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GENERATION OF TRANSGENIC MICE WITH A HEART-SPECIFIC DELETION OF THE JNK-1 & 2 GENES USING MHC-CRE

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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April 26, 2012

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ABSTRACT

The purpose of this project was to generate a mouse line containing a heart-specific deletion of genes encoding JNK-1 and -2, which may map to insulin-resistance, to create a model for studying the effects of type II diabetes on cardiomyocytes. The Cre/loxP recombination system was used in a C57BL/6 mouse, with Cre under the control of a MHC promoter to drive expression in cardiomyocytes. PCR analysis showed the creation of 3 Cre-positive mice for JNK1, and 3 for JNK2, providing mice which can be used to determine the potential role of JNK1 and JNK2 in insulin-resistance in heart cells.

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ACKNOWLEDGEMENTS

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BACKGROUND

Diabetes

Overview

Diabetes originates from the term Diabetes Mellitus; diabetes is a Greek word which means to siphon, referring to one of the most obvious symptoms of the disease, excessive urination. Mellitus is Latin, meaning "sweet like honey", and refers to an anonymous physician who upon tasting the urine of a diabetic concluded it had a sweet taste (Lundstrom and Rossini, 2004). Diabetes affects nearly 300 million people worldwide and is one of the leading causes of death in the most developed countries (Diabetes Atlas 2011). Diabetes is a disease resulting in glucose dys-regulation from a set of failures in metabolism relating to insulin and the cellular uptake of glucose.

General Description

Diabetes is a chronic disease in which the human system fails to maintain glucose homeostasis, resulting in high blood sugar levels from an absence or deficiency of insulin in the system, or an inability of the body to respond to it. Insulin is the main hormone that stimulates the conversion of sugar obtained from food into energy. In the absence or deficiency of this hormone (Type I diabetes, discussed below), glucose is unable to enter essential target tissues, resulting in hyperglycemia. When serum glucose levels increase, there is a flight or fight response by the body causing symptoms such as nausea and jitters (Marieb, 2002). During this fight or flight response, the body makes excess glucose to prepare for survival, and this excess glucose is expelled by urination from the body in a process called glycosuria (Marieb, 2002).

As a result of decreased insulin, there is a deficiency of cellular sugars needed for metabolism. Hence, fat production is activated to provide energy, and this state of very high levels of fatty acids is known as lipidemia (Marieb, 2002). The products of fat metabolism are known as ketone bodies, and they are very strong organic acids. The accumulation of these compounds in the serum leads to their excretion in the urine. In severe cases, known as ketonuria, it leads to a drop in the blood pH. This in turn leads to an occurrence known as ketoacidosis. Prolonged cases can be fatal as the low pH disturbs heart activity, transport of oxygen, and functioning of the nervous system (Marieb, 2002).

If left untreated or undiagnosed, hyperglycemia has complications that affect every organ in the body (Staff, 2012). The three main signs of diabetes are polyuria, polydipsia, and polyphagia. Polyuria (increased urine) results when the body reacts to excessive serum glucose in the kidneys (Marieb, 2002). As a result of polyuria, there is also a large loss of ketones and electrolytes in the urine, causing an electrolytic imbalance. This leads to Polydipsia (excessive thirst) from a dehydration of the hypothalamic thirst centers, and to Polyphagia (excessive hunger) (Marieb, 2002).

Main Types of Diabetes

Type I Diabetes Mellitus (TID)

There are two major forms of diabetes mellitus: type I and type II. Type I diabetes, also known as Insulin-dependent diabetes mellitus (IDDM), formerly called juvenile onset diabetes, which usually occurs before the age of fifteen (Marieb, 2002). Type I results when the pancreatic β-cells of the Islets of Langerhans that generate insulin are destroyed by the body's immune system, so the body cannot produce insulin. Insulin normally binds to insulin receptors

on the surface of cells to upregulate signal transduction pathways that increase the presence of glucose transporters (GLUT) on the cell surface. The GLUTs bind glucose releasing it in the cytoplasm, lowering serum glucose levels (**Figure-1**).

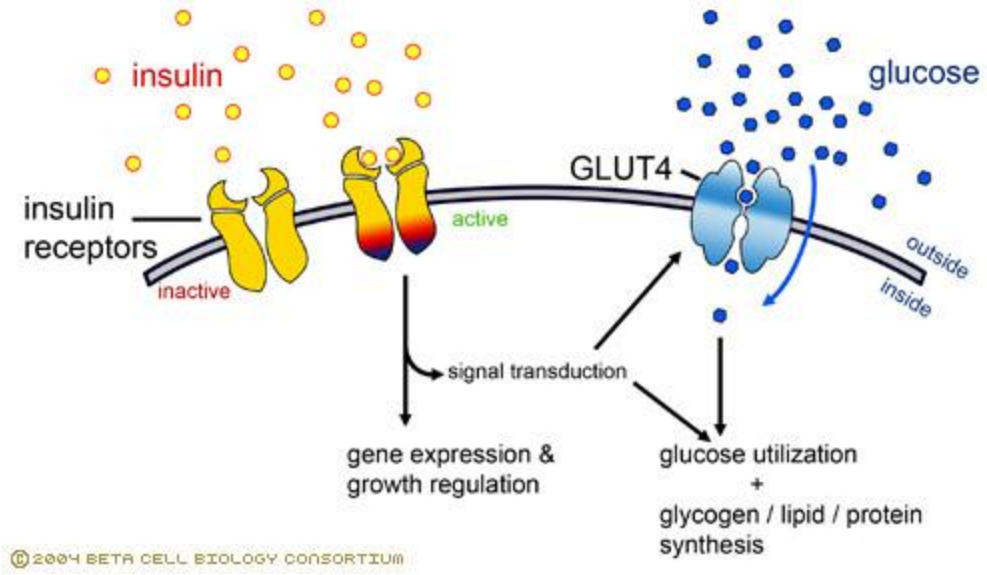

Figure 1: Diagram of Insulin-Mediated Glucose Uptake. Serum insulin binds to the insulin receptors on the cell surface to induce a signal transduction cascade which upregulates GLUTs on the cell surface to transport glucose into the cytoplasm (Beta Cell Biology, 2012).

The cause of Type I diabetes remains unknown, but some factors such as genetics, the status of the immune system, and the environment, can affect susceptibility (Lundstrom and Rossini, 2004). Although less than 20% of the cases of IDDM have family history, there are some forms with familial correlations. For instance, if one of the parents has this form of Type I, there is a 7% chance that the child will also contract it. With identical twins, there is a 30-50% chance if one has it the other will too (Lundstrom and Rossini, 2004). Environmental factors and viruses such as Rubella, mumps and Coxsackie virus, are also associated with Type I. IDDM

can also develop when the immune system is misdirected to attack its own β-cells (**Figure-2**) (Lundstrom and Rossini, 2004).

In addition to the main symptoms of diabetes discussed above (polyuria, polydipsia, and polyphagia), other the symptoms of Type I include significant and sudden weight loss, slow-healing wounds, recurrent infections, and blurred vision (Diabetes Atlas, 2011).

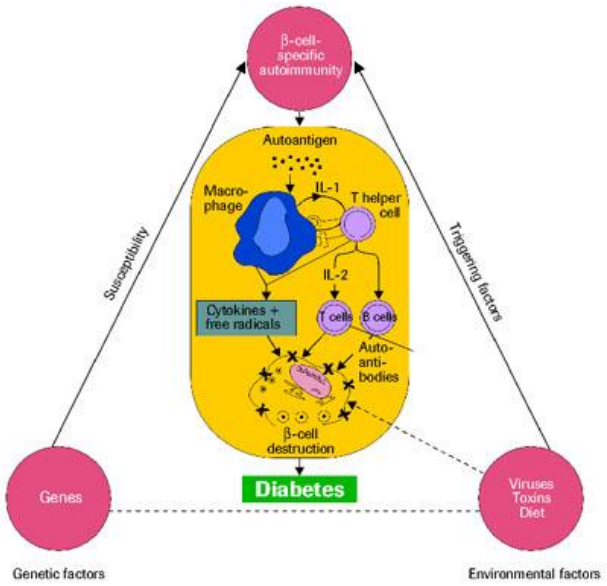

Figure 2: The Autoimmune Response in Type I Diabetes. As a result of either genetic or environmental factors, auto antigens in β-cells are recognized by the body's cytotoxic T cells which cause β-cell destruction (Roche Diagnostics, 2012).

Type II Diabetes Mellitus (NIDM)

The more common form of diabetes is known as non-insulin-dependent diabetes mellitus (Type II), and usually occurs in individuals after the age of 40; hence it was formerly regarded as adult-onset diabetes (Marieb, 2002). However, there is recently an increase in Type II cases in children and adolescents. Type II results from an increased resistance to insulin; the β-cells still produce insulin, but the cell does not respond to the insulin; the signal transduction

pathways become de-sensitized and do not upregulate GLUT (**Figure-3**) (Marieb, 2002). Due to the lack of sensitivity to insulin, and lack of GLUT upregulation, there is a build-up of glucose in the blood (Lundstrom and Rossini, 2004).

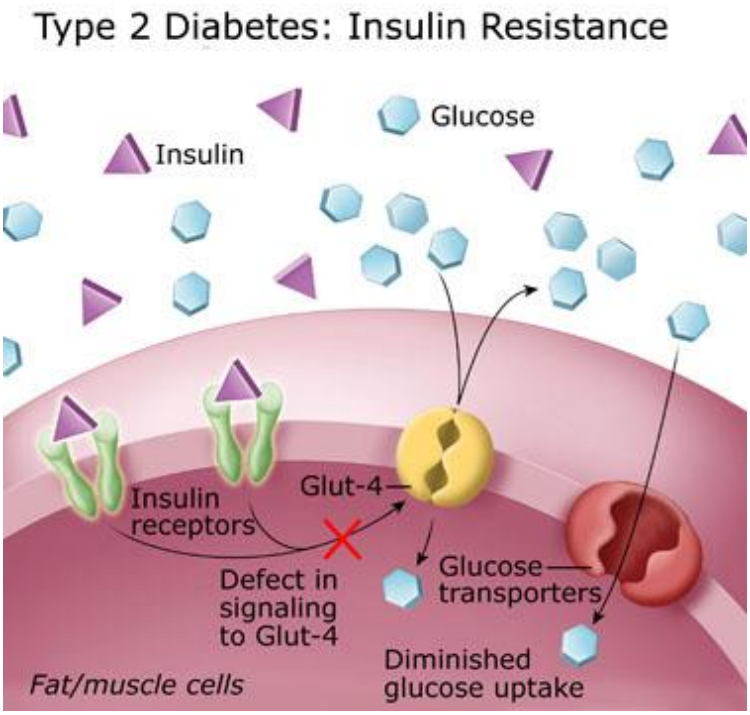

Figure 3: Mechanism of Insulin Resistance in Type II Diabetes. Prolonged cell stress or unknown factors lead to a desensitization of the insulin and GLUT pathways, so GLUT is not increased on the cell surface and glucose is not internalized. (dtc.ucsf.edu)

Individuals with Type II may not be aware of their disease for a long period because the damage caused to the body by excess blood glucose may take a while to surface. Diagnosis is usually made only after complications become evident (Diabetes Atlas, 2012). Type II can be caused by stressors such as pregnancy, excessive weight gain, specific prescription drugs, or other illnesses. However, some factors that may lead to insulin resistance are hereditary (Lundstrom and Rossini, 2004). Some Type II risk factors include obesity, poor diet, physical

inactivity which leads to premature aging, a genetic history of the disease, ethnicity and poor nutrition during pregnancy which affects the embryo (Diabetes Atlas, 2012).

Prevalence

Diabetes affects close to 300 million people worldwide and is one of the leading causes of death in the world's most developed countries (Diabetes Atlas, 2011). It is estimated that 366 million people have developed diabetes in 2012 (**Figure-4**), and this number will rise by almost 50 percent in the next 20 years. About 10 percent of the population of the United States is affected by diabetes, and about 3 percent of these are unaware they have it (Diabetes Atlas, 2011). Diabetes complications consume about 15% of the U.S. health care budget, which is at least 465 billion dollars (Diabetes Atlas, 2011). Diabetes is the leading cause of non-traumatic amputations, end-stage renal diseases, and increased morbidity via cardiovascular disease (Cavellerano, 2005). In 2004, about four people suffered diabetic amputations daily in Louisiana alone (Webster, 2005). Diabetes caused 4.6 million deaths in 2011 (Diabetes Atlas, 2011). Diabetes is also the leading cause of blindness, and patients are 25% more likely to become blind than their non-diabetic counterparts (Cavellerano, 2005). Diabetes is undoubtedly one of the most challenging health problems of the 21st century.

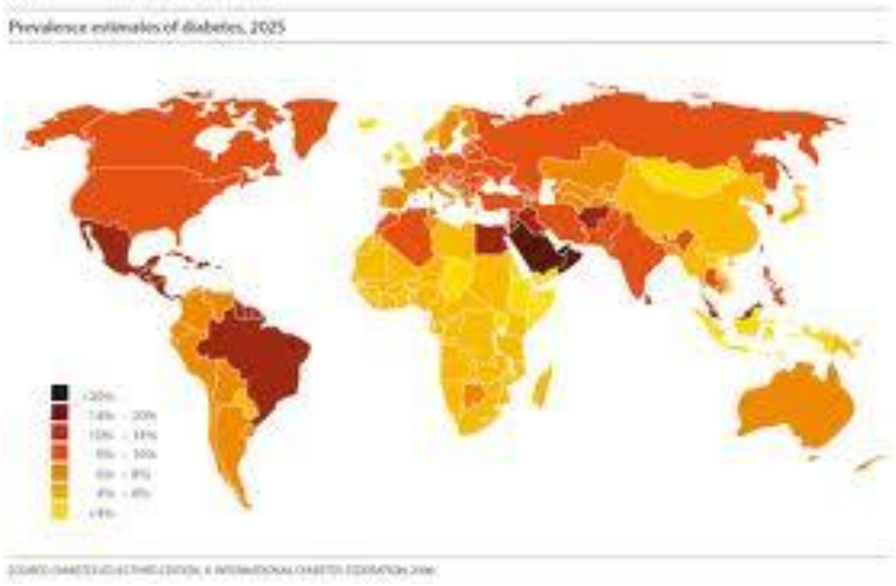

Figure-4: Prevalence Estimates of Diabetes by 2025. The darker the shade of yellow, the higher the prevalence estimate. (globalsherpa.org)

Treatment

Type I diabetes is chronic and lifelong, hence a lot of time and money is dedicated to treatment. The most common and effective treatment is insulin replacement therapy. An ideal form of therapy would be to implant a system capable of producing insulin as the pancreas normally does, however, this has yet to be perfected and has only been successfully achieved in mouse models. In insulin replacement therapy, subcutaneous injections of insulin, or insulin pumps, are used to increase serum insulin following a meal. The insulin is biomanufactured with the use of recombinant DNA technology from *E. coli* bacteria (Lidstone, 2005).

In type II diabetes, the body already produces insulin, so its treatment does not usually involve insulin injections. However, insulin may be prescribed in conjunction with exercise,

good diet, and other oral medications to form an adequate management technique (Marieb, 2002).

New research from Gray and Kim (2011) at the University of Massachusetts Medical School looks to investigate the impact of insulin-resistance on the diabetic heart. According to their findings, structural, metabolic, and cellular disorders can arise in the diabetic heart from type II diabetes. Structurally, Type II has been shown to damage the left ventricular wall of the heart, specifically through the thickening of the existing present cardiac tissue in the area. And metabolic changes have been documented in the heart, such as increased lipid oxidation and the accumulation of triglycerides in the myocardium of the heart. Cellular changes in the diabetic heart have been also shown to occur, including mitochondrial dysfunction by the increased uptake of lipid fatty acids, promoting the generation of reactive oxygen species.

The Cre Recombinase System for Mouse Knockouts

Cre recombinase is a protein about 38 kDa long, originally obtained from bacteriophage P1, which serves as a mediator for intramolecular and intermolecular site specific recombination between two or more loxP sites. When a particular gene is flanked by loxP sites (floxed), the binding of Cre recombinase to the sites results in an excision of the DNA intervening between them, which is subsequently ligated to leave one of the loxP sites behind. The system can be used to eliminate any particular gene that is floxed, so long as cre is expressed in that tissue. The Cre/loxP system is usually used to knock out normal chromosomal genes, but it can also be used to remove a transgene that has been overexpressed in specific tissues to study the effect of the downregulation.

One useful application of the Cre/loxP system involves two mouse strains bred to produce a deletion (**Figure-5**). One transgenic mouse line containing Cre targeted to a specific tissue is bred with another mouse that containing the floxed target gene. Recombination and knockout occurs in all the cells expressing Cre, while the target gene remains active in the cells and tissues that do not express Cre. The excising capability of Cre can also be useful in conditionally turning on foreign genes by removing stop sequences between the promoters and coding region of the transgene.

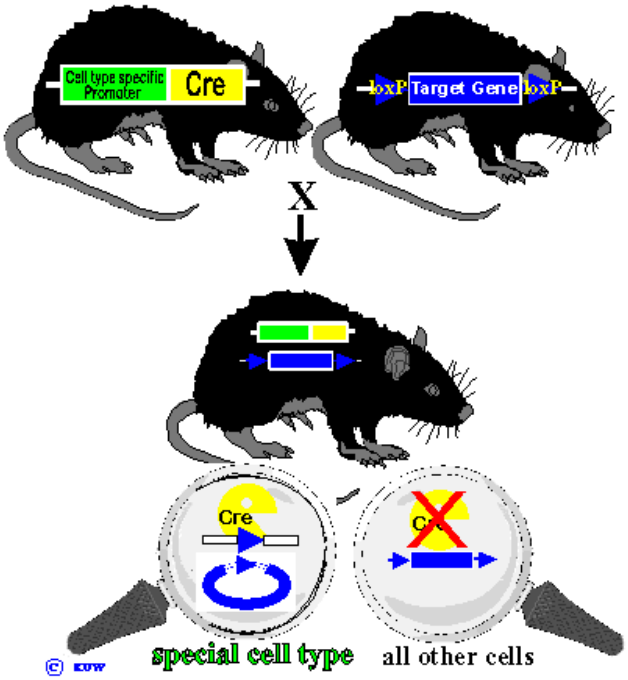

Figure 5: The Cre/loxP Recombination System in Transgenic Mice. One mouse strain expresses Cre recombinase (left) while the other strain contains the floxed target gene (right). Depending on the promoter used to drive the expression of the Cre, the offspring will express Cre in only a specific tissue, which will contain the knockout. (mammary.nih.gov).

In diabetes research, the Cre/loxP system has already proven useful for studying insulin resistance in specific tissues by knocking out the insulin receptor gene in a specific tissue to induce a cellular hypoglycemic state in that tissue. If expression of the Cre recombinase is driven by the muscle creatine kinase (MCK) promoter, the cre is expressed in skeletal muscle and cardiomyocytes. If the Cre is driven by the myosin heavy chain (MHC) promoter, the Cre is expressed mostly in cardiomyocytes. The "MHC-Cre mouse" has been used to study the effects of loss of the insulin receptor gene in mouse cardiomyocytes (Wagner, 1998), but has not yet been used to study the effects of deleting signal transduction genes related to insulin-resistance, such as JNK1 and JNK2, in the heart (see below).

MAPK/JNK Pathway

Mitogen-Activated Protein Kinases (MAPKs) are kinases activated as a result of extracellular stimuli. MAPKs are expressed in various cell types, including cardiomyocytes, vascular endothelial cells and vascular smooth muscle cells. c-Jun NH(2)-terminal protein kinases (JNKs), a type of MAPK, and are highly stimulated by environmental stresses and can be moderately stimulated by mitogens, inflammatory cytokines, oncogenes, and inducers of cell differentiation and morphogenesis (SABiosciences, 2010). All ten of the known JNK isoforms identified are encoded by three genes: JNK 1, JNK 2 and JNK 3. These JNK isoforms are very important in regulating stress responses, neural development, inflammation, and apoptosis (SABiosciences, 2010).

The most powerful inducers of JNK are stress or genotoxic agents. These forms of stress serve to mediate JNK activation via cellular pathways. Growth factors also initiate JNK activation, however the pathway that leads to this activation is not fully known. The MAPK/JNK-stress pathway is linked to various pathological conditions, including Type II

diabetes and insulin resistance (**Figure-6**), therefore a proper understanding of this pathway could lead to potential therapeutic intervention and manipulation methods of these diseases (SABiosciences, 2010).

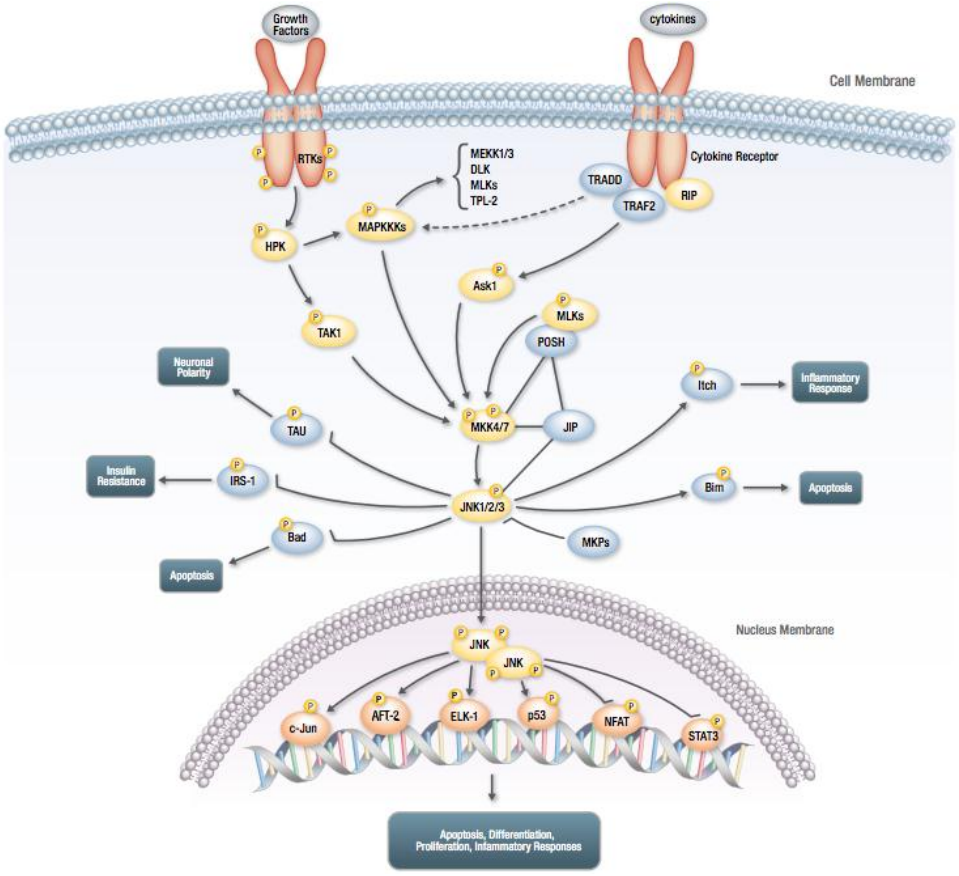

Figure 6: The MAPK-JNK Pathway is a Crucial Mediator of Insulin-Resistance in Type II Diabetes. MAPKs are a family or serine-threonine protein kinases that participate in a major signaling system by which cells transduce extracellular stimuli into intracellular responses. Both JNK1 and JNK 2 protein kinases have been shown to be involved in obesity-related metabolic regulation and insulin resistance (SABiosciences, 2010).

PROJECT PURPOSE

JNK1 and JNK2 are kinases that function in the MAPK proinflammatory pathway, and are important for mediating cellular stress and insulin-resistance in Type II diabetes. In this project, the JNK1 and JNK2 genes will be deleted in mouse cardiomyocytes through the use of the MHC-Cre/LoxP recombination system. In this system, in one strain of mice the Cre recombinase is driven by the myosin heavy chain (MHC) promoter which drives Cre expression in cardiomyocytes. This strain is bred to another strain in which the JNK1 and JNK2 genes have been floxed to support their removal. The F1 progeny will be heterozygous floxed for the JNK1 or JNK2 allele, and either Cre-positive or Cre-negative. Inter-crossing of the F1 progeny will produce F2 mice that are homozygous floxed for JNK1 or JNK2, and Cre-positive or negative (the latter must be determined by genotyping).

This analysis will help determine whether the use of cell-specific genetic recombination in transgenic mice is a viable method for studying signal transduction components in Type II diabetes in specific tissues. Following the mouse breeding, DNA will be isolated from the mice and genotyped by PCR to determine the status of the JNK1, JNK2, and Cre genes. The percent of Cre positive mice will then be determined to facilitate subsequent experimental designs. This mouse model can be used to investigate insulin-resistance and pro-inflammatory MAPK pathway contributions to Type II diabetes in cardiomyocytes.

METHODS

Cre/LoxP Recombination

In the Sauer and Henderson (1988) study, the basic parameters for performing cell-specific gene deletion through Cre/LoxP recombination were established. **Figure-7** depicts the general mechanism by which the target genes, in their case MAPK8 and MAPK9, were deleted. In this mechanism, the LoxP 34 bp nucleotide sequence, flanks or "floxes" the target gene of interest. Under the heart-specific promoter Myosin Heavy Chain, Cre recombinase is expressed in cardiomyocytes, and is able to attach to the LoxP sites in and excise the floxed target gene from the genome. The excised gene contains one of the LoxP nucleotide recognition sequences, with the other sequence remaining on the genome as a reporter gene.

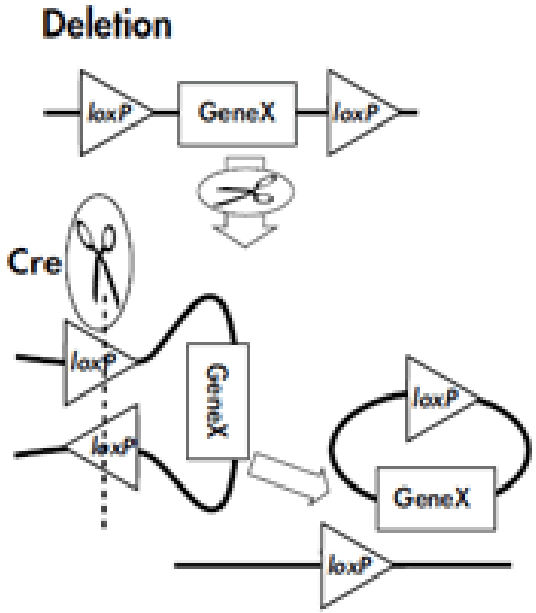

Figure 7: Strategy for Gene Knockout: The Outcome of the Cre-LoxP Recombination is Determined by the Orientations and Location of Flanking LoxP Sites. In this segment, the two LoxP sites are oriented in the same direction (cis arrangement), mediating a deletion of the floxed segment.

Generation of Cre-Positive Transgenic Mice

Generation of Cre-positive, homozygous transgenic mice involved the breeding of mice in the F0 and F1 generation, resulting in the target progeny of the F2 generation. In the F0 generation, male C57BL/6 mice expressing Cre recombinase under the heart specific myosin heavy chain (MHC) promoter were crossed with female mice containing the heterozygous floxed JNK1 or JNK 2 allele. The resulting F1 progeny are heterozygous floxed for the JNK1 or JNK2 allele, and either Cre-positive or Cre-negative. For the F2 generation, heterozygous JNK1 and JNK2 F1 progeny were crossed to obtain homozygous floxed, Cre-positive (JNK null) mice.

Hot Alkaline Lysis of Mouse Tails

Tail section DNA was used for genotyping. A 0.5 mm section of each mouse tail was taken and mixed with 75 µL of alkaline lysis reagent. After this was done, the mouse tails were incubated for 30 minutes at 95°C in a dry bath to allow lysis of the tissue samples. After the dry bath, the specimens were cooled on ice for about 5 minutes to allow for proper DNA lysis optimization. Subsequently, 75 µL of neutralization reagent was added to each tube to stop the alkaline lysis reaction completely. Crude DNA lysates (not phenol extracted) were obtained by vortexing the mixture and centrifuging the sample at 10,000 rpm for 2-3 minutes. The supernatant was then transferred to newly labeled tubes and stored in -20°C until PCR analysis.

PCR Genotyping

The purpose PCR analysis was to test for the presence of the MHC-Cre gene in F2 mice. This was accomplished through the use of specific 5’ and 3’ Cre-specific DNA primers. After the

hot alkaline mouse tail lysis had been completed, 1 µl of the purified DNA supernatant was used for PCR analysis. The primers used in the analysis were MHC-cre 5': (ATG ACA GAC AGA TCC CTC) and MHC-cre 3': (CTC ATC TCG TTG CAT C). The primer triplets were used in amplification of genes for the F2 generation, JNK1 and JNK 2 homozygous mice. Promega 2X Green PCR master mix was added in a 20 µl volume to the product. The PCR Master Mix is a premixed, ready-to-use solution containing Taq DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. Conditions for PCR analysis were 40 cycles (30 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C).

Gel Electrophoresis

Separation of the amplicon DNA in the gel was accomplished through a standard agarose gel electrophoresis protocol. A 1% agarose gel was used as the analysis medium and was prepared using a microwave heat source. After gel cooling, 5 µl of DNA marker ethidium bromide was added and the gel was prepared for electrophoresis using casting combs. Loading buffer was added to PCR/DNA solution, after which the DNA and ladder was loaded onto the gel, and electrophoresed at 100V for 1 hour. Amplicons were visualized by UV trans-illumination.

RESULTS

In order to generate transgenic mice with a heart-specific deletion of JNK1 and JNK2 genes, a series of breeding procedures were followed (**Figure-8**). Male mice that expressed Cre recombinase under a heart-specific myosin heavy chain (MHC) promoter were crossed with female mice containing a floxed JNK 1 or JNK 2 allele (upper part of diagram). The resultant F_1 progeny were heterozygous for floxed JNK 1 or JNK 2 alleles, and were either cre positive or cre negative. The F_1 progeny were subsequently intercrossed, and the resultant F2 progeny were homogygous for floxed JNK 1 or JNK 2 alleles, and remained either cre positive or Cre negative (the latter state must be determined by genotyping). It is important to note that there were no expected significant phenotype changes in the resultant progeny.

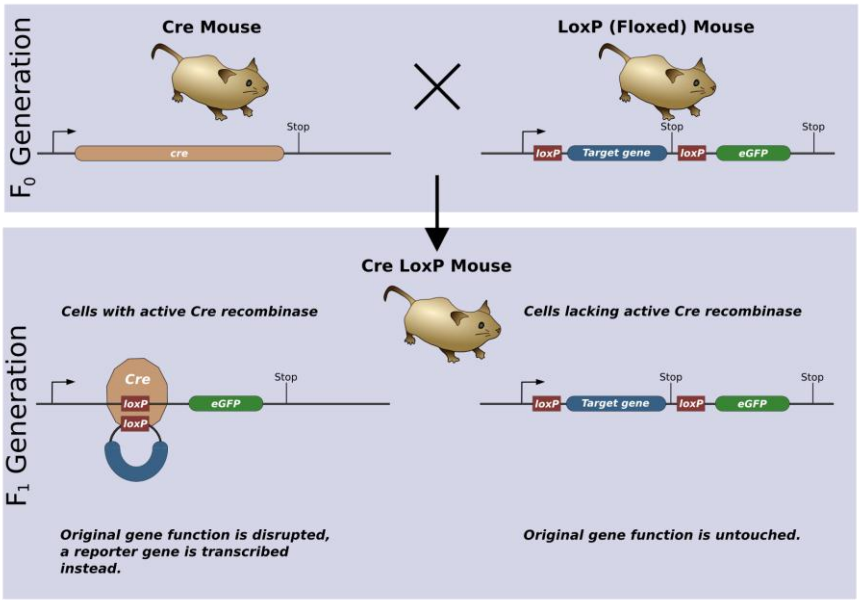

Figure 8: The Cre/loxP Mating Strategy Used in This Project. Male mice expressing cre under the control of an MHC promoter were crossed with female mice containing floxed JIN1 or JNK2. The F1 offspring are heterozygous for floxed JIN1 or JIN2, and either cre positive or negative. Interbreeding of F1's produces F2's that are homozygous for floxed JNK1 or JNK2, and either cre positive or negative (the latter is determined by genotyping).

After producing the resultant homozygous floxed JNK1 and JNK2 F2 progeny, a hot alkaline lysis was performed on mouse tail sections, and a small 1 µL aliquot was used for Cre PCR genotyping. The results were visualized on two agar gels, for JNK1 mice (**Figure-9)** and JNK2 mice (**Figure-10**). Each gel showed the presence of the MHC-cre recombinase gene in three of the mice, so in total six of the mice were Cre positive. However, one of the JNK2 homozygous mice unexpectedly showed a shorter amplicon (lane-7 Figure-10) while one of the JNK1 homozygous mice showed a longer amplicon (lane-13, Figure-9).

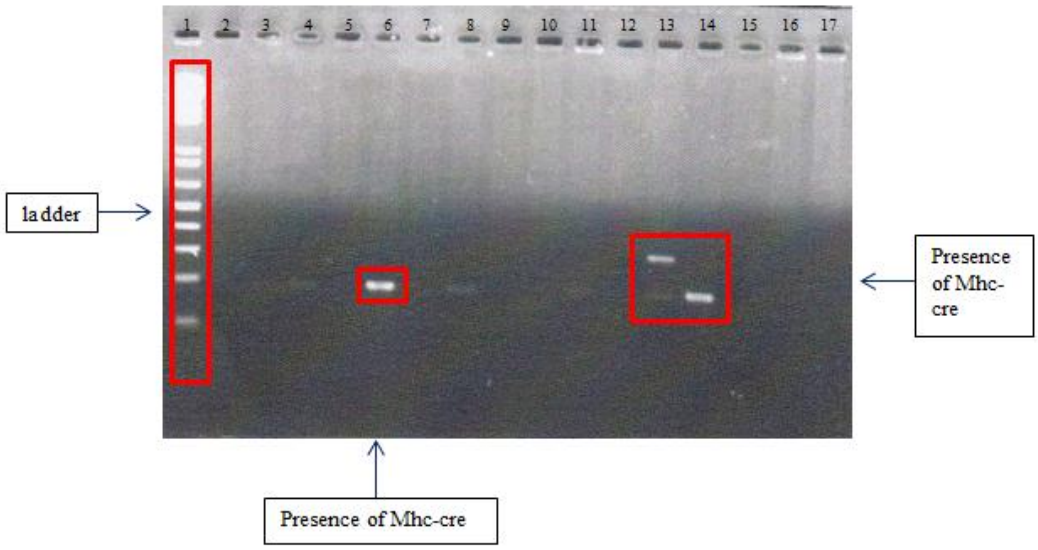

Figure 9: PCR Analysis for the Presence of the MHC-Cre Gene in Floxed JNK-1 Homozygous Mice. MHC-Cre DNA amplified by PCR was visualized by agarose (1%) gel electrophoresis Each lane contained 5 µl of the PCR reaction. Lanes 6, 13 and 14 showed successful amplification of the MHC-cre transgene, although the amplicon was unexpectedly longer in lane-13.

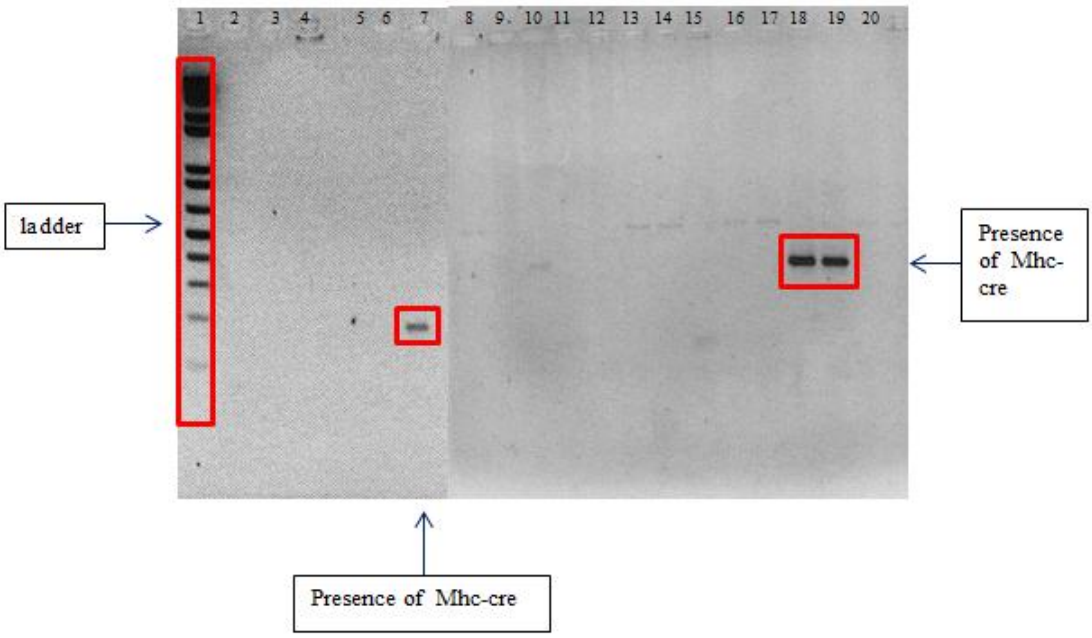

Figure 10: PCR Analysis of the Presence of the MHC-Cre Gene in Floxed JNK-2 Homozygous Mice. The legend is as described above, except the mice are homozygous for floxed JNK-2. Lanes 7, 18 and 19 show successful presence of the MHC-cre gene, however the amplicon in lane 7 is unexpectedly short.

DISCUSSION

From this study, it was determined by PCR that the MHC-Cre gene was present in both floxed JNK1 and JNK2 homozygous mice. Amplicon signals from the MHC-Cre positive mice showed that the PCR design to detect the Cre gene was valid, and produced results consistent with the breeding experimental plan. The results from a PCR analysis performed by other Kim lab personnel (data not shown) also confirm that the Cre/LoxP recombination system was successful in producing a heart-specific deletion on the JNK1 and JNK2 gene in the experimental mice.

During this project, several problems were encountered. One of the main problems was the existence of unexpected amplicon lengths in two of our MHC-Cre positive mice. It was concluded that the unexpected amplicon lengths likely result from a mutation in the Cre DNA during the PCR process. This random mutation through the addition or deletion of nucleotides from the target amplified DNA fragment could have caused the unexpectedly longer amplicon in a MHC-Cre positive JNK1 mouse, as well as an unexpectedly shorter amplicon in a MHC-Cre positive JNK2 mouse. The use of known Cre positive DNA could have been used to show the reliability of the alkaline lysis and PCR method for genotyping.

The project was successful in detecting MHC-Cre in the F2 generation of our transgenic mice. However, several questions were still left unanswered by our research. Our experiment used a Myosin Heavy Chain (MHC) promoter to direct cardiac tissue-specific expression of the Cre, and thus tissue-specific knockouts of the JNK1 and JNK2 genes using the Cre/LoxP method. However, we did not use RT-PCR to verify tissue-specific *expression* of the Cre. Our experimental PCR protocol from mouse-tail samples was designed only to determine the

presence of the Cre gene in the genome to distinguish Cre positive from Cre negative mice. The MHC promoter has long been used in research to drive expression in cardiomyocytes (reviewed in Wagner, 1998), so it is logical to assume that our six Cre positive mice are expressing Cre in cardiomyocytes, but we did not perform RT-PCR.

Future studies can use the mice to determine whether JNK1 and JNK2 KO mice, missing these key components of the MAPK pathway related to cell stress, show phenotypes for inflammation and insulin-resistance in cardiomyocytes. Other studies could knockout other members of the MAPK pathway to determine whether they are important for mediating cell stress in cardiomyocytes. While the MAPK/JNK pathway has been shown to be one of many pathways that stimulate Type II diabetes, its effect in this process is not well understood, mostly due to the complexity involved in the signaling process. Research should be done to investigate the specific signal that obesity uses to activate the MAPK/JNK pathway and how this could be stopped.

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