Generation of Knockout Mice for GRP78 in Adipose Tissue as a Model for Studying Cellular Stress and Insulin Resistance

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ABSTRACT

Glucose Regulated Protein-78 (GRP78) is an ER stress chaperone whose decreased expression is associated with Type II diabetes by inhibiting AKT activation in the GLUT signaling pathway to facilitate insulin resistance. Although mice fully lacking GRP78 die early in development, mice missing GRP78 in only one tissue can be viable. The goal of this project was to create a knockout mouse for GRP78 in adipocytes to allow allow future studies on the effects of GRP78 absence in that tissue. Mice hemizygous for Cre-recombinase under the control of the adipocyte-specific and tamoxifen-inducible promoter adiponectin (Ad-Cre) were bred with mice heterozygous for floxed GRP78 to produce a subset of pups of the desired genotype (homozygous floxed GRP78, hemizygous for Ad-Cre). Pups were screened by PCR genotyping tail snip DNA for Ad-Cre and GRP78. Two of the F1 male pups (4366 and 4368) were of the exact desired genotype. In the future, the mice will be treated with Tamoxifen to generate the GRP78 knockout in adipose tissue, and then analyzed by a hyper-insulinemic-euglycemic clamp test to determine whether insulin resistance has resulted.

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BACKGROUND

Diabetes Overview

Diabetes, also known as "the illness of the rich", is a disease that affects roughly twenty five million Americans. Diabetes is a metabolic disorder in which the absorption of glucose in the body is altered (Ali, 2011), and comes in two different forms, Type I and Type II. Type I diabetes is also known as insulin-dependent diabetes mellitus, while Type 2 diabetes is known as noninsulin-dependent diabetes mellitus. Glucose is absorbed into the bloodstream following digestion, and is normally taken up by cells with the help of the hormone insulin. Insulin is produced by the pancreas, a large gland at the back of the stomach responsible for the secretion of digestive juices. Diabetes is characterized by a high level of glucose in the bloodstream due to an impairment of insulin production (Type I) or activity (Type II) (NDIC, 2013).

Glucose Homeostasis

The pancreas is comprised of α and β cells, contained within the Islets of Langerhans, that work together to maintain a balanced level of glucose in the blood. The β cells produce insulin which is responsible for the uptake of glucose from the bloodstream. The α cells, on the other hand, release the hormone glucagon, which releases glucose from the liver directly into the bloodstream (NDIC, 2013). Normally when foods are ingested, blood glucose levels increase. Glucose is then utilized by muscle or fat tissue for energy, and stored in the liver and muscle as glycogen, or in adipose tissue as fat. Together insulin and glucagon work to maintain a balanced level of

sugar in the blood. Impairment of insulin production, or response to it, results in glucose

energy stored as fat, and protein acting as the body's main source of energy (Ali, 2011).

The liver releases glucose into the bloodstream by one of two pathways. The liver

can breakdown glycogen (carbohydrate) into glucose via a process known as

glycogenolysis, or it can break down amino acids and non-carbohydrate sources into

glucose. Carbohydrates normally act as the main source of glucose for the body. They

can be simple sugars that are readily absorbed by the body, or can be complex requiring

degradation into simpler disaccharides and monosaccharides before they can be utilized

for energy and storage purposes (Ali, 2011). The liver also works to decrease glucose

levels in the bloodstream by absorbing glucose-rich blood via the portal vein that is

directly attached to the gastrointestinal tract. The liver is therefore the main organ

involved in glucose maintenance (NDIC, 2013).

Other factors that affect glucose levels include diet, as well as additional glycogen

breakdown in muscle tissue (short-term storage) and fat tissue (long-term storage). Once

glycogen is broken down into glucose, it undergoes glycolysis, a process that converts

glucose into pyruvate while simultaneously producing ATP in muscle and fat tissue. In

order for glucose to enter the cell and undergo glycolysis the polar molecule must be

absorbed by one of five different transport proteins in the cell membrane, the glucose

transporters (GLUTs). The most prominent GLUTs are GLUT2 and GLUT5 (Ali, 2011).

Figure 1 summarizes the effects of insulin and glucagon. During hyperglycemia

and even following a meal, the β cells release insulin, which converts glucose into

glycogen, its storage form, in a process called glycogenesis, resulting in a decline in

blood glucose levels and subsequent insulin levels, achieving a balance of glucose and

insulin in the blood (Gropper et al., 2009).
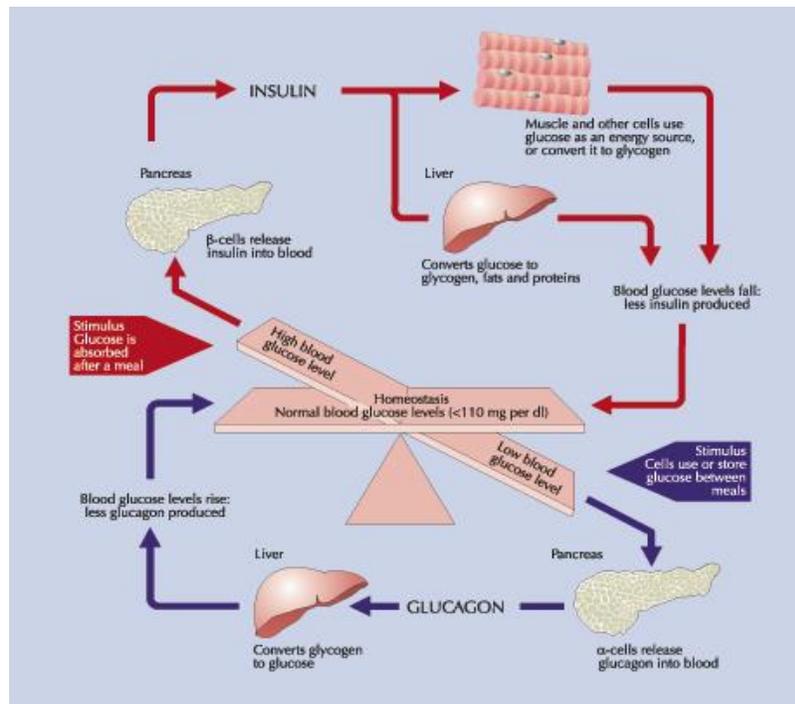
**Figure 1: Overview of Insulin and Glucagon Effects on Glucose Blood
Levels.** An increase in blood glucose leads to release of insulin (upper left),
while a decrease in blood glucose leads to the release of glucagon (lower right).
(wordpress.com, 2012)

Type I Diabetes

Type I diabetic patients are usually diagnosed with the condition before the age of

thirty. These patients do not produce enough insulin in the body to effectively absorb

glucose from the bloodstream. Of the millions of American diagnosed with diabetes, five

to ten percent suffer from the Type I diabetes (Ali, 2011). The disease can occur at any

stage of life, but is especially prevalent among young adults and children. It is caused by

genetic and environmental factors that induce an autoimmune disorder in which a

patient's immune system attacks the pancreatic β cells responsible for insulin synthesis.

Treatment of Type I diabetes currently requires insulin injections to replace the diminished levels in the body (NDIC, 2011).

Type II Diabetes

The vast majority (90-95%) of diabetic patients are Type II. Obese individuals over the age of thirty with a family history of diabetes are more prone to becoming Type II diabetics. This form of diabetes is highlighted by the body's insensitivity or resistance to insulin. At the onset of the disease patients are advised to modify their diets and practice regular exercise to attempt to reverse the condition. Type II diabetics suffer from hyperglycemia even with normal levels of insulin because the hormone's receptors or downstream signaling pathways do not function properly. Doctors therefore additionally prescribe hypoglycemic drugs (Ali, 2011). As the disease progresses, the increased demand by the body for useful insulin stresses and eventually destroys the cells responsible for its synthesis, the β cells (NDIC, 2011).

Treatment and Prevention of Diabetes

The normal glucose level in the bloodstream is 100 mg/dL (Ali, 2011). Fasting glucose levels of 110-125 mg/dL are indicators of pre-diabetes. Diabetic patients can work with health care providers to reverse the disease during its early stages via a strict diet and regular exercise. Negative effects of diabetes could range from hypertension to heart and kidney disease. Complications readily escalate if not monitored. Diabetic treatment involves the control of blood pressure, glucose, and lipid levels. Type II diabetics do not usually need insulin injections, and patients are thus often deceived to

believe they do not have diabetes. Treatment of Type II diabetes is highlighted by diet

management and drug intervention to control glucose levels (ADA, 2013).

Normal Insulin Signaling

After insulin is released into the blood, it binds to the insulin receptor located on

the cell membrane (**Figure-2**, upper right**)** (Fort et al., 2010). This binding initiates

intracellular cell signaling by phosphorylating specific tyrosine residues on the

cytoplasmic side of the receptor (Chang et al., 2004). The phospho-tyrosine residues are

recognized by Insulin Receptor Substrates (IRS-1, IRS-2, IRS-3, and IRS-4) that also

become phosphorylated (Bevan, 2001). The activated IRS proteins then serve as docking

proteins for three main signaling molecules that have Src-homology or SH2 domains.

Each of these signaling molecules determine the resulting signaling pathway, and include

PI3K (phosphatidyl inositol 3 kinase), SHP2 (a tyrosine phosphatase), and Grb2 (a small

adaptor molecule) (Fort et al., 2010). The binding of PI3K to the activated IRS proteins

is the main pathway that activates a kinase cascade that leads to the translocation of

GLUT4 transporters form inside the cell to the plasma membrane and subsequent insulin-

mediated glucose uptake from the blood (**Figure-2**, upper left). It also promotes the

synthesis of glycogen, the storage form of glucose, in skeletal muscle and the liver

(**Figure-2**, lower left) (Bevan, 2001). In short, insulin binds to insulin receptors on the

surface of cells to upregulate the insulin signal transduction pathway whose main

outcome is the upregulation of glucose transporters and the internalization of glucose

from the blood. The GLUT then transports the glucose from the blood into the cytoplasm

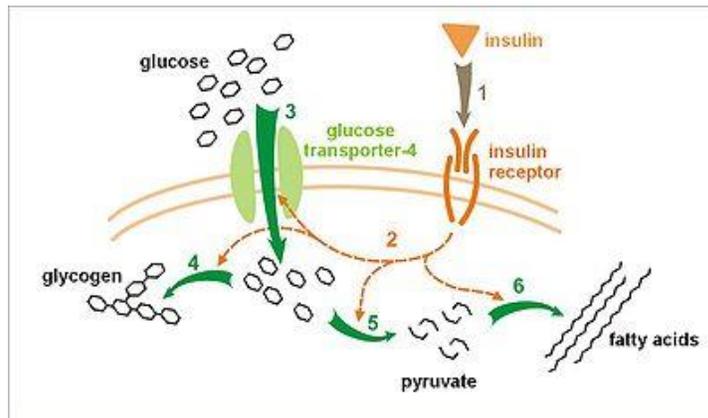of the cells, reducing blood glucose levels (Saini, 2010).

Figure 2: Insulin Signaling Pathway Mediating Glucose Uptake. Insulin binds to the insulin receptor activating signal transduction pathways whose outcome is the upregulation of the glucose transporter on the cell surface and the internalization of glucose. The glucose then undergoes glycogenesis (Source: php.med.unsw.edu.au).

Insulin Resistance

Insulin resistance is a condition that occurs when the body makes insulin but does not efficiently respond to it. It is also known as Syndrome X and Metabolic Syndrome (Haynes, 2012). It is the impaired ability of insulin to stimulate glucose usage (Kim, 2009). When the insulin signaling pathway becomes desensitized in a tissue, due to cellular stress or the production of too much insulin, the cell fails to upregulate GLUT or to internalize glucose, causing hyperglycemia (Kahn et al., 2006; Saini, 2010; Haynes, 2012). The development of insulin resistance in liver, adipose, and muscle tissues causes the pancreas to produce more insulin in an effort to reduce blood glucose levels (Haynes, 2012). Insulin resistance is a major characteristic of type II diabetes, and can lead to metabolic abnormalities (Ye, 2010).

In adipose tissue, insulin resistance and a reduction of cellular glucose results in an excessive breakdown of stored fat in the form of triglyceride into fatty acids (Gray and

Kim, 2011). This, in turn, leads to hyperlipidemia (increased serum lipids) in obese

individuals, which can lead to atherosclerosis or hardening of the arteries and cause

future medical complications such as stroke and cardiovascular disease.

Measuring Insulin Resistance

Three main tests have been used to measure insulin resistance. The first test is the

glucose tolerance test (GTT) that examines the clearance of injected glucose from the

blood. The limitation of this test is that it does not account for the subsequent insulin

secretion that occurs as a response to the hyperglycemia (Kim, 2009).

The second test is called the insulin tolerance test (ITT), which examines glucose

removal from the blood in response to injected insulin. However, administration of this

test can induce severe hypoglycemia (Kim, 2009).

The third test is the hyperinsulinemic-euglycemic clamp, and is the most accurate

of the three tests for insulin resistance (Kim, 2009). This test is considered the "gold-

standard method for assessing insulin sensitivity" in vivo, and addresses both limitations

of the GTT and ITT tests (Kim, 2009). This test determines the amount of glucose that

must be infused into the body, during a hyperinsulinemic state, to maintain

normoglycemia.

Diabetes and Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is an organelle responsible for protein

processing and packaging. Pancreatic β cells contain a high amount of ER due to their

primary function of secreting insulin. One theory about insulin resistance in type II

diabetes is that prolonged ER stress results in desensitization of the insulin signaling pathway (Kim, 2012). ER stress might result from prolonged exposure to environmental stressors, alteration of ER stress response proteins (Balasubramanyam, 2010), or prolonged activation of the secretion pathway for insulin which alters the balance between proinsulin and insulin. According to the theory, the ER stress desensitizes the signaling components of the cell so it no longer efficiently responds to insulin, or the prolonged stress in pancreatic β cells results in permanent cell damage that depletes the number of viable cells in the pancreas. Thus, the biochemical pathways induced by ER stress are potentially target pathways for the cure of Type 2 diabetes (Kim et al., 2012).

Within the ER, premature polypeptide chains undergo a variety of post-translational modifications that produce mature active proteins. The post-translational processing of insulin begins with the transcription of preproinsulin at chromosome 11 to produce a protein that is delivered to the ER co-translationally. A signal polypeptide 24 amino acids long interacts with a signal recognition particle (SRP) that inhibits translation until the protein and ribosome are properly seated on the ER membrane. The signal peptide is then removed from preproinsulin by signal peptidase, allowing the synthesis of proinsulin to continue in the ER lumen. Proinsulin is matured within the ER lumen by folding the chain and forming three disulfide bridges, and then it is packaged into secretory granules to be delivered to the Golgi apparatus. Under hyperglycemic conditions, the insulin granule is secreted into the bloodstream by exocytosis. Problems with proinsulin folding during ER stress inhibit insulin production early in the biosynthesis process and cause ER stress (Kim et al., 2012). ER stress results from an imbalance between the number of pro-insulin entering the ER and the amount of

processed insulin delivered to the Golgi. In addition, metabolic dysregulation associated with obesity predisposes pancreatic β cells to ER stress.

The ER Stress Response

The ER responds to stress by activating a series of biochemical pathways that attempt to decrease preproinsulin and proinsulin synthesis and degrade improperly folded synthesized proteins to alleviate the source of stress at both ends of the ER. Collectively, these methods of ER stress control are induced by numerous pathways including the activation of activating factor 6 (ATF6) that is responsible for the expression of ER chaperone genes. ER chaperones aid in properly folding the overload of proinsulin product packed in the ER lumen (Kim, 2012). The following sections discuss some of the ER stress response proteins.

Activating Transcription Factor 6 (ATF6)

ATF6 is an ER trans-membrane protein responsible for transcriptional induction of ER chaperones and folding enzymes. The trans-membrane protein initiates the transcription of ER chaperones glucose-regulated proteins (GRP) 78 and 94, and Bisphosphate (BiP). Under normal conditions, ATF6 is bound to BiP rendering the chaperone inactive (Ye, 2000). Under stressful conditions, BiP dissociates from ATF6 and binds to the surrounding misfolded proteins to assist their folding and prevent their aggregation. This frees ATF6 from BiP and yields its active conformation.

BiP also associates with Golgi localizing signals that bind to ATF6 to translocate it to the Golgi (Shen et al., 2002). In the Golgi, ATF6 is cleaved by site 1 and site 2

proteases. ATF6 is a type 1 protein in which the N-terminus faces the cytoplasm and the C-terminus faces the lumen of the organelle. Site 2 protease cleaves the N-terminal cytoplasmic domain and moves it to the nucleus where it initiates the transcription of chaperone molecules and enzymes essential for protein folding (Ye et al., 2000). ATF6 activation is rapid because it is produced from a preexisting precursor protein, implying that ATF6, unlike other unfolded protein response (UPR) proteins, is activated early on during ER stress (Shen et al., 2002).

ATF6 also induces the expression of a nuclear receptor called small heterodimer partner (SHP). SHP affects promoter activity of the insulin gene and results in the down regulation of insulin gene expression under chronic hyperglycemic conditions. This suppression of insulin gene expression results in the eventual dysfunction of pancreatic β-cells (Kim et al., 2012).

ATF6 and XBP-1

In addition, ATF6 splices and activates of X-box-binding-protein-1 (XBP-1). XBP-1 is a protein responsible for suppression of insulin transcription and the degradation of insulin mRNA (Ye et al., 2000). Prolonged activation of ATF6 leads to simultaneous splicing and activation of XBP-1. The activation of XBP-1 inhibits insulin gene expression and thus leads to the eventual apoptosis of pancreatic β-cells.

ATF6 and GRP78

Alternatively, ATF6 can be advantageous to pancreatic cell survival during ER stress by activating the synthesis of chaperone and enzymatic proteins responsible for

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protein folding, such as GRP78/BiP. This is especially true in adipocytes that secrete

large amounts of peptides. GRP78 is more readily observed in adipose tissue than in liver

and muscle cells (Ye et al., 2010). In adipocytes, GRP78 production is known to help

alleviate obesity resulting from high-fat diets (HFD) and its associated insulin resistance.

GRP78 heterozygosity induces an adaptive UPR that protects the cell from cell death.

The unfolded protein response resulting from chronic hyperglycemia results in a

prolonged block of mRNA transcription. During adaptive UPR, the increased expression

of chaperone proteins such as GRP78 lessens the extent of the block on mRNA

translation.

GRP78 deletion has been shown to induce insulin resistance by inhibiting the

activation of AKT kinase, a component of the insulin signaling pathway (Pfaffenbach and

Lee, 2010). The role of AKT in the pancreas has been investigated. Inhibition of AKT

kinase leads to the development of type II diabetes. The role of GRP78 in adipocyte

tissue remains unknown, and is the subject of this project.

Cre Recombinase System for Mouse Knockout

The Cre recombinase system is a powerful tool used for the analysis of gene

function in transgenic mice (Sauer, 1998). This technique can create tissue-specific

knockout of genes in mice without inducing early embryonic lethality. The *cre* gene

stands for cyclization recombination, and encodes a site-specific DNA recombinase

called Cre recombinase, a 38 kDa recombinase protein that can recombine specific DNA

sites called loxP sites (Sauer, 1998; Kuhn and Torres, 2002). A loxP site consists of two

inverted repeats 13 base pairs long each with an 8 base pair long spacer between them

(Sauer, 1998). The Cre recombinase recognizes these loxP repeats sites and binds to

them, so two Cre recombinase enzymes bind each loxP site (Kuhn and Torres, 2002).

Once bound, the recombinase cuts the double stranded DNA at the loxP site so that the

spacer (nonpalindromic 8 base pair sequence) is deleted (Ghosh and Van Duyne, 2002).

The Cre recombinase then joins the remaining DNA ends together.

Flanking a specific gene by loxP sites (floxing the gene) allows the gene to be

removed by Cre recombinase (**Figure-3**). Mice that have Cre recombinase expressed in a

specific tissue (under the control of a tissue-specific promoter; Sauer, 1998) (**Figure-3**,

upper left) are bred with mice that have a target gene flanked by loxP sites (**Figure-3**,

upper right) (Kuhn and Torres, 2002). The offspring will express Cre in the desired

tissue which will remove the targeted gene and join the DNA ends together (lower center)
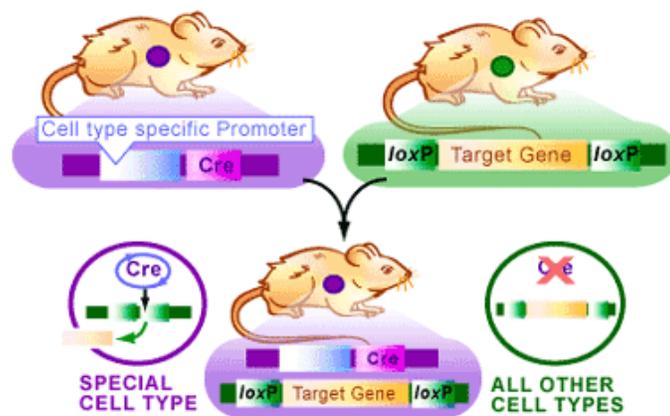
(Ghosh and Van Duyne, 2002).

Figure 3: Diagram of the Cre/loxP Recombinase System For Gene Knockouts in Breeding Mice. Mice that have a Cre recombinase expressed in a specific tissue (upper left) are bred with mice that have a target gene flanked by loxP sites (upper right). The offspring will express Cre in the desired tissue which will cut the DNA at the loxP sites to remove the target gene and rejoin the DNA ends. (Source: http://www.scq.ubc.ca/targeting-your-dna-with-the-crelox-system/)

PROJECT PURPOSE

As discussed in the Background, glucose response protein-78 (GRP78) is an ER chaperone activated during the ER stress response that helps prevent the inhibition of AKT kinase activation in the insulin signaling pathway, and helps alleviate the stress by increasing ER protein folding. In adipocytes, GRP78 production has been associated with helping alleviate ER stress resulting from high fat diets (Hotamisligil, 2010). We hypothesize that GRP78 helps alleviate ER stress and prevent insulin resistance in adipose tissue. The purpose of this project is to generate a mouse line with the GRP78 gene deleted in adipocytes to study the role of adipose tissue GRP78 in insulin resistance.. Previously constructed mice completely lacking GRP78 in heart tissue died early during development, so to study a decrease in GRP78 in that tissue mice had to be created that were heterozygous for the GRP78. This study focused on knocking out GRP78 in adipose tissue where the mice might remain viable even when containing a homozygous knockout (KO) of GRP78. The KO will be achieved using the Cre recombinase system. Mice hemizygous for the Cre recombinase gene under the control of a tamoxifen-inducible adipose-specific adiponectin promoter (Ad-Cre mice) (that express Cre recombinase in adipocyte tissue in mice treated with Tamoxifen) will be bred with mice heterozygous for floxed GRP78 to create a subset of pups with the desired genotype (hemizygous for Ad-Cre, homozygous for floxed GRP78). Polymerase chain reaction (PCR) for Ad-Cre and for GRP78 will be performed on tail snip DNA to identify positive pups. Future experiments will treat the mice with tamoxifen to induce the knockout in adipose tissue, will isolate adipose tissue from F2 pups to verify the

homozygous GRP78 KO in that tissue, and will use the mice to determine the importance

of GRP78 on insulin resistance, metabolism, and diabetes.

METHODS

Mouse Strains

Mice containing Cre recombinase under control of adiponectin were obtained from Evan Rosen at Harvard Medical School. Mice containing GRP78 flanked by loxP sites were obtained from Amy S. Lee at the University of Southern California, Keck School of Medicine.

Mouse Breeding/Cre Recombinase System

The Cre/loxP recombinase system was set up to create a mouse line containing a knockout for GRP78 in adipocyte tissue based on previous Cre procedures (Sauer, 1998). For this project, the Cre recombinase gene is under the control of the adipocyte-specific promoter adiponectin (Ad). The promoter is induced by tamoxifen, so the knockout can be induced at any age of development. The Ad-Cre mice were bred with homozygous mice containing the GRP78 gene flanked by loxP sites. Four cages were set up containing a male and female pair in each on January 11, 2013. For the first cage, the female was ear tagged #3585 and the male was #3314; the second cage, the female #3581 and the male #3577; the third cage, the female #3582 and the male #3744; and the fourth cage, the female #3583 and the male #3414. The dates of birth of the offspring were: first cage January 31, 2013; second cage February 2, 2013; third and fourth cage January 31, 2013. In total, all four cages produced 7 male pups in the F1 generation: #'s 4365-4371. The pups had their tails snipped at the ends for lysing and performing PCR to determine their genotype.

Parent mice in the F0 were crossed to obtain pups in the F1 generation. Further studies will cross the F1 generation to obtain positive results in the F2 generation, which would contain the Ad-Cre allele and be homozygous for the GRP78 floxed allele. The F0 generation consisted of male mice (3314, 3577, 3744, and 3414) containing the homozygous GRP78 allele and female mice (3585, 3581, 3582, and 3583) expressing Cre recombinase under the adiponectin promoter. The resulting F1 progeny sought in this project are homozygous floxed GRP78 mice that are Ad-Cre-positive.

Hot Alkaline Lysis Preparation of DNA Lysates from Tail Sections

This procedure was performed to obtain genomic DNA from mouse tail snips for use in PCR. Tail snips about 3 mm thick were prepared from Ad-GRP78 potential positive male mice labeled 4365-4371. The snips were placed in 1.5 mL eppendorf tubes for a total of 7 tubes. 75 µL of alkaline lysis reagent (25 mM NaOH, 0.2 mM Na$_2$EDTA) was added to each tube and placed in a 95°C dry bath for 30-45 minutes, until the tails were mostly dissolved. The tubes were then placed on ice for 5 minutes. Afterwards, 75 µL of neutralizing agent (40 mM Tris-HCl pH 7.5) was added to each tube to stop the alkaline lysis reaction. The tubes were then vortexed and centrifuged at 10,000 rpm for 2-3 minutes to pellet any undissolved tissue. The supernatant was transferred to new tubes and stored at -20°C until PCR.

PCR Genotyping

PCR was used to ensure that the parent mice had intact floxed GRP78 alleles throughout the body (heart and adipose tissue) that can be passed on to the F1 pups and to

assay whether the Grp and Cre genes were both present in the pups. DNA samples from the parent tail snips were subjected to myosin heavy chain- Cre PCR (MHC-Cre) and adiponectin- GRP78 (Ad-GRP78) PCR. Pup DNA samples were subjected to Ad-Cre and Ad-GRP78 PCR.

Two PCR protocols were utilized for all 7 pup DNA samples: adiponectin-Cre (AdCre) and floxed GRP78. The DNA from the tail snips was analyzed for the presence of Ad-Cre and GRP78 genes. The target mice must contain Ad-Cre and homozygous floxed GRP78 (F/F) allele in their genotype.

For the Ad-Cre protocol, Primers 1 (5-'GGT CAG CCT AAT TAG CTC TGT-'3), 2 (5'-GAT CTC CAG CTC CTC CTC TGT C-'3), 3 (5'-AGC GAT GGA TTT CCG TCT CT-'3), and 4 (5'-CAC CAG CTT GCA TGA TCT CC-'3) were utilized. Primers 1 and 2 would bind to the WT allele while primers 3 and 4 would attach to the Ad-Cre allele. 18 µL of Master Mix (2x Go Taq Green, Primers 1, 2, 3, and 4, and distilled water) was added to each of the 8 PCR tubes. 2 µL of DNA from each of the 7 different DNA samples (4365-4371) were added. A negative control sample was prepared by the replacement of DNA with 2 µL of water in the 8th PCR tube. Conditions for PCR were: 3 minutes 94°C (initial denaturation), and then 34 cycles of 30 seconds at 94°C, 1 minute at 61°C, and 1 minute at 72°C. This was followed by a polishing step for 2 minutes at 72°C, and then 4°C and stored in similar temperature conditions.

For the GRP78 protocol, upstream primer PF3:3'-CGC CAT CCC AAC TGG TAT-'5 and downstream primer PR3: 3'-GAT GAG GCG TCT TGA AAG-'5 were utilized. 9 µL of Master Mix (2x Go Taq Green, Primers PF3 and PR3, and distilled water) was added to each of the 8 PCR tubes. 1 µL of DNA from each of the 7 different

DNA samples (4365-4371) were added. A negative control sample was prepared by the replacement of DNA with 1 µL of water in the 8th PCR tube. Conditions for PCR were: 5 minutes at 95°C (initial denaturation), then 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C. The reaction was incubated for 5 minutes at 72°C for a polishing step, then at 4°C until taken out and stored at similar temperature conditions.

Parent mice DNA samples were subjected to an MHC-Cre (myosin heavy chain [heart-specific] cre recombinase) PCR in coincidence with the GRP78 PCR. For the MHC-Cre protocol, upstream primer MHC-Cre forward: 3'- GCA TTA CCG GTC GAT GCA ACG AGT GAT GAG -'5 and downstream primer MHC-Cre reverse: 3'GAG TGA ACG AAC CTG GTC GAA ATC AGT GCG -'5 were utilized. 9 µL of Master Mix (2x Go Taq Green, forward and reverse primers, and distilled water) was added to each of the PCR tubes along with 1 µL of DNA from each of the large pool of parents. A negative control sample was prepared as before by the replacement of DNA with 1 µL of water. Conditions for PCR were: 3 minutes at 95°C (initial denaturation), then 30 seconds at 95°C, 40 seconds at 58°C, 40 seconds at 72°C for 35 cycles. The reaction was incubated for 5 minutes at 72°C for a polishing step, then at 4°C until taken out and stored at similar temperature conditions.

Gel Electrophoresis/UVB Imaging

PCR amplicons were analyzed by agarose electrophoresis. 1% agarose gels were prepared, for the AdCre, GRP78, and MHC-Cre reactions. 1 gram of agarose was added to 50 mL of 1X TAE buffer and microwaved until completely dissolved with frequent stirring. 5 µL of ethidium bromide was added, and the mixture was poured into a mold

with casting combs. The gels were allowed to cool for about 20 minutes. The casting

combs were then removed. PCR reaction sample was loaded onto each lane. The gels

were electrophoresed at 100V for about 45 minutes (Ad-Cre and MHC-Cre) or for 1.5 hrs

(GRP78). The amplicons were visualized using a UVB imaging device.

RESULTS

Unlike in heart tissue, the complete deletion of the GRP78 allele (GRP78 F/F) in adipose tissue induced during adult life does not compromise normal growth and development in mice (Luo et al., 2006). Parental mice must be homozygous for the floxed GRP78 allele to ensure its continued presence in subsequent generations. This was assessed in the F0 generation using GRP78 PCR and MHC-Cre PCR. GRP78 PCR is indicative of the genotype of the allele throughout the body. One parent must contain two floxed GRP78 alleles while the other must contain one GRP78 floxed allele and a Cre allele. This will ensure maximum yield of F1 pups with floxed GRP78 alleles. The second parent is heterozygous for the floxed allele because it also must contain a Cre allele. In order for the mice to grow past their embryonic stages, GRP78 cannot be completely deleted in either adipose or heart tissue. Therefore, after testing the genotype of GRP78 in both adipose and heart tissue using GRP78 PCR, a MHC-Cre PCR was also conducted that could verify the presence or absence of the Cre allele. To ensure the floxed GRP78 gene is passed on to the F1 generation, and to ensure the mice do not die early in the embryonic stages of life, parental mice must be homozygous for the floxed GRP78 allele and must lack the Cre allele (otherwise the GRP78 is removed and the mice die). In contrast, parents that were heterozygous for the floxed GRP78 allele must contain the Cre allele to ensure that the Cre gene is successfully passed on to the F1 pups.

Unlike the Ad-Cre PCR which is specific to Cre in adipose tissue and was performed on DNA sample of the F1 pups, MHC-Cre is specific to heart tissue and was performed on the parents. Ad-Cre PCR has two sets of forward and reverse primers.

Primers 1 and 2 would attach to the WT allele while primers 3 and 4 would attach to the Cre allele in adipose tissue. The Cre allele is hemizygous and as a result, when the Ad-Cre amplicons are run on a gel, DNA samples positive for the Cre allele would contain two bands, the WT and the Cre allele. This is because the Ad-Cre gene has its own engineered primers that render the PCR protocol site specific.

In contrast, the MHC-Cre PCR can indicate the presence or absence of the Cre allele in the heart tissue. Since the parent generation were needed for breeding purposes that would potentially lead to successful GRP78 knockout in adipose tissue it was necessary that the MHC-Cre allele be absent in the parent generation. To produce the target F1 pups, a cross was set up between male mice homozygous for the floxed GRP78 allele (F/F) and absent of the MHC-Cre allele with female mice heterozygous for floxed GRP78 and hemizygous for the Ad-Cre allele.

The target F1 mouse must contain the hemizygous Ad-Cre allele to allow the KO to be induced only in adipose tissue, and only under tamoxifen stimulation. This will allow a study of Endoplasmic Reticulum (ER) stress in adipocyte tissue. Induction with tamoxifen will generate the transgenic mouse line knocked out for the GRP78 allele in adipose tissue. ER stress studies could then be performed on these mice via hyperinsulinemic-euglycemic clamps. In addition to the production of mouse models for ER studies, F1 pups lacking the Ad-Cre gene can be utilized to setup F2 crossings to once again produce the target mouse model. Therefore, mice homozygous for the floxed GRP78 allele and simultaneously lacking the Ad-Cre allele (F/F) can be saved and crossed with mice homozygous for the floxed GRP78 allele and hemizygous for the Ad-Cre allele (F/F, Cre).

Five weeks after the parent generation were bred, the F1 generation pups were weaned. The females were then euthanized because hormonal cycles in females may affect metabolism and generate construed results. Seven male pups were produced which were ear tagged for identification purposes (4365-4371). The genotypes were performed on endothelial cells retrieved from individual tail clips. DNA was extracted from these tail clips via hot alkaline lysis and amplified via PCR for Ad-Cre (**Figure-4**) and for floxed GRP78 (**Figure-5**).

Figure 4: PCR Analysis for the Presence of the Ad-Cre Gene. Lane 1 contains the DNA ladder, lanes 2- 8 represent DNA samples from mouse pups 4365-4371, and lane 9 is the WT control. The Ad-Cre gene was positively present in 3 of the 7 pups tested: mouse pups 4366, 4368, and 4369 (boxes). Positives are identified by the presence of two bands: one at the wild type (WT) position of ~900 bp and the Ad-Cre around 200 bp lower at 700 bp. The Ad-Cre DNA results in two bands and is subsequently thinner than the single WT band**.** The amplicons were analyzed on 1% agarose gels.
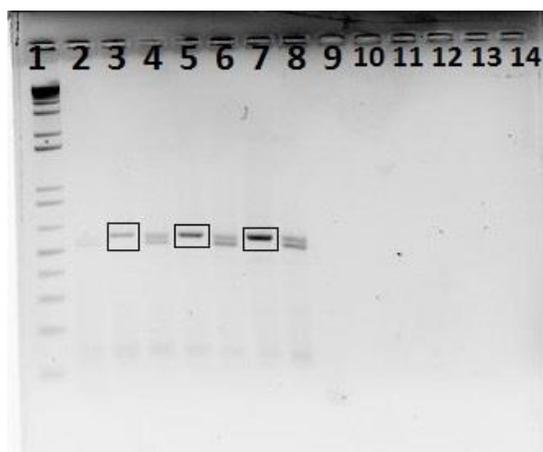
Figure 5: PCR Analysis for Floxed GRP78. Lane 1 contains the DNA ladder, lanes 2-8 represent DNA samples from 7 mouse pups 4365-4371, and lane 9 is the control. Pups 4366, 4368, and 4370 (boxes) contain two floxed GRP78 alleles (homozygous). Positives are identified as the single upper band at 568 bp. Mouse pups 4367, 4369, and 4371 were all heterozygous for floxed GRP78; the upper band at 568 bp represents floxed GRP78, and the lower band at 524 bp represents WT GRP78. The genotype of 4365 is WT.

Table-I shows a summary of the PCR results for seven pups. Three of the seven pups were positive for Ad-Cre (4366, 4368, 4369), two pups were homozygous for floxed GRP78 (4366, 4368, 4370), and 3 were heterozygous for GRP-78 (one allele WT, one allele floxed) (4367, 4369, and 4371). Thus, only two pups (4366 and 4368) were of the desired genotype, positive for both Ad-Cre and homozygous for floxed GRP78. In addition, one mouse pup 4370 was homozygous for floxed GRP78 and lacked the Ad-Cre allele, and will be bred with a female mouse homozygous for the floxed GRP78 allele and hemizygous for Ad-Cre to continue production of positive mouse models in the F2 generation.

Table-I: Summary of F1 PCR Data

Mouse Pup	Ad-Cre	GRP-78
4365	WT	WT
4366	**Ad-Cre**	**F/F Homozygous Floxed**
4367	WT	Heterozygous floxed and WT
4368	**Ad-Cre**	**F/F Homozygous Floxed**
4369	**Ad-Cre**	Heterozygous floxed and WT
4370	WT	**F/F Homozygous Floxed**
4371	WT	Heterozygous floxed and WT

Table-II shows a summary of the crossings of the parent F0 generation that resulted in the F1 generation. Four different breeding cages were set up that crossed a male homozygous for the floxed GRP78 allele with a female heterozygous for the flox GRP78 allele and hemizygous for the Ad-Cre allele in both adipose and heart tissue. Four cages were setup to increase the likelihood of successful mouse models since female mice were automatically eliminated from the screen. The strains were genotyped via PCR and gel electrophoresis on tail snip DNA (**Figures 6-10**).

Table-II: Summary of Parental Mice PCR Data

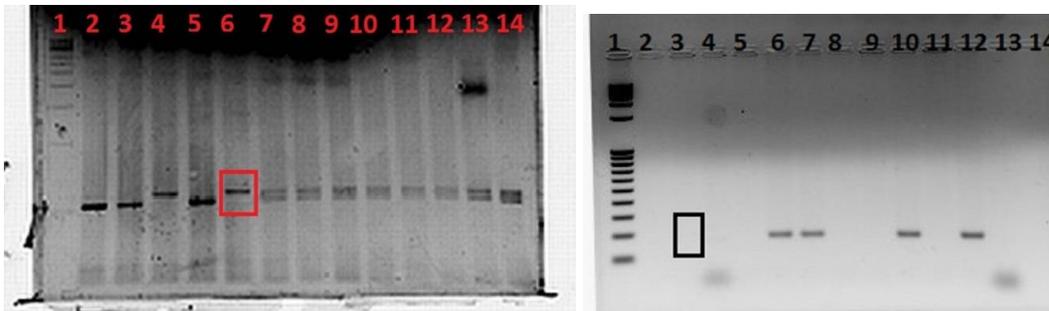| Mouse #	Sex	Strain	Date of Birth	Genotype	Set up Date
3314	M	GRP78 F/F	9/16/2012	F/F	1/11/2013
3585	F	AdCre GRP78	10/18/2012	F/+, Cre	
3577	M	GRP78 F/F	10/15/2012	F/F	1/11/2013
3581	F	AdCre GRP78	10/18/2012	F/+, Cre	
3744	M	GRP78 F/F	11/19/2012	F/F	1/11/2013
3582	F	AdCre GRP78	10/18/2012	F/+, Cre	
3414	M	GRP78 F/F	9/24/2012	F/F	1/11/2013
3583	F	AdCre GRP78	10/18/2012	F/+, Cre	

Figure 6: PCR Analysis of Parental Mouse 3744 for Floxed GRP78 (left) and MHC-Cre (right). Lane 6 of the left image (boxed) shows a positive signal for homozygous floxed GRP78 PCR for DNA from mouse parent #3744. Lane 3 of the right image (boxed) shows the lack of a positive band for MHC-Cre. Thus, mouse #3744 is homozygous for the flox allele (GRP78 F/F) and simultaneously lacks the Cre allele.
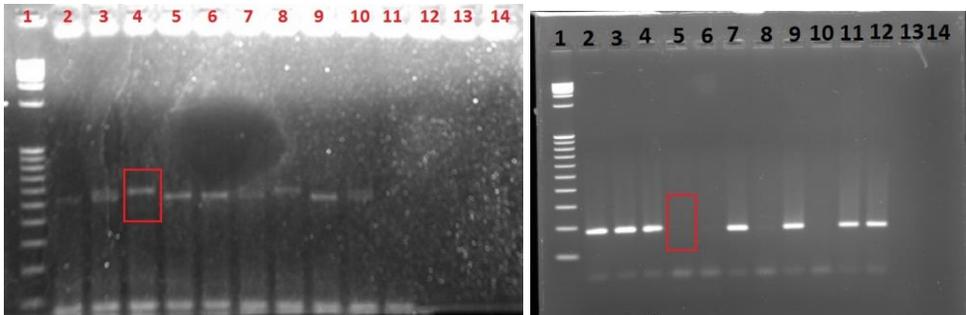

Figure 7: PCR Analysis of Parental Mouse 3414 for Floxed GRP78 (Left Panel) and MHC-Cre (Right Panel). Left Panel: Lane 4 (boxed) contains a homozygous floxed GRP78 PCR DNA sample from mouse parent # 3414. **Right Panel:** Lane 5 (boxed) contains a DNA sample from mouse parent # 3414, and shows no signal. Thus, mouse #3414 is homozygous for the flox allele (GRP78 F/F) and simultaneously lacks the Cre allele.
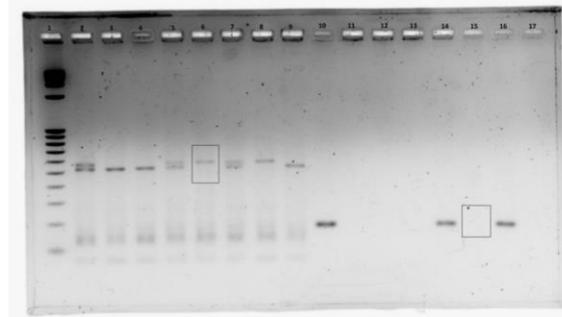

Figure 8: PCR Analysis of Parental Mouse 3314 for Floxed GRP78 (left half) and MHC-Cre (right half). Lane 6 left (boxed) shows a positive signal for homozygous floxed GRP78. Lane 15 shows a negative signal for MHC-Cre. Thus, mouse 3314 is homozygous for the flox allele (GRP78 F/F) and simultaneously lacks the Cre allele.
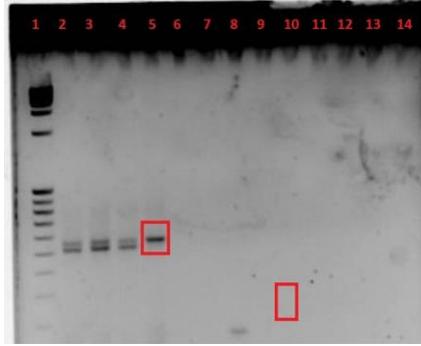
Figure 9: PCR Analysis of Parental Mouse 3577 for Floxed GRP78 (left half) and MHC-Cre (right half). Lane 5 (boxed left side) shows a positive signal for homozygous floxed GRP78. Lane 10 (boxed right side) shows a negative signal for MHC-Cre. Thus, mouse 3577 is homozygous for the flox allele (GRP78 F/F) and simultaneously lacks Cre.
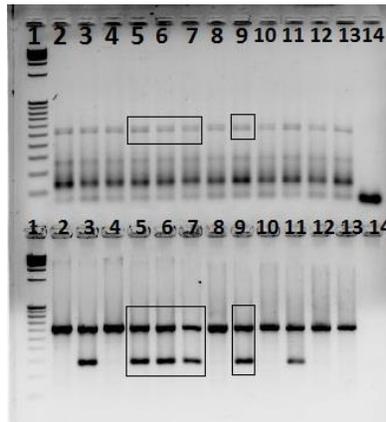

Figure 10: PCR Analysis for Floxed GRP78 (upper gel) and Ad-Cre (lower gel). Lanes 5, 6, 7 and 9 of the top row contains DNA for mouse pups 3581, 3582, 3583 and 3585, and shows heterozygous signals for floxed GRP78. Lanes 5, 6, 7 and 9 of the lower gel show the same pups as positive for Ad-Cre. Thus, the four mice are hemizygous for the Cre allele and heterozygous for floxed GRP78 (F/+).

DISCUSSION

The collected data shows that three mouse pups (4366, 4368, and 4370) out of the seven pups produced were homozygous for the floxed GRP78 allele. Two of them (4366 and 4368) also were positive for Ad-Cre, while the remaining pup (4370) was not. This indicates that two mice were of the desired genotype, and once induced should contain a knockout of GRP78 in adipose tissue. Although 4370 lacked Cre, it may still be used to yield positive results in a future F2 generation because the male homozygous for floxed GRP78 allele (4370) can be crossed with a female homozygous for floxed GRP78 allele that is hemizygous for the Cre allele.

The F0 or parent generation had male mice homozygous for the floxed GRP78 allele and female mice heterozygous for the floxed GRP78 allele and hemizygous for the Cre allele. When the cages were set up for breeding, the following male and female pairs were crossed: #3314 with #3585, #3577 with #3581, #3744 with #3582, and #3414 with #3583. It was predicted that 25% of the F1 progeny would be homozygous for the floxed GRP78 allele, 25% would be heterozygous for the floxed GRP78 allele, 25% would be homozygous for the floxed GRP78 allele and hemizygous for the Cre allele, and the 25% would be heterozygous for the floxed GRP78 allele and hemizygous for the Cre allele. The target of this study is the 25% of the mice that are homozygous for the floxed GRP78 and hemizygous for Cre. However, of all the mice pups born in the F1 generation, only the male mice were obtained because female mice undergo the estrous cycle which can affect metabolism and result in inconsistent results. The genotypes of the parent F0 mice were shown in **Figures 6-10** and summarized in **Table-II**.

The GRP78 gene is one that occurs naturally in the studied mouse line. The Cre allele, however, is a genetically engineered allele that is added into an organism of choice if a specific gene is targeted for deletion, which in this study's case is GRP78. To do this the GRP78 must be also be genetically engineered to be flanked by two lox p sites on either side of the allele.

GRP78 is known to be vital for survival during the embryonic stages of mouse growth (Luo et al., 2006). Therefore, one or both of the GRP78 alleles must be present in the heart and adipose tissue of the mice at all times during early stages of growth. Complete GRP78 knockout can however be generated in the adipose tissue of adult male mice without causing lethality. In addition, heterozygous knockout of the GRP78 allele in heart tissue can be generated to study 50% deletion of the gene and the subsequent diabetic side effects studied accordingly. The focus of this study was to generate GRP78 knockout in adipose tissue (Luo et al., 2006).

To create these mice models the parent mice must be comprised of a male that is homozygous for the floxed GRP78 allele (lacking Ad-Cre) and a female heterozygous for the floxed GRP78 allele and hemizygous for the Ad-Cre allele. To verify these genotypes in the parent mice MHC-Cre and GRP78 PCR were conducted and run through a gel. The GRP78 PCR was done on both the male and female parent mice to verify whether the floxed allele is heterozygous or homozygous. An MHC-Cre (Myosin Heavy Chain) PCR was also run to screen for the presence of the MHC-Cre allele that has been genetically engineered in the mice. MHC-Cre is a PCR that is run to check whether the Cre allele is present in the heart tissue.

The female parent mice of the F0 generation were heterozygous floxed for the GRP78 allele and hemizygous for the Ad- Cre allele. This is necessary to ensure that the F1 generation of mice will have the Cre allele present in adipose tissue because the Cre allele present in the female parent mice is Ad-Cre (specific to adipose tissue). The male mice lacked the MHC-Cre (specific to heart tissue) allele to ensure that the F1 pups only contained Ad-Cre activity.

The Cre alleles are engineered to be recognized by different tissue-specific promoters in heart and adipose and hence amplification of the gene requires different protocols. In adipose tissue, the gene is attached to the promoter activity adiponectin and is additionally under control of the estrogen receptor (ER) which is induced by tamoxifen, an estrogen analog. In heart tissue, the Cre gene is attached to transcriptional machinery found in cardiomyocyte that recognize Myosin Heavy Chain (MHC) promoter and transcribe the Cre recombinase without any intervention.

The results verified that the MHC-Cre allele is not present in any of the parent mice and consequently ensured that the male F0 generation will not pass on an Ad-Cre gene to the F1 generation. Instead, the Cre allele will be passed on to the F1 pups from the female F0 parent mice that contain Ad-Cre. Expression of the Cre allele specific to adipose tissue (Ad-Cre) is driven by treating the mice with Tamoxifen. Recombinase protein can then cut out GRP78 (F/F) and generate positive mouse models- knockouts of GRP78 in adipose tissue.

When performing PCR for floxed GRP78, there are three possible results: 1) homozygous floxed GRP78 (loxP in both alleles; both GRP78 amplicons would be of the larger 560 bp size), 2) homozygous WT (no loxP sites flanking GRP78; both GRP78

amplicon bands are 520 bp), or 3) heterogygous floxed GRP78 (one band at 560 bp and a second band at 520 bp).   When performing the Ad-Cre PCR, the presence of a band at 700 bp indicates the presence of Cre.

The purpose of the F2 generation is to optimize the yield of mice homozygous for the floxed GRP78 allele and hemizygous for the Cre allele. Since two of the seven mice (#4366 and #4368) had this genotype, they can be bred with a female that is only homozygous for the floxed GRP78 allele to obtain 50% of F2 progeny that are homozygous for the floxed GRP78 allele and Cre negative, and 50% homozygous for the floxed GRP78 allele and hemizygous for the Cre allele.

One F1 mouse (#4370) was homozygous for the floxed GRP78 allele, but lacked the Cre allele, so it can be crossed with a female mouse that is hemizygous for the Cre allele to create a 50-50 chance in the F2 progeny as obtained by using mice #4366 and #4368. As a result, three mouse pups were successfully bred in the F1 generation to be used in breeding for the F2 generation to obtain the desired genotype.

One problem encountered in this project was the low yield from the breeding, producing only seven F1 pups, of which two had the exact desired genotype, and three had a useful genotype. In the future, several breeding pairs might be required to generate a sufficient number of F1 positives to do tamoxifen experiments. This project successfully produced three useful mice, demonstrating the feasibility of the approach, but more work needs to be done in the future to generate more mice, and to study the effects of a decreased GRP78 expression in adipose tissue on ER stress and insulin resistance.

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