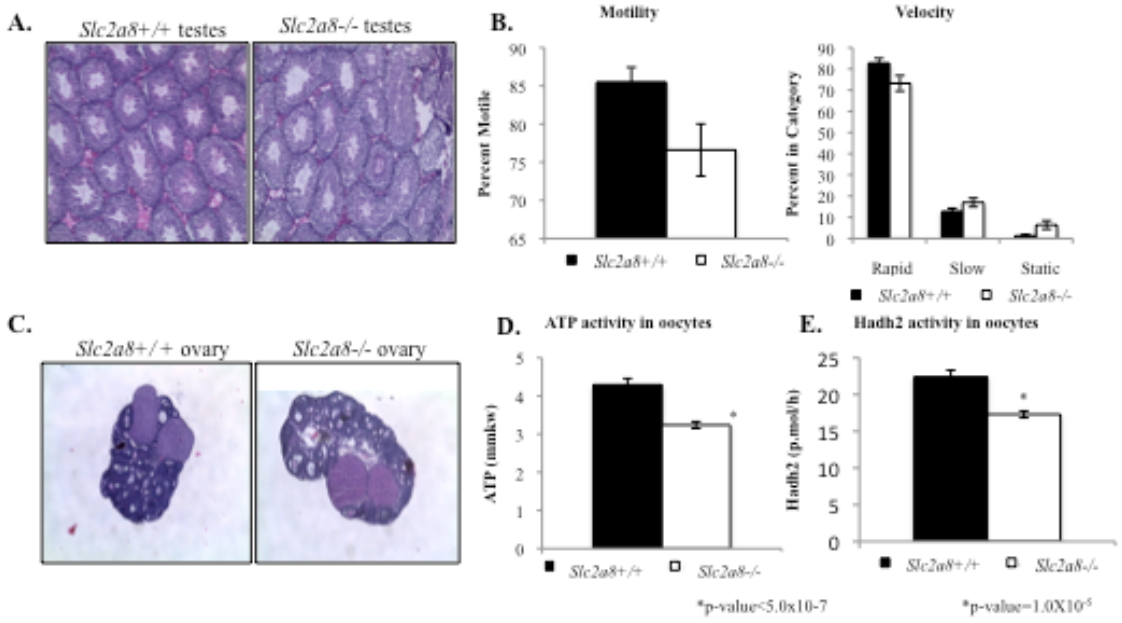

A.
Slc2a8+/+ testes
Slc2a8-/- testes
B.
Motility
Percent Motile
90
85
80
75
70
65
Slc2a8+/+
Slc2a8-/-
Velocity
Percent in Category
90
80
70
60
50
40
30
20
10
0
Rapid
Slow
Static
Slc2a8+/+
Slc2a8-/-
C.
Slc2a8+/+ ovary
Slc2a8-/- ovary
D.
ATP activity in oocytes
ATP (mmkw)
5
4
3
2
1
0
Slc2a8+/+
Slc2a8-/-
*p-value<5.0x10-7
E.
Hadh2 activity in oocytes
Hadh2 (p.mol/h)
25
20
15
10
5
0
Slc2a8+/+
Slc2a8-/-
*p-value=1.0X10-5

**Figure 3.3**

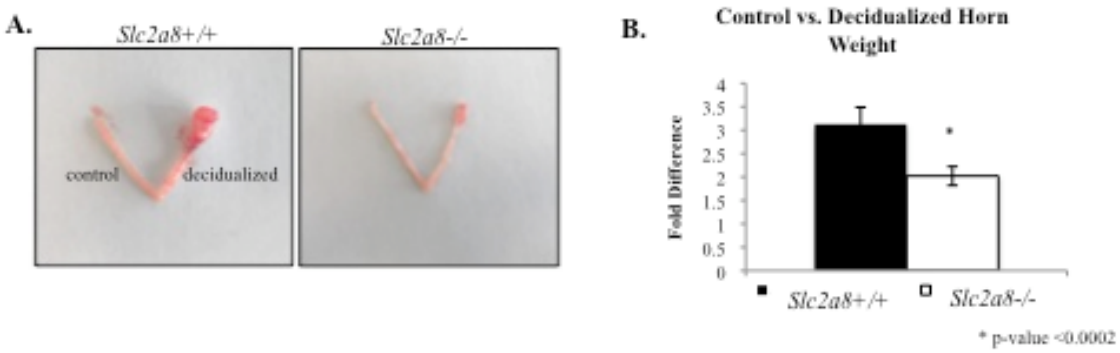

A.
Slc2a8+/+
Slc2a8-/-
control
decidualized
B.
Control vs. Decidualized Horn Weight
Fold Difference
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Slc2a8+/+
Slc2a8-/-
* p-value <0.0002

**Figure 3.4**

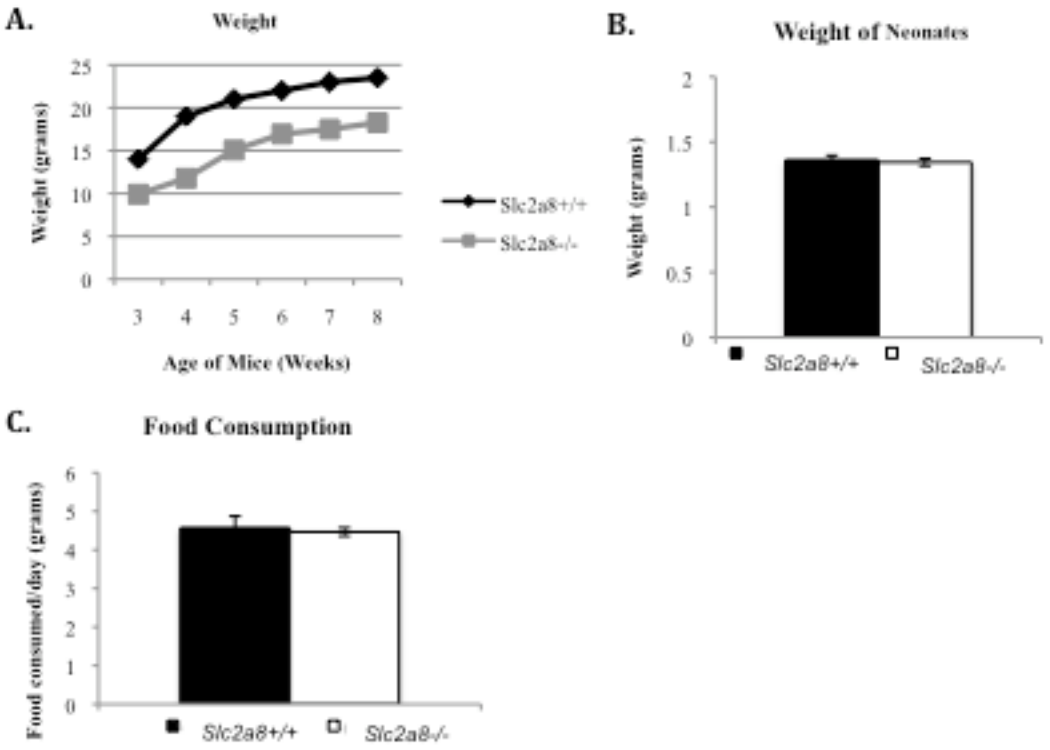

A.
Weight
Weight (grams)
25
20
15
10
5
0
3
4
5
6
7
8
Age of Mice (Weeks)
Slc2a8+/+
Slc2a8-/-
B.
Weight of Neonates
Weight (grams)
2
1.5
1
0.5
0
Slc2a8+/+
Slc2a8-/-
C.
Food Consumption
Food consumed/day (grams)
6
5
4
3
2
1
0
Slc2a8+/+
Slc2a8-/-

**Figure 3.5**

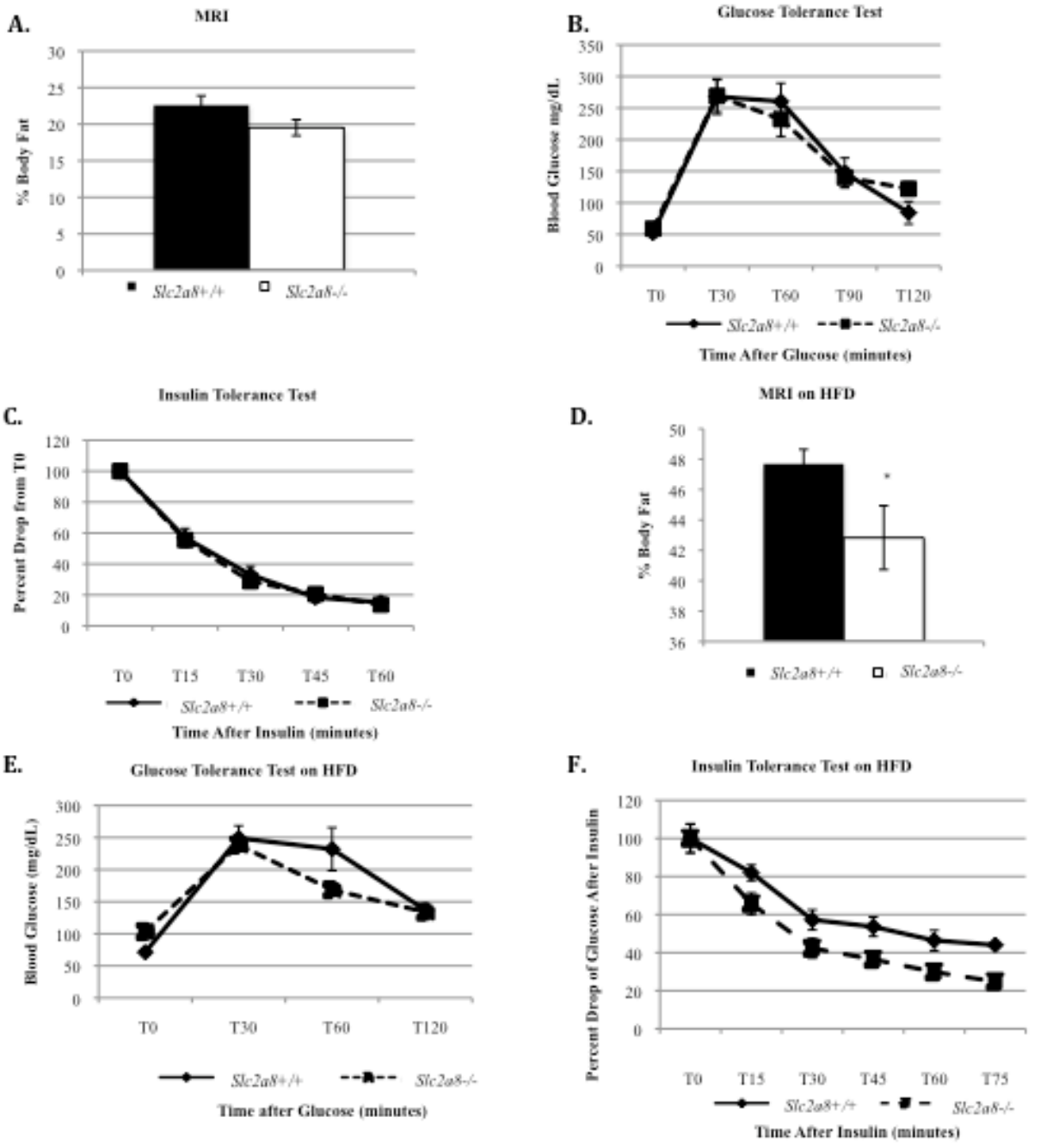

A.
MRI
% Body Fat
30
25
20
15
10
5
0
Slc2a8+/+
Slc2a8-/-
B.
Glucose Tolerance Test
Blood Glucose mg/dL
350
300
250
200
150
100
50
0
T0
T30
T60
T90
T120
Slc2a8+/+
Slc2a8-/-
Time After Glucose (minutes)
C.
Insulin Tolerance Test
Percent Drop from T0
120
100
80
60
40
20
0
T0
T15
T30
T45
T60
Slc2a8+/+
Slc2a8-/-
Time After Insulin (minutes)
D.
MRI on HFD
% Body Fat
50
48
46
44
42
40
38
36
*
Slc2a8+/+
Slc2a8-/-
E.
Glucose Tolerance Test on HFD
Blood Glucose (mg/dL)
300
250
200
150
100
50
0
T0
T30
T60
T120
Slc2a8+/+
Slc2a8-/-
Time after Glucose (minutes)
F.
Insulin Tolerance Test on HFD
Percent Drop of Glucose After Insulin
120
100
80
60
40
20
0
T0
T15
T30
T45
T60
T75
Slc2a8+/+
Slc2a8-/-
Time After Insulin (minutes)

**Figure 3.6**

| Male Genotype | Female Genotype | Pups per Litter (average) | S.E.M. | p-value |
|---|---|---|---|---|
| wild-type | wild-type | 6.0 | 2.2 | - |
| Slc2a8-/- | Slc2a8-/- | 4.3 | 1.7 | 0.00016 |
| wild-type | Slc2a8-/- | 3.7 | 1.7 | 0.0035 |
| Slc2a8-/- | wild-type | 4.4 | 1.9 | 0.0067 |
| Slc2a8+/- | Slc2a8+/- | 4 | 2 | 0.0029 |

**Table 3-I.**

# Chapter 4

## Further Characterization of Facilitative Glucose Transporter 8

Adastra K, Sands M, Moley K

## 4.1 Introduction

While *Slc2a8* deficiency has been studied in several knockout model models, a physiological role for this glucose transporter remains elusive. Due to the unique localization of SLC2A8 on the late endosome and lysosome, it is hypothesized that SLC2A8 could function as a facilitative lysosomal transporter[1].

Previously, the literature has recognized the existence of a facilitative lysosomal transporter specifically in studies done in rodent liver[2-5]. The transporter described in these studies has characteristics similar to SLC2A8. One apparent difference is the proposed affinity for glucose. The transporter described in the literature has a lower affinity for glucose with a Km=22-77mmol/L, while the proposed affinity of SLC2A8 for glucose is reported to be around 2mmol/L[2, 5, 6]. However, if SLC2A8 represents a lysosomal facilitative transporter for hexoses, then deficiency in this protein could lead to detrimental effects manifesting as lysosomal degradation dysfunction.

The lysosome is the primary organelle responsible for degradation; therefore, several other pathways, including endocytosis, phagocytosis and autophagy converge with this pathway[7, 8]. Dysfunction in lysosomal degradation leads to the accumulation of non-degraded substrates within the lysosome. This disturbance disrupts cellular homeostasis leading to secondary disruptions in biochemical and cellular processes. These secondary disruptions result in the severe pathology observed in patients with lysosomal storage disorders.

Lysosomal Storage Diseases are a class of metabolic disorders that stem from a dysfunction in lysosomal degradation. Although often these disorders are a result of mutations or loss in lysosomal hydrolases, other disorders have been linked to dysfunction of vesicular trafficking or lysosomal biogenesis. To date, over 50 different genetic diseases have been classified as lysosomal storage disorders[7, 8].

Several mouse models have been created to investigate lysosomal storage disorders in the laboratory. Despite differences in enzyme deficiencies, several of these mouse models including MPSI, MPSIIIB, MPSVII, Niemann-Pick type A/B and infantile neuronal ceriod lipofuscinosis exhibit similarities between phenotypes. For example, these mouse models display a reduced adiposity with seemingly normal adipocyte function[9]. Instead, the reduction in adiposity is hypothesized to be a result of a negative energy balance stemming from the disruption in lysosomal degradation. It is proposed that the energy required to maintain the dysfunctional lysosomes, as well as the energy required to replenish substrates that are inaccessible to the cell, leads to a negative balance in energy and a loss of adipose stores[9]. This phenotype of a reduction in adiposity with normal adipocyte function is observed in the *Slc2a8-/-* mice created by our lab.

Due to these similarities in phenotype and the proposed function of SLC2A8, we have investigated the intersections between the autophagic and lysosomal pathways and SLC2A8 using our *Slc2a8-/-* mouse model. Our data is the first to describe SLC2A8 co-localization with ATG5, a known autophagosomal protein, in the presence of autophagy activation. This co-localization occurs in murine endometrial stromal cells, which have been hypothesized to undergo glycogen autophagy to supply the necessary energy to

undergo the dynamic processes of decidualization. Therefore, this co-localization of SLC2A8 with the autophagosome, may suggest a role for SLC2A8 in autophagy during conditions requiring an abundance of glucose. Furthermore, using histological, microscopic and enzymatic evaluation we have concluded that the *Slc2a8* deficient mouse model does not display common characteristics of a lysosomal storage disorder under basal conditions. Overall, our data suggests a role for SLC2A8 in energy utilization, however, requirement of this facilitative glucose transporter become apparent in stress conditions.

## 4.2 Material and Methods

*Isolation and culture conditions of primary endometrial stromal cells*

For *in vitro* decidualization of endometrial stromal cells (ESC), female glucose transporter 8 transgenic global over-expressing mice were mated and then sacrificed 4 days post conception (dpc), and pregnancy was confirmed by flushing dissected uterine horns and ostia with Hanks' buffered salt solution and observing for presence of blastocysts. The procedure for ESC isolation is described elsewhere[10]. Briefly, the uteri positive for pregnancy were cut into $1mm^3$ pieces and digested in DMEM:F12 without phenol red and 2g/L Collagenase Type I (Life Technologies, Inc., Gathersburg, MD) for 90 min at 37°C, vortexing every 15 min during the incubation. The solution was then passed through a 40µm sieve (BDFalcon, BD Biosciences, San Jose, CA) and centrifuged at 1100 rpm for 5 min. The isolated ESCs were then resuspended in culture media (DMEM:F12 without phenol red supplemented with 2% heat-inactivated charcoal dextran-stripped calf serum (HyClone, Logan, UT) and 50µg/mL penicillin/streptomycin

(Cambrex Bio Science, Walkersville, MD)) and plated in a six-well Costar plate at $7.5x10^5$ cells per well. The ESCs were allowed to adhere overnight and then treated for 24 hours with either culture media or culture media with 5μM Rapamycin to induce autophagy activation or DMSO for vehicle control.

*Immunofluorescence staining*

After isolation and culture in the treatment groups for 24 hours, the cells were fixed in 3% paraformaldehyde in PBS/2%BSA(v/v) for 20 minutes at room temperature, then permeabilized in 0.5% TritonX-100 in PBS/2%BSA for 30 minutes. To block, 5% normal goat serum in PBS/2%BSA was used for one hour. For co-localization studies, staining was performed sequentially. The cells were incubated in the primary antibody (1:250) in PBS/2%BSA overnight at 4°C (GLUT8 antibody, created by KHM, Atg5 antibody, Novus Biologicals, Littleton, Colorado). The slides were washed three times with PBS/2%BSA and then incubated with secondary antibody (Alexa Fluor goat-anti-rabbit IgG 488, goat-anti-mouse IgG 546, Molecular Probes, Eugene, Oregon) for 45 minutes at room temperature. The slides were rinsed three times again and placed in To-pro®-3-iodide (Molecular Probes, Eugene, Oregon) (1:500) in PBS for 15 minutes. After three more rinses in PBS, the embryos were mounted on slides using Vectashield (Vecta, Burlingame, California) and visualized by confocal microscopy.

*Immunolocalization by transmission electron microscopy*

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 primary antibody (SLC2A8: 1:100, ATG5: 1:200) followed by 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.

*Autophagosomal number by Electron Microscopy*

For starvation groups, mice were restricted from chow for 48 hours prior to sacrifice. Fed and starved mice were then sacrificed and interscapular brown adipose tissue was collected. The tissue was processed as described previously in the Molecular Microbiology Imaging Facility at Washington University in St. Louis[11]. The number of mitochondria were counted in 25 cells and expressed as the number of mitochondria per nucleus.

*b-glucoronidase Assay*

Details concerning the construction of *Slc2a8-/-* mice have previously been reported (chapter 3). The b-glucuronidase assay was performed in the laboratory of Mark Sands at

Washington University, as described previously[12]. Briefly, 500uL of homogenization buffer (150mM NaCl, 10mM Tris (pH7.5), 0.2% TritonX-100, 1mM DTT) was used to homogenize samples. After centrifugation, the supernatant was collected into a new tube and 2μL of the sample was used. 100μL of 4-methylumbulliferel-B-glucoronidase substrate was added to the sample and incubated for 1 hr at 37°C. After 1 hr, 1mL of stop buffer (0.1M Sodium Carbonate) was added. Fluorescence was read at 365nm excitement/448nm emission.

## 4.3 Results

### *4.3.1 Co-localization of SLC2A8 and ATG5*

While autophagosomes have been described in the literature beginning in the 1960s, the field of autophagy has been an active field of investigation the last five years due to the identification of the genes responsible for this process[13]. While localization studies of SLC2A8 have been undertaken, the co-localization of SLC2A8 with autophagic markers has not yet been elucidated in the literature[14].

Previously our lab has reported the presence of SLC2A8 in the high- and low-density intracellular membrane fractions of murine primary endometrial stromal cells using cellular fractionation[15]. Furthermore, in the *Slc2a8-/-* mice detailed in the previous chapter, we observe an impairment of the uterine decidualization process, indicating a role for SLC2A8 in this process. Therefore, we have investigated the localization of SLC2A8 with a known autophagic marker, ATG5 in endometrial stromal cells of *Slc2a8* transgenic mice that overexpresses SLC2A8 globally. Using

immunofluorescence techniques with antibodies specific to SLC2A8 and ATG5, a protein critical for autophagosome formation, we observed a diffuse expression pattern of ATG5 under basal conditions, suggesting a low level of autophagosomal formation **(Figure 4.1)**. However, upon activation of the autophagic pathway by treatment of rapamycin, the expression pattern of ATG5 changed to a more punctate expression pattern signifying the formation of autophagosomes. Rapamycin suppresses mTOR, a negative regulator of the autophagic pathway, thus activating the autophagic pathway. Furthermore, upon activation of autophagy, SLC2A8 co-localizes with the ATG5 marker in a punctuate patterning. This co-localization of SLC2A8 with the autophagic pathway was confirmed by immuno-electron microscopy. Using gold-conjugated secondary antibodies we again observed SLC2A8 and ATG5 co-localized to autophagosomes in endometrial stromal cells treated with rapamycin. These data suggest that in conditions of activated autophagy pathway, such as stress conditions, SLC2A8 is localized to the autophagosomal membrane, indicating a possible role for SLC2A8 in autophagy. One explanation for this localization is that SLC2A8 is responsible for hexose transport out of the autolysosome after degradation during conditions of elevated autophagy, such as stress or nutrient starvation. This finding, of SLC2A8 on the autophagosomal membrane is novel and warrants further investigation into the role of SLC2A8 in autophagy.

### *4.3.2 Slc2a8-/- mice have increased mitochondria after starvation*

While SLC2A8 is associated with the autophagosomal membrane, a role for SLC2A8 in the autophagosomal pathway remains elusive. Recently, the phenotype of an adipose-specific knockout of *Atg7* in mice was detailed[11]. This mouse displays similar characteristics to *Slc2a8-/-* mice with reduced adiposity and resistance to high fat diet.

Furthermore, the adipose tissue of the mice is distinct from wild-type as it shows a significant increase in mitochondria. The authors attributed these characteristics to a dysregulation of adipogenesis, presumably due to aberrations in autophagy[11, 16]. Furthermore, a lack of mitochondrial degradation is observed in the hepatocytes of *Atg7* deficient mice upon starvation[16]. These results overall indicate a need for autophagy in the clearance of organelles, in particular mitochondria.

Due to the phenotypic similarities of the autophagy deficient mice and our *Slc2a8-/-* mice and the possible interplay between SLC2A8 and autophagy, we investigated the *Slc2a8-/-* mice for a dysregulation in mitochondrial degradation. Briefly, wild-type and *Slc2a8-/-* mice were either sacrificed under fed conditions or starved to induce autophagy for 48 hours and then sacrificed. Interscapular brown adipose tissue was dissected, then sectioned and viewed using electron microscopy. The number of mitochondria were counted and expressed per nucleus **(Figure 4.3)**. While the control and *Slc2a8-/-* mice display a similar number of mitochondria during basal conditions, upon starvation significantly more mitochondria are observed in the *Slc2a8-/-* mice, suggesting a disruption in degradation.

### *4.3.3 Slc2a8-/- mice do not display classical characteristics of lysosomal storage disorders*

As previously reported in chapter 3 of this manuscript, *Slc2a8-/-* mice weigh significantly less than their wild-type counterparts at wean age and continue to remain smaller into adulthood (Figure 3.5). This weight difference is associated with a trend towards decreased body fat percentage and an increase in lean mass (Figure 3.6 and data

not shown, respectively). Several studies have shown that leptin is circulated at levels proportional to body fat. In accordance, the *Slc2a8-/-* mice have significantly lower leptin levels than control mice **(Figure 4.4)**.

While the *Slc2a8-/-* mice have deficiencies in adiposity, characterized by less body fat and lower leptin levels, the mice exhibit normal whole body glucose homeostasis, as glucose and insulin tolerances were similar to wild-type (Figure 3.6). Additionally, fasting insulin levels are normal compared to wild-type mice (data not shown). Furthermore, no significant differences are observed from plasma levels of non-esterified fatty acids, glucose, triglycerides or cholesterol **(Figure 4.5)**. This suggests that the decreased adiposity of the *Slc2a8-/-* mice is not a result of dysfunctional adipocytes.

To verify that the reduction in adiposity was not due to a reduced caloric intake, food consumption was measured daily for several weeks. As reported in the previous chapter, the *Slc2a8-/-* mice had a similar daily food intake as their wild-type counterparts (Figure 3.5). Additionally, while *Slc2a8-/-* mice were more resistant to the effects of a high fat diet, as described in chapter 3, displaying less body fat and improved insulin tolerance tests compared to control mice fed a high fat diet, the *Slc2a8-/-* mice still gained more weight than animals fed normal chow, suggesting normal adipocyte function in *Slc2a8-/-* mice.

Because SLC2A8 is localized to the lysosome and the phenotype of the *Slc2a8-/-* mice phenocopies mouse models of lysosomal storage disorders, with a reduced adiposity and normal whole body glucose homeostasis, we investigated our *Slc2a8-/-* mice for

characteristics of lysosomal storage disorder (LSD). The phenotypes of lysosomal storage disorders often do not arise until later in life as the accumulation of non-degraded substrates causes strain on the normal cell function, which then results in the severe phenotype, exhibited in these models. Due to this often late onset, we studied the possibility of lysosomal storage disorder phenotypes in *Slc2a8-/-* mice and wild-type mice that were one year of age or older.

In some mouse models with lysosomal storage defects, an exaggerated response to acoustic startling is reported. Often times these loud noises will trigger seizures in the affected mice[17, 18]. Observation of the *Slc2a8-/-* mice does not reveal any abnormal response to acoustic startling. However, other abnormal behavioral traits are reported in mouse models of LSD, such as altered anxiety[19]. While further behavioral tests were not conducted on our model of *Slc2a8* deficiency, another *Slc2a8-/-* mouse model was reported to exhibit hyperactivity and a trend towards elevated anxiety[20]. These findings along with data detailing increased proliferation in the hippocampus, suggests a disruption in normal brain activity in this mouse model. Therefore, studies are warranted to obtain a clear understanding of the role of SLC2A8 in normal brain function.

As previously mentioned, the accumulation of substrates within lysosomes leads to secondary effects in the cell resulting in the severe pathology present in patients with lysosomal storage disorders. In mouse models of lysosomal storage disorders one of the common phenotypes present is enlarged organs or hypertrophy[21]. We examined our mice for both cardiac hypertrophy and hepatomegaly upon necropsy. The mice did not display signs of cardiac hypertrophy, as the heart size and morphology were similar to the wild-type group (data not shown). To determine if the *Slc2a8-/-* mice displayed

hepatomegaly, we weighed the livers of both groups and reported it as a percentage of total mouse weight **(Figure 4.6)**. The liver weights of the *Slc2a8-/-* mice were similar to the control group. Therefore, we conclude that our *Slc2a8-/-* mice do not show signs of hypertrophy.

In addition to the necropsy, ultra-thin sections of several tissues were examined for the presence of lysosomal storage material by transmission electron microscopy. Accumulation of non-degraded substrates within the lysosome over time leads to lysosomal distention due to an increase in storage material, which is visible by transmission electron microscopy[22]. The tissues that were prepared for examination included the brain, eye, liver, kidney, heart and testes. Upon inspection of these tissues, the lysosomes did not appear distended but rather similar in size to the wild-type mice (Mark Sands, personal communication, data not shown). This suggests that under basal conditions an abundance of storage material is not observed in the lysosomes of *Slc2a8* deficient mice.

Lastly, we measured the enzyme b-glucuronidase in the brain, liver, spleen and testes of the *Slc2a8-/-* mouse compared to the control mouse. B-glucuronidase is an enzyme responsible for the degradation of glycosaminoglycans[23]. If there were a dysfunction of the lysosomal degradation, a secondary elevation in this enzyme may be exhibited. However, in the *Slc2a8-/-* mice, no differences in b-glucuronidase levels were observed comparing the control and *Slc2a8-/-* groups in the brain, liver and spleen. However, in the testes this enzyme was significantly elevated. Studies of b-glucuronidase in testes function are limited, however it has been suggested to be important in sperm to aid in fertilization, in particular to digest the cumulus cells

surrounding the oocyte[24]. The mechanism of elevation in the testes is unclear and warrants further investigation.

### 4.4 Discussion

While SLC2A8 has been localized to the lysosomal membrane, localization of SLC2A8 with the autophagosome has not been described. In the data presented in Figures 4.1 and 4.2, co-localization with the autophagosomal marker, ATG5 is shown by both immunofluorescence and immuno-labelled transmission electron microscopy during conditions of activated autophagy. Localization of SLC2A8 to the autophagosomal membrane is novel and implies the necessity for further investigation of SLC2A8 in relation to autophagy activation. One possible explanation for SLC2A8 on the autophagosomal membrane in conditions of activated autophagy is to facilitate the transport of hexoses, such as glucose, out of the autolysosome for expedited return to the cytosol, particularly in conditions that require a high demand for glucose.

One process, which requires an abundance of energy in the form of glucose, is uterine decidualization. SLC2A8 may be important in supplying glucose via autolysosomal degradation for the decidualization process. It is evident that glycogen is accumulated by the decidual cells prior to decidualization[25]. Furthermore, during the decidualization process, an increase in autophagosomal and lysosomal structures are observed[26]. Therefore, specifically in the murine endometrial stromal cells, the co-localization of SLC2A8 during conditions of activated autophagy, may be mimicking this physiologically need for an abundance of glucose recycling.

It is clear that autophagy is increased in starvation conditions, presumably as a pro-survival mechanism. This increase is considered random, as organelles degraded are proportional to protein degradation[27]. In the *Slc2a8-/-* mice levels of mitochondria are similar to fed conditions and significantly more abundant than wild-type starved conditions. It is not clear whether this is an increase in mitochondria or a lack of degradation of the organelle. However, in another *Slc2a8-/-* mouse model, defective mitochondria displaying a lack in condensation and increase in membrane potential were evident in the sperm of the mice[29]. Therefore, removal of SLC2A8 may lead to an energy imbalance allowing damaged mitochondria to not be degraded[28].

Due to the unique localization of SLC2A8 on the lysosome, it has been suggested that SLC2A8 might represent a lysosomal hexose transporter. Concurrent with that hypothesis, our *Slc2a8* deficient mouse described in chapter 3 of this manuscript phenocopies several mouse models of lysosomal storage disorders. These include MPSI, MPSIIIB, MPSVII, Niemann-Pick type A/B and infantile neuronal ceriod lipofuscinosis, which exhibit reduced adiposity not due to adipocyte dysfunction. In addition to other characteristics including reproductive failures, resistance to effects of high fat diet and an overall normal whole body glucose homeostasis. Therefore, we investigated our *Slc2a8* deficient mouse model using classical lysosomal transporter disorder assays.

Despite these similarities, the *Slc2a8-/-* mice do not display the classical characteristics present in lysosomal storage disorders such as an increase in storage material or b-glucuronidase. While the *Slc2a8-/-* mice do not display apparent dysfunctions in lysosomal storage under basal conditions, during conditions that require an abundance of energy in the form of glucose and other hexoses, a role of SLC2A8 may

be revealed. Therefore, studies investigating SLC2A8 in lysosomal storage should be conducting by challenging the *Slc2a8-/-* mice, particularly in conditions of nutrient deprivation.

However, it is possible that the *Slc2a8-/-* mice do not display characteristics of lysosomal storage disorders, because SLC2A8 is not required for lysosomal hexose transport. In this case, the phenotype of reduced adiposity may be due to another reason. For example, there could be an upregulation of other glucose transporters in the lysosome or upregulation of SLC2As in the gut, leading to changes nutrient absorbency. In fact, studies conducted in our laboratory suggest an upregulation of SLC2A12 in the gut in the absence of *Slc2a8*, which leads to an increase uptake of fructose. Ongoing studies are being conducted to investigate this possibility.

### 4.5 Acknowledgements

The authors would like to acknowledge Wandy Beatty in the Molecular Microbiology Imaging Facility at Washington University in St. Louis for the transmission electron microscopy imaging and The Bakewell Neuroimaging Laboratory for the use of the confocal microscopy for imaging.

## 4.6 References


1. Augustin, R., *The protein family of glucose transport facilitators: It's not only about glucose after all.* IUBMB Life. **62**(5): p. 315-33.
2. Mancini, G.M., C.E. Beerens, and F.W. Verheijen, *Glucose transport in lysosomal membrane vesicles. Kinetic demonstration of a carrier for neutral hexoses.* J Biol Chem, 1990. **265**(21): p. 12380-7.
3. Docherty, K., G.V. Brenchley, and C.N. Hales, *The permeability of rat liver lysosomes to sugars. Evidence for carrier-mediated facilitated diffusion.* Biochem J, 1979. **178**(2): p. 361-6.
4. Chou, H.F., et al., *Genistein inhibits glucose and sulphate transport in isolated rat liver lysosomes.* Br J Nutr. **103**(2): p. 197-205.
5. Jonas, A.J., P. Conrad, and H. Jobe, *Neutral-sugar transport by rat liver lysosomes.* Biochem J, 1990. **272**(2): p. 323-6.
6. Ibberson, M., M. Uldry, and B. Thorens, *GLUTX1, a novel mammalian glucose transporter expressed in the central nervous system and insulin-sensitive tissues.* J Biol Chem, 2000. **275**(7): p. 4607-12.
7. Parkinson-Lawrence, E.J., et al., *Lysosomal storage disease: revealing lysosomal function and physiology.* Physiology (Bethesda). **25**(2): p. 102-15.
8. Schultz, M.L., et al., *Clarifying lysosomal storage diseases.* Trends Neurosci. **34**(8): p. 401-10.
9. Woloszynek, J.C., et al., *Lysosomal dysfunction results in altered energy balance.* J Biol Chem, 2007. **282**(49): p. 35765-71.

10. Frolova, A., et al., *Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells.* Endocrinology, 2009. **150**(3): p. 1512-20.

11. Zhang, Y., et al., *Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis.* Proc Natl Acad Sci U S A, 2009. **106**(47): p. 19860-5.

12. Freeman, B.J., et al., *Behavior and therapeutic efficacy of beta-glucuronidase-positive mononuclear phagocytes in a murine model of mucopolysaccharidosis type VII.* Blood, 1999. **94**(6): p. 2142-50.

13. Kundu, M. and C.B. Thompson, *Autophagy: basic principles and relevance to disease.* Annu Rev Pathol, 2008. **3**: p. 427-55.

14. Augustin, R., J. Riley, and K.H. Moley, *GLUT8 contains a [DE]XXXL[LI] sorting motif and localizes to a late endosomal/lysosomal compartment.* Traffic, 2005. **6**(12): p. 1196-212.

15. Flessner, L.B. and K.H. Moley, *Similar [DE]XXXL[LI] motifs differentially target GLUT8 and GLUT12 in Chinese hamster ovary cells.* Traffic, 2009. **10**(3): p. 324-33.

16. Kim, J.S., et al., *Impaired autophagy: A mechanism of mitochondrial dysfunction in anoxic rat hepatocytes.* Hepatology, 2008. **47**(5): p. 1725-36.

17. Christopher, R., G.R. Rangaswamy, and K.T. Shetty, *GM2 gangliosidoses: a review of cases confirmed by beta-N-acetylhexosaminidase assay.* Indian J Pediatr, 1995. **62**(4): p. 479-83.

18. Labate, A., et al., *Startle epilepsy complicating aspartylglucosaminuria.* Brain Dev, 2004. **26**(2): p. 130-3.

19. Pan, D., et al., *Progression of multiple behavioral deficits with various ages of onset in a murine model of Hurler syndrome.* Brain Res, 2008. **1188**: p. 241-53.

20. Schmidt, S., et al., *Deletion of glucose transporter GLUT8 in mice increases locomotor activity.* Behav Genet, 2008. **38**(4): p. 396-406.

21. vom Dahl, S. and E. Mengel, *Lysosomal storage diseases as differential diagnosis of hepatosplenomegaly.* Best Pract Res Clin Gastroenterol. **24**(5): p. 619-28.

22. Galvin, N., et al., *A murine model of infantile neuronal ceroid lipofuscinosis-ultrastructural evaluation of storage in the central nervous system and viscera.* Pediatr Dev Pathol, 2008. **11**(3): p. 185-92.

23. Gwynn, B., et al., *Intracisternal A-particle element transposition into the murine beta-glucuronidase gene correlates with loss of enzyme activity: a new model for beta-glucuronidase deficiency in the C3H mouse.* Mol Cell Biol, 1998. **18**(11): p. 6474-81.

24. Tulsiani, D.R., et al., *The biological and functional significance of the sperm acrosome and acrosomal enzymes in mammalian fertilization.* Exp Cell Res, 1998. **240**(2): p. 151-64.

25. Frolova, A.I. and K.H. Moley, *Glucose transporters in the uterus: an analysis of tissue distribution and proposed physiological roles.* Reproduction. **142**(2): p. 211-20.

26. Katz, S. and P.A. Abrahamsohn, *Involution of the antimesometrial decidua in the mouse. An ultrastructural study.* Anat Embryol (Berl), 1987. **176**(2): p. 251-8.

27. Komatsu, M., et al., *Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice.* J Cell Biol, 2005. **169**(3): p. 425-34.

28. Gottlieb, R.A. and R.S. Carreira, *Autophagy in health and disease. 5. Mitophagy as a way of life.* Am J Physiol Cell Physiol. **299**(2): p. C203-10.

29. Gawlik, V., et al., *Targeted disruption of Slc2a8 (GLUT8) reduces motility and mitochondrial potential of spermatozoa.* Mol Membr Biol, 2008. **25**(3): p. 224-35.

## 4.7 Figure Legends

**Figure 4.1 SLC2A8 co-localizes with ATG5 upon activation of autophagy.** In primary endometrial stromal cells obtained from SLC2A global over-expressing transgenic mice, SLC2A8 and ATG5 do not co-localize in basal conditions or in cells treated with DMSO (A-B). However, upon treatment with 5uM Rapamycin for 24hours, a localization of SLC2A8 with ATG5 in a puntuacted pattern is observed (C-D).

**Figure 4.2 SLC2A8 and ATG5 are localized to autophagosomes upon autophagy activation.** Using immuno-electron microscopy, we observed co-localization of SLC2A8 and ATG5 in autophagosomes of murine primary endometrial stromal cells obtained from *Slc2a8* over-expressing transgenic mice.

**Figure 4.3 Mitochondrial number in *Slc2a8-/-* brown adipose tissue is not decreased upon starvation.** Brown adipose tissue was collected from fed and starved, wild-type and *Slc2a8-/-* mice and examined using electron microscopy. Mitochondria from 25 different cells in each group were counted and expressed per nuclei.

**Figure 4.4 Leptin is decreased in *Slc2a8-/-* mice.** Plasma of *Slc2a8-/-* mice shows a significant decrease in leptin levels compared to wild-type mice. n=wild-type(15), *Slc2a8-/-*(20)

**Figure 4.5 *Slc2a8-/-* mice do not display hepatomegaly.** Weights of whole liver were recorded at necropsy and compared to total body weight. The *Slc2a8-/-* mice did not exhibit a difference in liver weight/total body weight compared to wild-type mice. n=wild-type(2), *Slc2a8-/-*(5)

**Figure 4.6 b-glucuronidase is elevated in the testes, but not other tissues.** The enzyme b-glucuronidase was measured in tissues of one-year old wild-type and *Slc2a8-/-* mice. Elevations of this enzyme compared to wild-type mice were not elevated in the brain, liver or the spleen, however, significant elevations was observed when comparing the enzyme amount in testes.

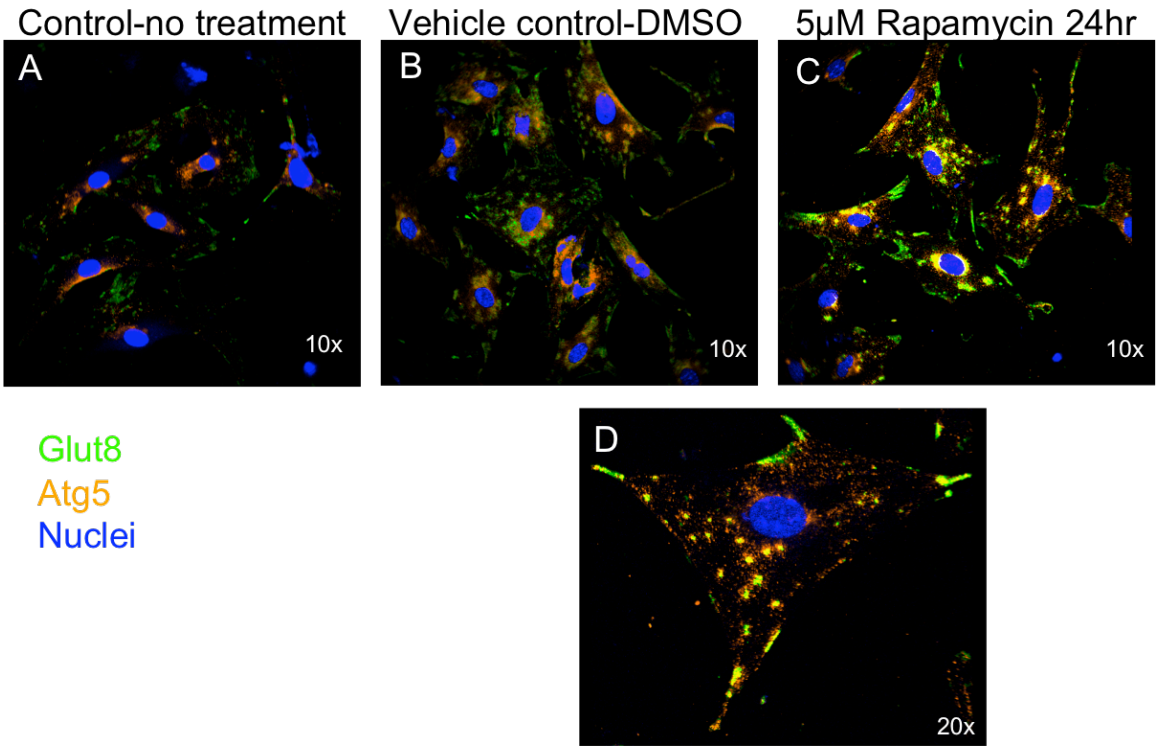

Control-no treatment
Vehicle control-DMSO
5μM Rapamycin 24hr
A
B
C
10x
10x
10x
Glut8
Atg5
Nuclei
D
20x

**Figure 4.1**

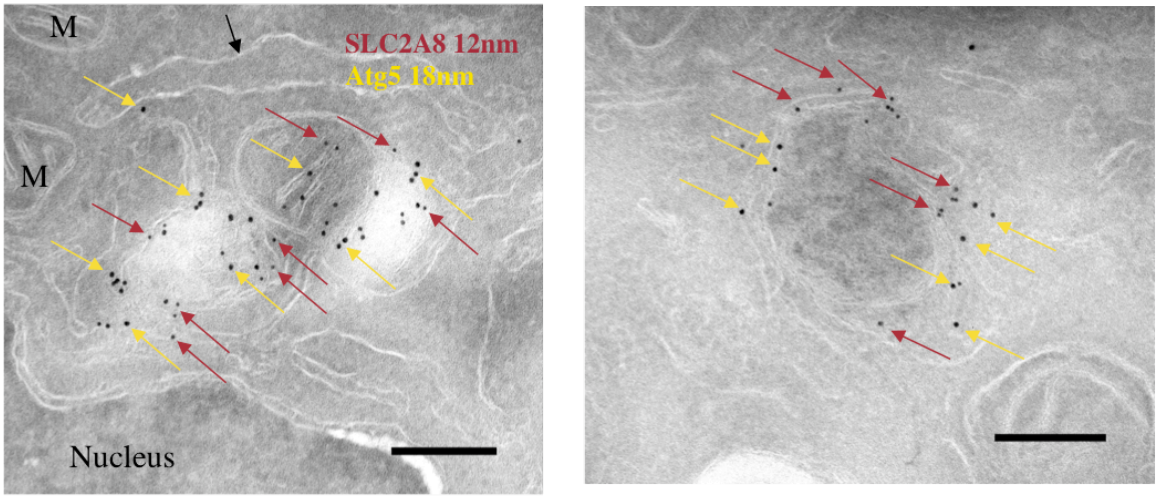



**Figure 4.2**

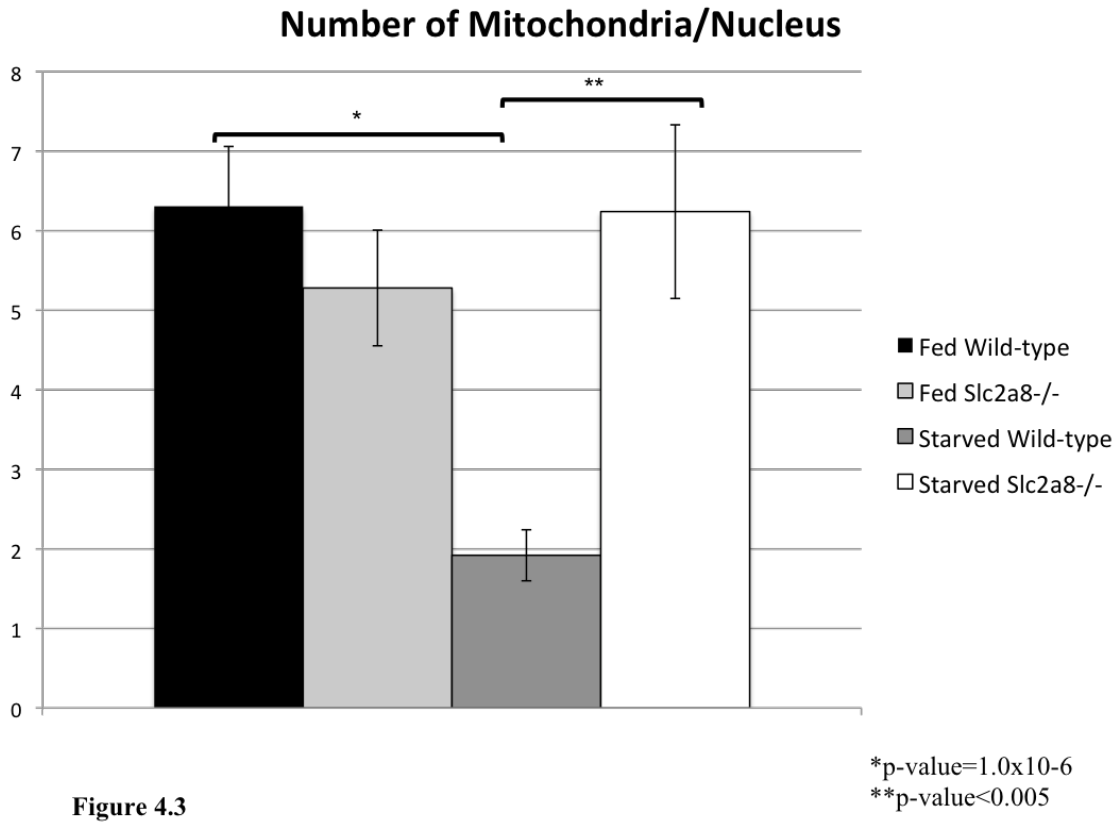

Number of Mitochondria/Nucleus
8
7
6
5
4
3
2
1
0
*
**
Fed Wild-type
Fed Slc2a8-/-
Starved Wild-type
Starved Slc2a8-/-
*p-value=1.0x10-6
**p-value<0.005

**Figure 4.3**

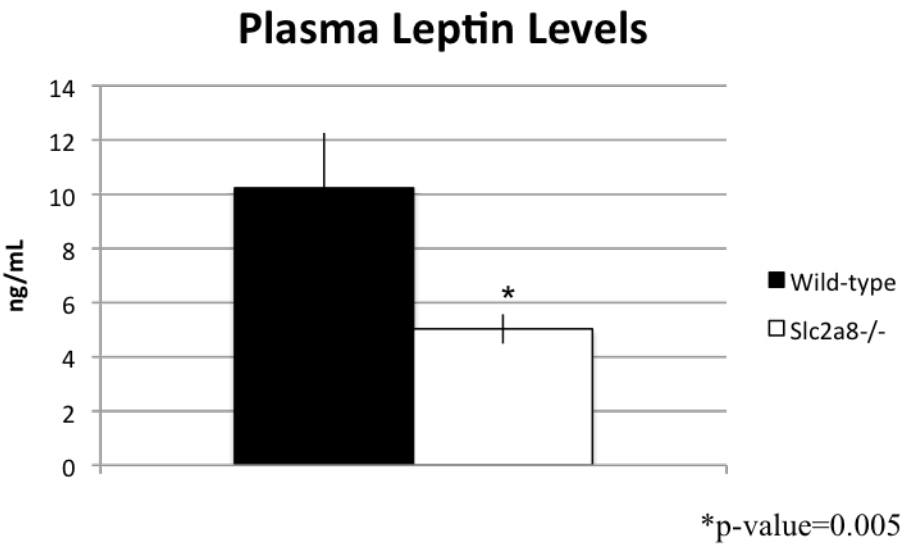

Plasma Leptin Levels
14
12
10
8
6
4
2
0
ng/mL
*
Wild-type
Slc2a8-/-
*p-value=0.005

**Figure 4.4**

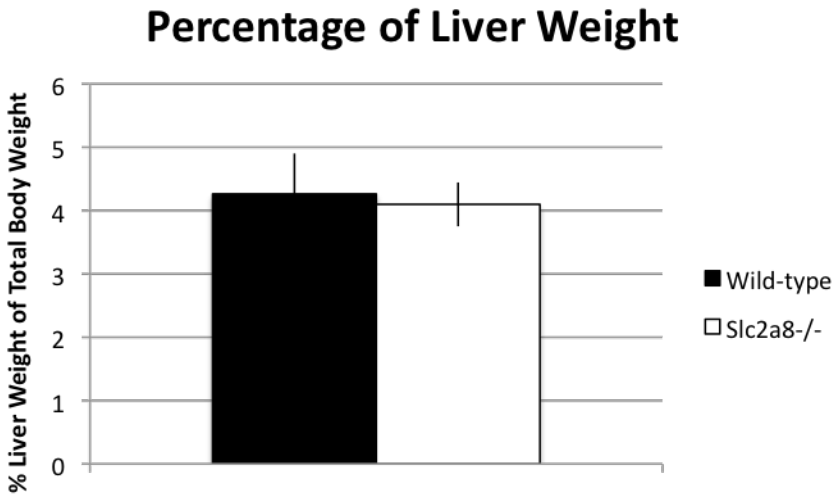

Percentage of Liver Weight
% Liver Weight of Total Body Weight
6
5
4
3
2
1
0
Wild-type
Slc2a8-/-

**Figure 4.5**

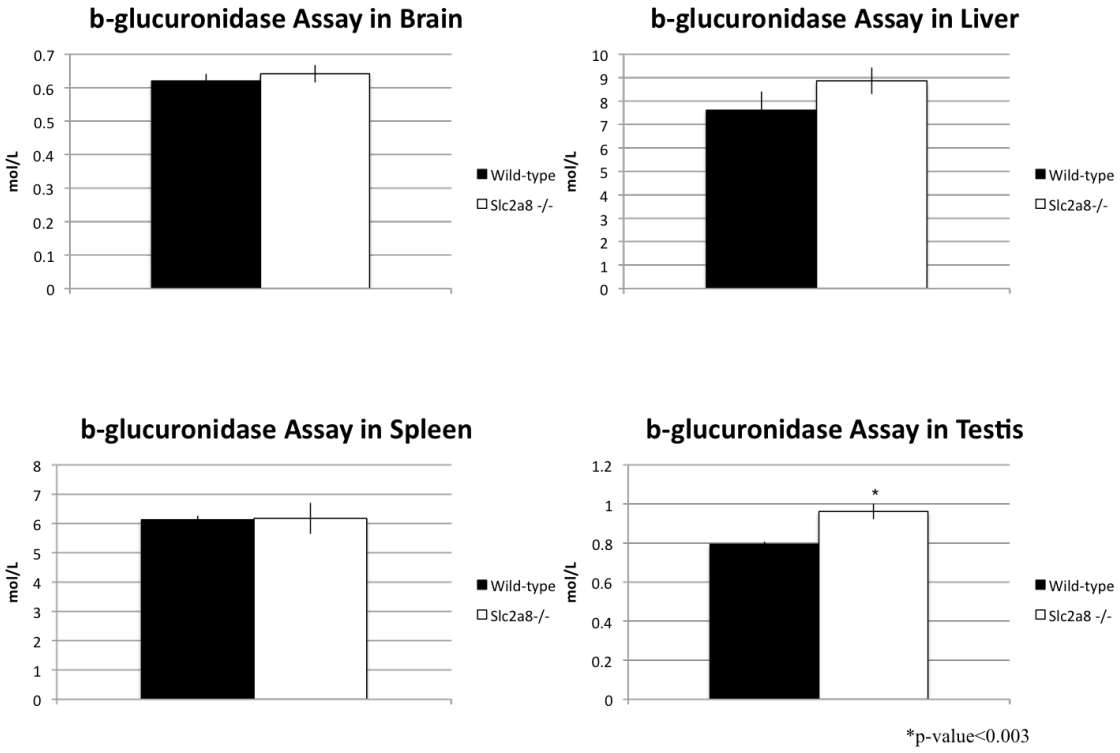

b-glucuronidase Assay in Brain
mol/L
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Wild-type
Slc2a8 -/-
b-glucuronidase Assay in Liver
mol/L
10
9
8
7
6
5
4
3
2
1
0
Wild-type
Slc2a8-/-
b-glucuronidase Assay in Spleen
mol/L
8
7
6
5
4
3
2
1
0
Wild-type
Slc2a8-/-
b-glucuronidase Assay in Testis
mol/L
1.2
1
0.8
0.6
0.4
0.2
0
*
Wild-type
Slc2a8 -/-
*p-value<0.003

**Figure 4.6**

# Chapter 5

# Conclusions and Future Directions

## 5.1 Conclusions and Future Directions

Successful pregnancy relies on the orchestration of many events, several of which occur during early pregnancy. In the field of reproductive biology, it is recognized that aberrations in early pregnancy events can lead to adverse reproductive outcomes. Of importance, homeostasis of the surrounding environment of the pre-implantation embryo is critical to fetal outcome and the success of a pregnancy. This is evident in studies of diabetes, obesity and poor maternal diet. Exposure to any of these milieus during the pre-implantation time period has been shown to have a negative impact on pregnancy and fetal outcome[1-3]. Therefore, investigation into the effect of these conditions on pre-implantation biology is necessary, as the prevalence of these health risks is rising in the United States[4].

Overall, the work presented has expanded our knowledge of the importance of the pre-implantation milieu, specifically concerning glucose utilization and energy homeostasis as they relate to pregnancy outcome. In chapter 2, we conclude that activation of autophagy results in disparate responses between the cells of the embryo. This finding supports the hypothesis of micro-environments within the embryo[5]. Furthermore, this suggests that the malformations observed in fetuses born to diabetic mothers may have resulted from aberrations in autophagy starting as early as the oocyte. Further studies to validate this hypothesis should be conducted and are outlined below.

We present evidence that the pre-implantation embryo alters autophagy in response to the external environment. The embryos respond to stressors including rapamycin, a known activator of autophagy, and a hyperglycemic environment, by

increasing expression of autophagic proteins, identifying autophagy as a pathway for survival in the blastocyst. Furthermore, the embryonic response is differentially activated in what appears to be specific cell niches within the embryos. We hypothesize that this differential autophagic response in single cells in the embryo during pre-implantation development could affect the subsequent lineage allocation in that cell and its resulting daughter cells. We postulate that this early change in lineage patterning may result not only in the growth abnormalities of infants and placentas, but also in malformations commonly observed in fetuses from diabetic mothers.

To test this hypothesis, embryos exposed to rapamycin, an activator of the autophagic pathway could be transferred into pseudo-pregnant control female mice and the fetal outcome assessed. In particular, investigation on 14.5 days post-conception should include successful pregnancy rate, resorption rate, apparent growth malformations and placenta defects. If maladies were observed similar to the outcomes found with embryos exposed to diabetic conditions, this would suggest that aberrant autophagy levels during pre-implantation development leads to adverse pregnancy outcomes.

Previously LC3 (microtubule-associated protein1A/B light chain3), a protein associated with the membrane of newly formed autophagosome has not been detected in oocytes until after fertilization, however we show a significant difference in activation of autophagy in the unfertilized oocyte from diabetic mice[6]. This raises the possibility that maternal proteins at this very early stage may be prematurely degraded by autophagy, resulting in oocytes deficient in protein and perhaps predisposed to increased autophagy and other abnormalities, as we see in the blastocysts from diabetic mice. Further studies investigating the effects of autophagic activation in the oocyte should be

undertaken. By treating oocytes with activators of autophagy, such as rapamycin, and by performing *in vitro* fertilization, followed by zygote transfers and then assaying reproductive outcome, we can determine whether this increase in autophagy observed in diabetic oocytes leads to adverse effects. Data suggests that cell death can be attributed to excessive autophagy[7], therefore, in the oocytes treated with these activators, cell death should also be assessed. Oocytes exposed to rapamycin could be transferred into pseudo-pregnant control females and pregnancy outcome should be assessed at 14.5dpc including resorption rate, growth malformations and placental defects. It would be expected that aberrant autophagic activation in the oocyte would lead to similar defects apparent in diabetic conditions.

In addition to the investigation of external metabolic changes on the oocyte and embryo we also characterized changes in phenotype due to genetic deletion of facilitative glucose transporter 8 (*Slc2a8-/-*). Unlike classical SLC2As, SLC2A8 is localized intracellularly, observed on the endoplasmic reticulum or the lysosome depending on the cell type[8, 9]. While research into the function of SLC2A8 is ongoing, a physiological role for this facilitative glucose transporter has yet to be elucidated. In the work detailed in chapters 3 and 4, we have uncovered a novel role for SLC2A8 in both reproduction and normal growth.

The *Slc2a8-/-* mice exhibit a reduction in number of pups per litter. We conclude that loss of *Slc2a8* results in affected gametes, embryo and uterine decidualization leading to this reduction in litter size. These findings support the hypothesized role of SLC2A8 in energy mobilization as a lysosomal transporter. Suggesting that during processes requiring an abundance of energy, such as early pregnancy events, SLC2A8

plays role in recycling the necessary energy in the form of glucose and potentially other hexoses to the cell. On the other hand, these characteristics could stem from an overarching global dysregulation in glucose utilization leading to the phenotype exhibited. Proposed experiments to further understand the reproductive phenotype follow.

As mentioned previously, SLC2A8 displays an intracellular localization, which is a unique characteristic of class III facilitative glucose transporters. Provided that this localization is unique to these transporters, compensation for loss of SLC2A8 by plasma membrane targeted proteins is unlikely. Furthermore, studies of other *Slc2a8-/-* mice have reported no changes in expression of other studied SLC2As[10]. Cellular fractions of mouse endometrial stromal cells (ESC) have confirmed SLC2A8 is not localized to the plasma membrane but instead found in the high- and low-density intracellular membrane fractions, although the specific intracellular localization is not known[11]. In order to understand why the impairment of decidualization is occurring with lack of *Slc2a8*, we should determine intracellular localization of the protein in the ESCs, as this will provide insight into the function of SLC2A8 during decidualization. While decidualization is impaired in the *Slc2a8-/-* mice, pregnancy does occur, therefore the possibility exist that other SLC2As may be altered to compensate for the loss of this protein. For instance, upregulation of SLC2A1, a facilitative glucose transporter shown to be critical to the decidualization process, may allow for decidualization to continue, albeit at an impaired level. Furthermore SLC2A12, reported to increase in the human endometrial stroma during decidualization (but not in the mouse), may be further increased in the event of

*Slc2a8* deficiency to compensate for this loss[12]. This could explain why the loss of *Slc2a8* does not result in complete loss of pregnancy.

Furthermore, the resultant *Slc2a8-/-* mice display a growth phenotype, as the pups are smaller at wean age despite normal whole body glucose homeostasis as evident by normal glucose and insulin tolerance tests. There are several hypotheses for this aberrant growth pattern. One plausible hypothesis is that SLC2A8 is necessary for lysosomal transport of glucose, and without it there is a lag or halt of recycling glucose to the cell. This theory was considered in chapter 4, in which we found no evidence for lysosomal storage defects in the *Slc2a8-/-* mice under basal conditions, using classical assessments.

While the Sl*c2a8-/-* mice are not smaller at birth, by wean age, the weights compared to wild-type mice are significantly reduced. Therefore, another viable hypothesis is that SLC2A8 functions in normal lactation. This is proposed in bovine, as SLC2A8 is increased 10-fold in the mammary gland in late pregnancy and early lactation periods[13]. This hypothesis could be addressed by switching litters of wild-type and *Slc2a8* deficient mice and recording growth by comparison of body weight. If the *Slc2a8-/-* mice suckling wild-type milk displayed a growth pattern similar to *Slc2a8-/-* suckled mice, then it is unlikely that lack of SLC2A8 is leading to a phenotype in lactation.

Lastly, studies conducted recently indicate that lack of SLC2A8 leads to an upregulation of SLC2A12 in the intestine. This upregulation of SLC2A12 leads to an increase uptake of fructose in these intestinal cells, suggesting a role for SLC2A8 in regulating SLCA12 protein (DeBosch and Moley, personal communication). Therefore,

a possible hypothesis remains that SLC2A8 is leading to an altered expression of other facilitative glucose transporters and abnormal absorption in the intestine. Research on this hypothesis is currently ongoing.

In the last chapter, we show evidence for SLC2A8 co-localization with autophagosomes during conditions of activated autophagy. This is a novel and intriguing finding which suggests a role for this facilitative glucose transporter in the autophagic pathway during activation. A hypothesis for this interaction is that in conditions of activated autophagy, in particular, nutrient deprivation, SLC2A8 is recruited to the autophagosome membrane to transport glucose out of the autolysosome. Further studies are needed to determine if SLC2A8 is recruited to the autophagosome under conditions of nutrient deprivation. Additionally, we could investigate autophagic marker expression in basal and stressed conditions in the *Slc2a8-/-* mice to determine if lack of SLC2A8 causes aberrant autophagy.

Due to the unique localization of SLC2A8 on the lysosome, lysosomal transport is potentially altered in *Slc2a8-/-* mice. The *Slc2a8-/-* mice display similar phenotypes to several LSD model mice including a reduced adiposity with seemingly normal adipocyte function and normal whole body glucose homeostasis. Data in the literature suggests that glucose and other hexoses may be transported out of the lysosome by free diffusion, however the presence of a facilitative lysosomal transporter is also been confirmed. Therefore, in instances that require an abundance of recycled glucose, such as differentiation and growth or in aberrant conditions such as starvation, the need for a facilitative transporter of glucose out of the lysosome may be required. So, while our findings conclude that the *Slc2a8-/-* mice do not display classical characteristics of

lysosomal storage disorders in a basal state, investigation during conditions of stress may lead to perturbations in this pathway. Examination of the lysosomal degradation pathway after exposure to starvation or calorie restriction may reveal a role for SLC2A8 in lysosomal transport under these conditions.

In summation, the novel findings in this dissertation expand our knowledge of glucose utilization and energy homeostasis in early pregnancy events. These findings provide a more detailed picture of the importance of normal glucose transport to fetal outcome. Overall, this knowledge may one day be translatable to clinical management surrounding pregnancy and fertility issues.

## 5.2 References


1. Purcell, S.H. and K.H. Moley, *The impact of obesity on egg quality.* J Assist Reprod Genet. **28**(6): p. 517-24.
2. Wang, Q. and K.H. Moley, *Maternal diabetes and oocyte quality.* Mitochondrion. **10**(5): p. 403-10.
3. Jungheim, E.S., et al., *Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring.* Endocrinology. **151**(8): p. 4039-46.
4. Mokdad, A.H., et al., *Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001.* JAMA, 2003. **289**(1): p. 76-9.
5. Pampfer, S., *Apoptosis in rodent peri-implantation embryos: differential susceptibility of inner cell mass and trophectoderm cell lineages--a review.* Placenta, 2000. **21 Suppl A**: p. S3-10.
6. Tsukamoto, S., A. Kuma, and N. Mizushima, *The role of autophagy during the oocyte-to-embryo transition.* Autophagy, 2008. **4**(8): p. 1076-8.
7. Akazawa, H., et al., *Diphtheria toxin-induced autophagic cardiomyocyte death plays a pathogenic role in mouse model of heart failure.* J Biol Chem, 2004. **279**(39): p. 41095-103.
8. Piroli, G.G., et al., *Peripheral glucose administration stimulates the translocation of GLUT8 glucose transporter to the endoplasmic reticulum in the rat hippocampus.* J Comp Neurol, 2002. **452**(2): p. 103-14.

9. Augustin, R., J. Riley, and K.H. Moley, *GLUT8 contains a [DE]XXXL[LI] sorting motif and localizes to a late endosomal/lysosomal compartment.* Traffic, 2005. **6**(12): p. 1196-212.

10. Gawlik, V., et al., *Targeted disruption of Slc2a8 (GLUT8) reduces motility and mitochondrial potential of spermatozoa.* Mol Membr Biol, 2008. **25**(3): p. 224-35.

11. Flessner, L.B. and K.H. Moley, *Similar [DE]XXXL[LI] motifs differentially target GLUT8 and GLUT12 in Chinese hamster ovary cells.* Traffic, 2009. **10**(3): p. 324-33.

12. Frolova, A.I. and K.H. Moley, *Quantitative analysis of glucose transporter mRNAs in endometrial stromal cells reveals critical role of GLUT1 in uterine receptivity.* Endocrinology. **152**(5): p. 2123-8.

13. Zhao, F.Q., et al., *Bovine glucose transporter GLUT8: cloning, expression, and developmental regulation in mammary gland.* Biochim Biophys Acta, 2004. **1680**(2): p. 103-13.