The characterization of obesity and noninsulin dependent diabetes mellitus in Swiss Webster mice associated with late-onset hepatocellular carcinoma

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ABSTRACT

Despite increased awareness of the obesity epidemic and a higher incidence of the metabolic syndrome in humans, the incidence of obesity and its comorbidities—most notably, type II diabetes (T2D) and cardiovascular disease (CVD)—continues to increase. Although numerous animal models are available to study the molecular pathways, genetics and therapeutic/prevention strategies for T2D, no model completely recapitulates T2D or it's comorbidities in humans. Therefore, novel animal models represent valuable research tools in understanding T2D. To develop a novel mouse model of T2D, I characterized an outbred mouse discovered at MIT that displayed clinical signs of diabetes. Prevalence of glucosuria in the Swiss Webster colony reached 60% (n=70) in males 8 weeks to 6 months of age. Despite severe obesity in some females, no females were diabetic. Pathologic findings in affected males included cachexia, dilated gastrointestinal tracts with poor muscular tone, pancreatic islet degeneration and atrophy with compensatory metaplasia and/or neogenesis, bacterial pyelonephritis, membranous glomerulopathy, and late-onset hepatic tumors with macrosteatosis, microsteatosis and hydropic change in aged males. Serum insulin correlated with blood glucose in a nonlinear pattern suggestive of islet exhaustion. Circulating leptin levels showed a weak inverse correlation with glucose. Diabetic males were bred with obese colony females to produce 20 male and 20 female offspring. Prevalence of diabetes in male offspring was 80% (16/20) with a median age of onset of 18 weeks. In contrast, no diabetic females were identified, despite being significantly more obese than males. Male predominance is likewise a feature of T2D in humans. To our knowledge, this is the first documentation of hepatocellular carcinoma and islet metaplasia and/or neogenesis in a spontaneous outbred mouse model of T2D. The SW availability and histolopathologic features represent a promising new model for the study of T2D. Further studies are required for complete molecular and genetic characterization of the diabetic SW mouse. These studies are outlined in this thesis.

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Part 1: Type 2 Diabetes in Humans

Disease Epidemiology and Significance

Despite increased awareness of the obesity epidemic and a higher incidence of the metabolic syndrome in humans, the incidence of obesity and its comorbidities—most notably, type II diabetes (T2D) and cardiovascular disease (CVD)—continues to increase in both adults and adolescents (Rosenbaum, Nonas et al. 2004). In the United States, over 20.6 million people age 20 and over have T2D representing greater than 9.6% of the total population over age 20 (NIDDK, 2005). This number is expected to rise to over 30 million people by the year 2010. Together with diabetic complications, T2D accounts for more than 92 billion dollars in direct health care costs in the U.S. alone with an additional 40 billion in indirect medical expenses (Yach, Stuckler et al. 2006). Worldwide, T2D affects approximately 150 million people (WHO, 2002), making obesity and obesity-related disease a global epidemic. For these reasons, T2D is major public health concern and of significant importance to the biomedical research community.

Factors in the Development of T2D and the Metabolic Syndrome

T2D has been classically described as a "glucocentric" disease, whereby hyperglycemia is viewed as the primary disease resulting from a combination of insulin resistance and an insulin secretory defect (beta cell dysfunction) (Unger, 2008). In the glucocentric paradigm, the pathogenesis of diabetes centers around glucose metabolism; therefore, treatment and prevention strategies relied on reducing hyperglycemia (high-dose exogenous insulin therapy, etc). More recently, defects in lipid metabolism with subsequent ectopic lipid accumulation have been implicated as primary mechanisms in

the pathogenesis of T2D and the metabolic syndrome (lipocentric) (Unger, 2008). Recent studies have provided some evidence for the lipocentric paradigm of T2D directly linking lipid metabolism dysfunction with insulin resistance. In 2002, Boden and Shulman found that chronically elevated free fatty acids (FFA) coincided with the onset of peripheral and hepatic insulin resistance, inhibiting insulin signaling by inhibition of glucose uptake through the GLUT4 transporter in skeletal muscle. In the same study FFA also inhibited insulin secretion. Defects in lipid metabolism also result in the ectopic accumulation of lipids in pancreatic islets and other tissues (Lee, Hirose et al. 1994). This may result in the lipotoxic destruction of beta cells, explaining the insulin secretory defect that is seen with T2D (Lee, Hirose et al. 1994). Therapeutic approaches based on a lipocentric view of T2D require addressing the underlying defects in lipid metabolism in addition to treatment of hyperglycemia (which is primarily responsible for microvascular and macrovascular complications of T2D).

Dysfunctional lipid metabolism links the rising obesity epidemic with the increased prevalence of T2D and the metabolic syndrome. Metabolic syndrome (MetS) is characterized by the presence of at least three of the following risk factors: abdominal obesity, dyslipidemia, hypertension, insulin resistance, a pro-inflammatory state (elevated C-reactive protein), and a prothrombotic state (elevated plasma plasminogen activator inhibitor) (Grundy, Brewer et al. 2004). The diagnostic criteria for MetS have been outlined by the Adult Treatment Panel III (ATPIII) as listed in table 1 (Grundy, Brewer et al. 2004). Although the mechanisms of MetS are poorly understood, inflammatory mediators and altered molecular processes associated with obesity and

lipid metabolism, such as defective mitochondrial function, have been implicated in the pathogenesis.

TABLE 1. ATP III Clinical Identification of the Metabolic Syndrome (Grundy, Brewer et al. 2004)

Parameter	Risk Level
Abdominal obesity (waist circumference)*	
Men	>102cm (>40in)
Women	>88cm (>35in)
Triglycerides	>150mg/dL
HDL Cholesterol	
Men	<40mg/dL
Women	<50mg/dL
Blood pressure	≥130/≥85 mm Hg
Fasting glucose$^{\Psi}$	>110mg/dL

*Obesity is associated with insulin resistance and the metabolic syndrome. Abdominal obesity is more highly correlated with the metabolic syndrome than an elevated BMI. Therefore, waist circumference is used to identify the body weight component that puts an individual at risk for metabolic syndrome.

ψThe American Diabetes Association established a cutpoint of ≥100 mg/dL, Fasting blood glucose above this cutpoint is diagnostic of either prediabetes (impaired fasting glucose) or diabetes.

Insulin Resistance and Mitochondrial Dysfunction

Insulin resistance has been recognized as a fundamental underlying metabolic defect in the pathogenesis of metabolic syndrome, a clustering of risk factors for heart disease and T2D that include central obesity, dyslipidemia and hypertension (Grundy, Brewer et al. 2004). Recent studies have linked mitochondrial dysfunction to the development of insulin resistance and obesity in humans, demonstrating abnormal mitochondrial structure, number and gene expression in diabetic patients and their offspring (Wiederkehr and Wollheim 2006). Mitochondrial DNA (mtDNA) mutations are also

known to cause diabetes by affecting insulin secretion from pancreatic beta cells (Nomiyama, Tanaka et al. 2004). Furthermore, humans and animal models of obesity and insulin resistance show improvement in insulin sensitivity and fat metabolism whentreated with substances that enhance the function of mitochondria (Gumieniczek, Hopkala et al. 2006; Waugh, Keating et al. 2006) such as pioglitazone, a peroxisome proliferator-activated receptor gamma (PPAR γ) agonist.

Deciphering the role that mitochondria play in the development of insulin resistance is confounded by the influence of obesity on mitochondrial function. Obesity in rodent models has been shown to significantly reduce the numbers of mitochondria within adipocytes as a result of the expanding fat droplet displacing cellular organelles (Choo, Kim et al. 2006). Additionally, cellular lipid accumulation and changes in fatty acid metabolism that occur in the insulin resistant state leads to an increased cellular oxidative stress (Lin, Berg et al. 2005). The generation of free radicals further damages the highly sensitive mitochondria, resulting in further dysfunction and promotion of insulin resistance and thus, accumulation of cellular lipids. This leads to an augmentative cycle of increased cellular lipid accumulation, increased free radical damage and increased insulin resistance. Oxidative stress and the release of reactive oxygen species (ROS) promote inflammation through activation of NF-κB and have also been implicated in the progression of diabetes complications, including microvascular and macrovascular dysfunction and liver disease (Lin, Berg et al. 2005).

Genetic Determinants of Type 2 Diabetes

Although T2D is complex and influenced by environmental factors such as caloric intake and physical inactivity, it is well documented that genetic predisposition can attribute to disease susceptibility with age-adjusted concordance rates approaching 70-80% in monozygotic twins (Ghosh and Schork, 1996). The genetic basis for T2D is difficult to decipher as defects in a number of molecular pathways can lead to the similar phenotype of T2D—hyperinsulinemia, insulin resistance and hyperglycemia. Common forms of T2D in humans are polygenic: several genetic alterations are responsible for an increased susceptibility to T2D (Barroso, 2004). The more rare monogenic forms of T2D, such as the family of disorders known as Maturity Onset Diabetes of the Young (MODY), can be attributed to single mutations in known genes including HNF1b and HNF4. Although overt mutations in these genes are less common, polymorphisms and/or less penetrant mutations in these genes, have been attributed to increased susceptibility to polygenic T2D (Barroso, 2004). The discovery of new candidate genes involved in T2D may have an important impact on the understanding of molecular pathways involved in T2D pathogenesis and novel therapeutic approaches.

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Part 2: Mouse Models of Type 2 Diabetes

The continued investigation into the key factors and mechanisms involved in the pathogenesis of T2D in humans requires the use of animal models. Animal models of T2D also allow the evaluation of potential therapeutics and prevention strategies. Appropriate animal models relevant to the study of T2D in humans must either 1) have characteristics that are similar to the pathophysiology and natural history of T2D in humans and/or 2) should develop diabetic complications with a similar etiology as humans (Cefalu, 2006). For decades, researchers have used numerous animal models to fulfill these requirements including rodents, felines, swine and nonhuman primates; however, mouse models of T2D are particularly useful. Advantages to mouse models include a complete knowledge of the mouse genome, a short life cycle/breeding span, ease of genetic manipulation, lower costs and ability to do longitudinal studies and invasive testing (Cefalu, 2006).

Spontaneous Mouse Models of T2D

Mouse models used in the study of T2D include induced models, inbred mice, mice with spontaneous genetic mutations, and genetically engineered mice. Although genetically engineered mice are heavily represented in recent T2D literature, spontaneous mouse models are still widely used to study obesity and T2D. Two of the most commonly used mice are the db/db mouse (diabetes) and ob/ob mouse (obesity).

The db/db mouse (B6.Cg-m+/+Lepr^{db}/J) was originally developed by the Jackson Labs (1966) after discovery of a leptin receptor (Lepr db) mutation on a C57BLKS/J background. Lack of leptin signaling results in hyperphagia with increased efficiency of food utilization, obesity, and increased circulating leptin and insulin levels (Hummel, Dickie et al. 1966). The mutation is recessive and only homozygous mice develop signs of obesity or diabetes. These mice display severe obesity, insulin resistance, hyperinsulinemia and T2D by an early age (4-8wk). The sequence of disease progression (hyperinsulinemia → beta cell failure/distruction → hypoinsulinemia/hyperglycemia) parallels the human condition. Like human T2D, the clinical phenotype is variable dependent on genetic background and gender (males > females) (Jackson Labs, accessed 2008). The db/db mouse is particularly valuable in that it develops diabetic comorbidities that mirror the human conditions including diabetic glomerulopathy with mesangial matrix expansion, glomerular hypertrophy, altered glomerular function and albuminuria (Sharma, McCue et al. 2003). Other reported conditions include heart diseas and diabetic dyslipidemia (Kobayashi, Forte et al. 2000). Despite their widespread utility in diabetes research, db/db mice, unlike humans, do not develop marked pancreatic pathology or severe comorbid diseases and display complications of altered leptin metabolism such as infertility (hypogonadism), altered thermoregulation, marked hyperphagia and a shortened lifespan. Also, they represent a monogenic form of diabetes which is uncommon in human populations.

ob/ob Mice (obesity) (B6.V-*Lep*^{ob}/J) have a mutation at chromosome 4 and are similar to db/db mice in that they represent a model of altered leptin metabolism (truncated,

inactive leptin product), obesity and insulin resistance. The phenotype is considered much less severe than db/db mice with ob/ob mice exhibiting obesity with hyperphagia, glucose intolerance, hyperinsulinemia and transient hyperglycemia (Jackson Laboratories, accessed 2008). However, the phenotype is highly dependent on genetic background and mice with a more severe diabetic phenotype are available. ob/ob Mice also develop cormorbidities associated with obesity and diabetes including peripheral neuropathy (Drel, Mashtalir et al. 2006), heart disease, delayed wound healing and skeletal abnormalities (Jackson Laboratories, 2008), and fatty liver disease that may occasionally progress to neoplasia (Anstee and Goldin, 2006). Drawbacks to ob/ob mice as a model of T2D in humans are similar to db/db mice.

Other spontaneous mouse models of T2D include the Nagoya-Shibata-Yasuda (NSY), KK mouse and the NONcNZO10/LtJ mouse. These mice vary in age-of-onset and in their presentation of T2D but all share similar features of moderate obesity and insulin resistance, while the NSY and NONcNZ10/LtJ mice develop hyperglycemia. The NSY mouse, developed through the selective inbreeding for glucose intolerance in outbred Jcl:ICR mice, develops signs of T2D in an age-dependent manner with a higher prevalence of males affected than females (98% v 13% respectively) (Ueda, Ikegami et al. 1994). NONcNZO10/LtJ also develop signs of mild to moderate obesity and diabetes dependent on age (maturity-onset) (Cho, Kim et al. 2007). The development of hyperglycemia and diabetes in KK mice is body weight dependent (Ikeda, 1994). Notably the NSY, KK and NONcNZO10/LtJ mice are all polygenic models of T2D compared with the monogenic db/db and ob/ob mouse models, mirroring human T2D.

Despite the utility of mouse models in T2D research, none of these mice completely recapitulate T2D and it's comorbidities in humans. Importantly, a major drawback of mouse models of T2D is the apparent lack of islet pathology observed in humans (Cefalu, 2006). Therefore, novel animal models are needed. Part 3 in this paper describes one such model discovered by the Division of Comparative Medicine at MIT.

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Part 3: Obesity and noninsulin dependent diabetes mellitus in Swiss Webster mice associated with late-onset hepatocellular carcinoma

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Abstract

Genetic mutations resulting in obesity and type 2 diabetes mellitus (T2D) are described for both inbred and outbred mice. However, no known mouse model completely recapitulates human T2D and its comorbidities. We identified a cohort of obese, male, outbred Swiss Webster (SW) mice as polyuric, polydipsic, glucosuric and hyperglycemic. Prevalence of glucosuria in the SW colony reached 60% (n=70) in males 8 weeks to 6 months of age. Despite severe obesity in some females, no females were diabetic. Pathologic findings in affected males included cachexia, dilated gastrointestinal tracts with poor muscular tone, pancreatic islet degeneration and atrophy with compensatory metaplasia and/or neogenesis, bacterial pyelonephritis, membranous glomerulopathy, and late-onset hepatic tumors with macrosteatosis, microsteatosis and hydropic change in aged males. Serum insulin correlated with blood glucose in a nonlinear pattern suggestive of islet exhaustion. Circulating leptin levels

showed a weak inverse correlation with glucose. Diabetic males were bred with obese colony females to produce 20 male and 20 female offspring. Prevalence of diabetes in male offspring was 80% (16/20) with a median age of onset of 18 weeks. In contrast, no diabetic females were identified, despite being significantly more obese than males. Male predominance is likewise a feature of T2D in humans. To our knowledge, this is the first documentation of hepatocellular carcinoma and islet metaplasia and/or neogenesis in a spontaneous outbred mouse model of T2D. The SW availability and histolopathologic features represent a promising new model for the study of T2D.

Introduction

The incidence of obesity and its comorbities—most notably metabolic syndrome, type 2 diabetes (T2D) and cardiovascular disease (CVD)—continues to increase in both adults and adolescents (33). Metabolic syndrome is defined by the presence of at least three of the following risk factors: abdominal obesity, dyslipidemia, hypertension, insulin resistance, a proinflammatory state (elevated C-reactive protein), and a prothrombotic state (elevated plasma plasminogen activator inhibitor) (19). A majority of metabolic syndrome patients are obese and insulin resistant, conferring an increased risk for T2D(19). Humans with T2D have a functional insulin deficiency characterized by fasting hyperglycemia due to peripheral insulin resistance combined with an insulin secretory defect (i.e. inability to increase beta cell mass).

An estimated 5-10% of the U.S. population suffers from T2D (18, 37) and is at increased risk for developing microvascular and neuropathic complications, macrovascular disease, microbial infections and neoplasia. Recently, several epidemiological studies have shown an increased risk of hepatocellular carcinoma (HCC) in patients with T2D in the absence of viral hepatitis (11, 13). HCC in humans with diabetes is associated with underlying nonalcoholic fatty liver disease (NAFLD), a common finding in patients with obesity and metabolic syndrome. NAFLD can progress to steatohepatitis (NASH) with cirrhosis and eventual neoplastic transformation. While the direct link between diabetes and HCC remains unknown, underlying chronic liver disease has been implicated in the development of both diabetes and HCC.

Several spontaneous and induced genetic mutations resulting in varying degrees of obesity and diabetes have been described in mice, making them popular models in diabetes research (2, 29). Mutation of the leptin or leptin receptor gene produces obesity and T2D in the ob/ob and db/db mouse respectively. The db/db mutation is available on a variety of mouse strain backgrounds, is well characterized, and displays many of the comorbities of T2D found in humans, including cardiovascular disease, renal disease and neuropathies (31, 35, 42). However, these mice represent a monogenic model demonstrating features related to leptin resistance, including morbid obesity, hyperphagia, and alterations in thermoregulation and fertility. In contrast to popular mouse models of diabetes, common forms of human T2D are considered polygenic disorders, occurring through the simultaneous action of several genes and rarely involving alterations of the leptin/leptin receptor axis (47).

We recently identified a cohort of male, outbred Swiss Webster (SW) mice in a breeding colony that required frequent cage changes due to urine-soaked bedding, and frequent water bottle changes (i.e. polyuria/polydipsia). The SW breeding colony at the Massachusetts Institute of Technology (MIT) is maintained to provide mice for the embryo transfer program and occasionally for sentinel animals as part of the rodent health monitoring program. At the time of the study, the colony had been closed for approximately two years with a random breeding strategy in place. Five males were isolated from the colony for further study. Metabolic comparisons with age-matched, control males showed markedly increased urine production and water consumption in the affected mice; however, there were no significant differences in food consumption or body weight (data not shown). Additionally, affected mice were glucosuric, and serum chemistry analysis with insulin levels on one mouse revealed hyperglycemia, hypercholesterolemia, azotemia and hyperinsulinemia—signs consistent with both metabolic syndrome and T2D in humans. The affected mice were subsequently bred to females from the SW colony to produce 20 male and 20 female offspring. The current study describes the clinicopathologic features of T2D in these SW offspring as well as the prevalence and characterization of this disease in the SW breeding colony where the disease was first recognized.

Materials and Methods

Animal sources and husbandry

Five, 18-week-old, male, outbred Swiss Webster (SW) mice and 10, 8-week-old, female SW mice were obtained from the breeding stock of the MIT Division of Comparative Medicine mouse breeding program (closed breeding program for a minimum of 2 years), and subsequently bred to produce 20 male and 20 female offspring (F1s). SW offspring were weaned at 4 weeks of age and separated by sex into groups of four. These mice have continued to be inbred, and mice up to generation 7 are included in the aging part of this study. Additional diabetic and nondiabetic male, and nondiabetic female SW mice were selected from existing sentinel mouse cages placed in mouse rooms on the MIT campus.

Mice were housed in polycarbonate microisolator cages on hardwood bedding (PharmaServ, Framingham, MA) under specific pathogen free (SPF) conditions (free of *Helicobacter* spp., *Citrobacter rodentium*, *Salmonella* spp., endoparasites, ectoparasites and known murine viral pathogens) in an Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility. Mouse rooms were kept at constant temperature and humidity on a 12:12 hour light to dark cycle, and mice were provided standard rodent chow (Purina Mills, St. Louis MO) and water ad libitum. All protocols were reviewed and approved by the MIT Committee on Animal Care. Mice were euthanized by CO_2 inhalation in accordance with guidelines set by the American Veterinary Medical Association (1).

Clinicopathology of colony Swiss Websters

The presence of glucosuria was determined by collection of urine from nonanesthetized mice onto glucose urine dipsticks according to manufacturer's instructions (Diastix®, Bayer Corporation, Elkhart, IN). Based on the presence or absence of glucosuria, mice were defined as diabetic or nondiabetic.

Fifteen diabetic male colony SW mice and five nondiabetic colony SW mice (three males, two females) approximately 6 months of age, were selected for necropsy. Non-fasting blood glucose concentrations were measured from 10/15 glucosuric and 5/5 non-glucosuric mice using the OneTouch Basic glucometer (Lifescan, Inc. Milpitas, CA) from whole blood collected via terminal cardiac puncture. Whole blood was analyzed for complete blood count (CBC) and serum was submitted to a reference laboratory (IDEXX, North Grafton, MA) for biochemical analysis, insulin concentration and insulin to glucose ratio. Urine was collected via cystocentesis and submitted for complete urinalysis and aerobic culture.

Breeding experiments

To assess the reproductive performance of diabetic mice, five 6-month-old, diabetic male SW mice and ten nondiabetic females were selected from the breeding colony and bred to produce 20 male and 20 female F1 offspring.

Selected F1 brother-sister matings were undertaken to gauge reproductive viability and to establish diabetic Swiss Webster (dSW) and nondiabetic Swiss Webster (ndSW) lines. For diabetic mice, males were selected for breeding at the first positive test for

glucosuria and mated to the heaviest female from the same litter. In nondiabetic mice, the smallest male and female were selected for breeding. Subsequent litters were inbred using the same paradigm, with mice up to generation 7 included in this study.

Pathology and immunohistochemistry

Tissues were collected at scheduled necropsy from diabetic and nondiabetic parent colony SW, as well as selectively bred dSW and ndSW lines. For routine histopathology, tissues were fixed in 10% neutral buffered formalin, routinely processed and embedded, sectioned at 4μm, and stained with hematoxylin and eosin (H&E). Formalin-fixed, whole pancreata were sectioned at 4μm to include the gastric (head) and duodenal (tail) portions for immunohistochemical staining using guinea pig anti-swine insulin antibody (30) (DakoCytomation, Carpinteria, CA) at 1:150 on an automated immunostainer as previously described (32).

Bacteriology

Urine collected via cystocentesis, and aseptically collected tissue samples from gross lesions of the kidney, urinary bladder, liver, and/or gallbladder were submitted in trypticase-soy broth (TSB) for aerobic culture at 37°C when appropriate.

Establishment and characterization of diabetic/nondiabetic Swiss-Webster (dSW/ndSW)

Diabetic SW (dSW) offspring produced during breeding experiments with the diabetic colony SW males were characterized during development to determine the onset of clinical signs of obesity and diabetes. Mice were weaned at four weeks of age and

separated by gender into 10 groups of four mice/group to total 20 males and 20 females (at least four males and four females were included from each fertile sire). At weaning, body weight was recorded and mice were tested for the presence of glucosuria using the urine dipsticks. Urine glucose was reassessed at 10, 18, 26, and 30 weeks to determine the median age of onset of glucosuria. Body weight was recorded at one week intervals until week nine and then at week 17, 19, 22, 26 and 30. Blood was collected at 13 and 30 weeks via the retro-orbital sinus, from nonfasted mice anesthetized with 2% isoflurane in oxygen for determination of blood glucose using the OneTouch Basic glucometer (LifeScan Inc, Milpitas, CA). Serum insulin and leptin concentrations were measured from the 30 weeks serum samples using Rat/Mouse Insulin and mouse leptin ELISA kits respectively (LINCO Research, St. Charles, MO) according to the manufacturer's instructions.

Glucose tolerance testing was performed to confirm diabetic status in selected diabetic and nondiabetic male and female mice. After a 12-14hr fasting period, baseline blood glucose measurements were obtained using whole blood collected from the tip of the tail in unanesthetized mice. Then, 1.5mg glucose per gram body weight was administered via intraperitoneally injection (75mg/mL solution) and serial blood samples were obtained and analyzed at 15min, 30min, 60min, 90min and 120min post injection.

Statistical Methods

Body weights and serum chemistries were analyzed by one-way analysis of variance (ANOVA) with Tukey's post test. Median survival was calculated by Kaplan-Meier

survival curve analysis. Serum insulin and leptin concentrations were compared to blood glucose concentration by nonlinear and linear regression analysis respectively. Glucose tolerance testing was analyzed by determination of the areas under the curve (AUC). Mean AUCs for diabetic and nondiabetic groups were compared using an unpaired t test. P values <0.05 were considered significant. All analyses utilized GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA).

Results

Characterization of diabetes in dSW mice

Prevalence of diabetes in the male dSWs was 80% (n=20) with a median age of onset of 18 weeks (Figure 1a), and no diabetic females were identified. Mean, nonfasting blood glucose of diabetic male offspring at 30 weeks was 451.3±93.9mg/dl and differed significantly from nondiabetic male and female offspring (163.7±11.3 and 171.3±25.2mg/dl, respectively $P<0.01$) (Figure 1b). Female offspring were significantly

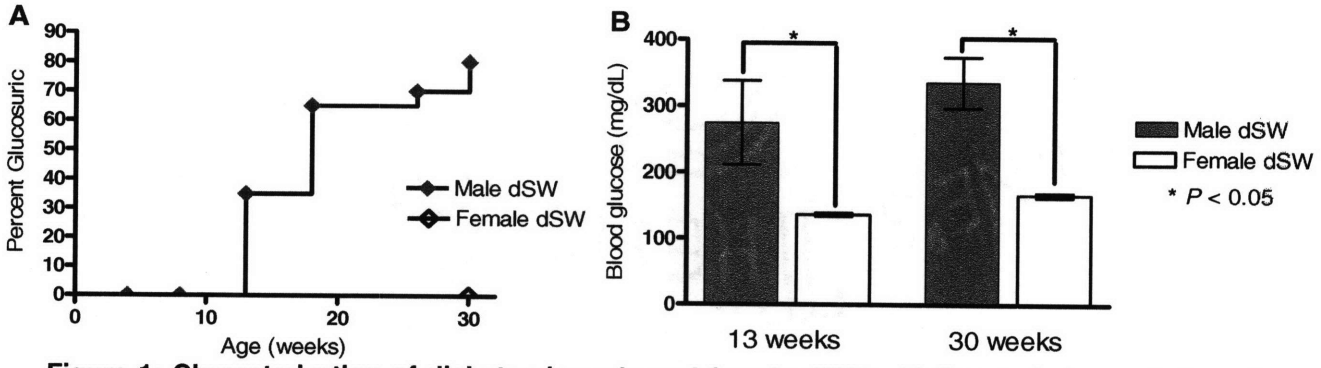

Figure 1: Characterization of diabetes in male and female dSWs. A) Onset of glucosuria in male dSWs. Kaplan-Meier survival curve where onset of glucosuria was considered as event. Median survival in male mice (n=20) was 18 weeks of age. Throughout the test period, no glucosuric females (n=20) were identified. **B)** Hyperglycemia in male dSWs. Nonfasting, whole blood glucose concentration was significantly higher in male dSWs (n=20) compared with in age-matched dSW females (n=20) at both 13 and 30 weeks of age as measured using a bedside glucometer. Blood glucose concentration was more variable in male mice compared with females with a greater proportion of males showing significant hyperglycemia at 30 weeks of age compared to 13 weeks. Mean values with standard error margins are displayed.

heavier than males at 53.9±5.7g and 48.2±6.0g respectively (P<0.05) at 30 weeks of age (Figure 2).

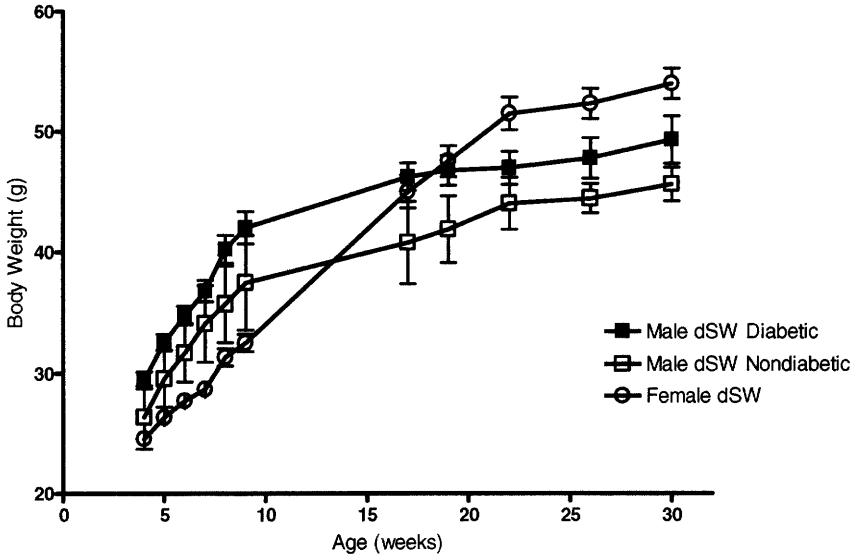

Figure 2: Growth rate in dSWs. Body weight was measured over time in male (n=20) and female (n=20) dSW mice. At 30 weeks of age, diabetic male dSW were significantly heavier than nondiabetic male dSW. Although weanling males were initially heavier than females, at 30 weeks of age, females were significantly heavier than males. Mean values with standard error margin are displayed.

Serum insulin concentrations for dSWs were measured using a rat/mouse insulin ELISA kit (Lincoplex). Serum insulin concentration was highly variable between mice, with no significant differences observed between the mean values for diabetic (males) and nondiabetic (male and female) mice. However, insulin concentrations showed a significant, nonlinear correlation with blood glucose concentrations in dSW males at 7-9mths of age (Figure 3). Insulin concentrations were increased in dSW with borderline and mildly elevated blood glucose concentrations, and

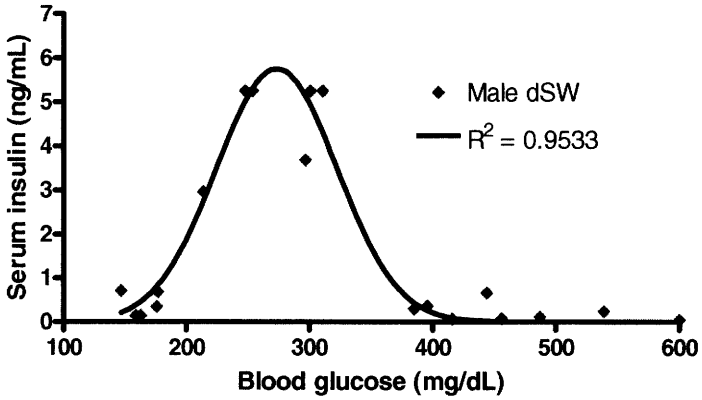

Figure 3: Insulin and blood glucose concentrations in male dSWs (n=20) at 7 months of age. Nonfasting serum insulin measured by ELISA showed a nonlinear correlation with whole blood glucose concentration. Male dSW with mild hyperglycemia showed elevated insulin concentrations compared to males with marked

were highest in those mice with a blood glucose concentration of approximately 300mg/dl (coincident with an onset of overt glucosuria). Diabetic mice with blood glucose concentrations above 300mg/dl, showed declining insulin concentrations that were inversely proportional to blood glucose concentration.

Glucose tolerance testing in fasted mice revealed elevated glucose curves in response to glucose challenge in diabetic male mice compared with nondiabetic mice. Mean area under the curve (AUC) for diabetic mice was significantly higher than nondiabetic mice (25,420±1,501 and 13,060±992 respectively $P<0.0005$; Figure 4), consistent with glucose intolerance in diabetic SW males. Blood glucose measurements in mice over 12 months of age were difficult to interpret due to the high prevalence of liver tumors in diabetic males. The presence of liver tumors occasionally coincided with a reversion to euglycemia or hypoglycemia in some mice with previously noted hyperglycemia.

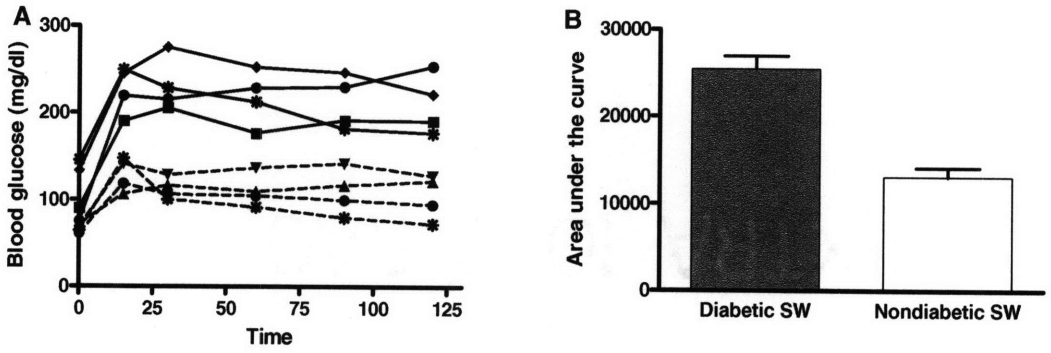

Figure 4: Glucose tolerance testing in aged dSW and ndSW. A) Glucose tolerance curves revealed elevated baseline with delayed return to baseline in diabetic males (solid lines) compared with nondiabetic males (broken lines). **B)** AUC measurements obtained from the curves in panel A were significantly higher in diabetic compared to nondiabetic males.

Serum leptin concentrations, measured by a mouse leptin ELISA kit (Lincoplex) were also highly variable between mice, and no significant differences in mean

concentrations were observed between diabetic and nondiabetic mice (6.821±6.920ng/ml and 8.766±4.385ng/ml respectively). However, serum leptin concentration showed a significant, inverse linear correlation with blood glucose concentration in diabetic 7-month-old, dSW males (Figure 5).

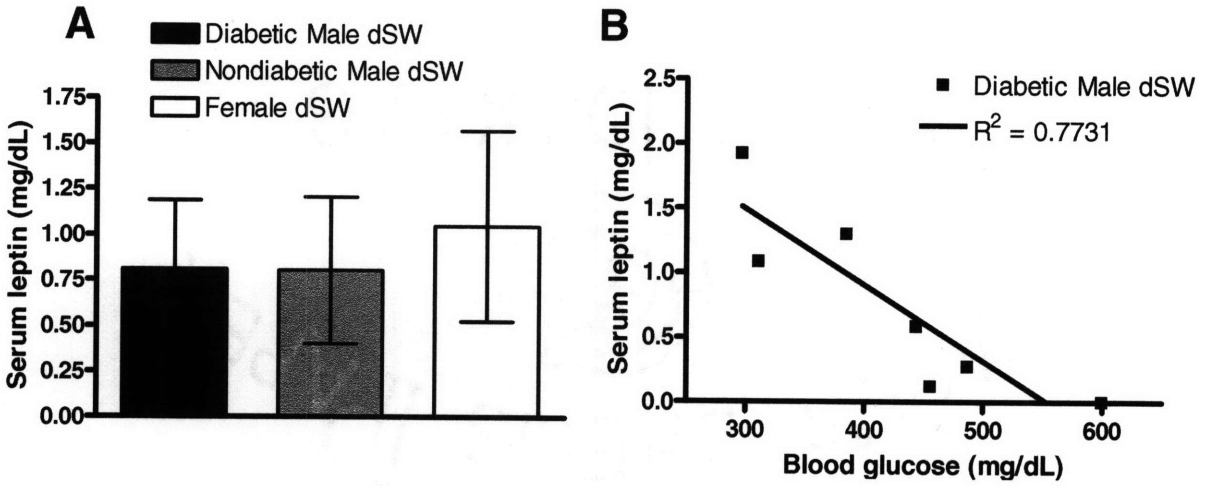

Figure 5: Serum leptin in dSW at 7 months of age. Nonfasting serum leptin concentrations as measured by ELISA were highly variable among diabetic male (n=17), nondiabetic male (n=7) and female (n=4) dSW with no significant differences observed. In diabetic male dSW, leptin concentrations showed a weak inverse correlation with whole blood glucose concentration. Markedly hyperglycemic males also showed decreased adipose stores and body condition which may explain decreased leptin concentrations.

Diabetic dSW male and female mice produced viable offspring (average 12 pups) when bred at less than 6 months of age and prior to the onset of moderate obesity in the females (<50g). No females became pregnant when bred after 6 months of age (n=3); however, dSW males remained reproductively viable past 8 months of age (8/8 tested males were capable of impregnating females and producing offspring).

Diabetic parent colony Swiss Webster (SW) mice

Over a sampling period of one week, the prevalence of diabetes (defined as blood glucose >300mg/dl with glucosuria) in the SW mouse breeding colony was 60% (42/70)

in males between 8 weeks and 12 months of age (average age >6 months). The prevalence of diabetes in male SW housed in a corridor separate from the breeding colony was 36% (17/47). However, the average age of males housed in the second corridor was <4 months and no breeding animals were tested. No females from either colony tested positive for glucosuria (n=40).

Serum biochemical data were analyzed in nine diabetic SW males and three nondiabetic SW males, approximately 6 months of age. The mean blood glucose concentration in diabetic (i.e. glucosuric) males was significantly higher than nondiabetic males at 396.2±14.4mg/dL and 235.3±35.59mg/dL respectively (P = 0.0005). Diabetic males had significant elevations in serum alanine aminotransferase (ALT), blood urea nitrogen (BUN), phosphorus and lipemic index compared with nondiabetic males (Figure 6). These alterations were consistent with early pre-renal or renal insufficiency, hepatocellular injury and hyperlipidemia.

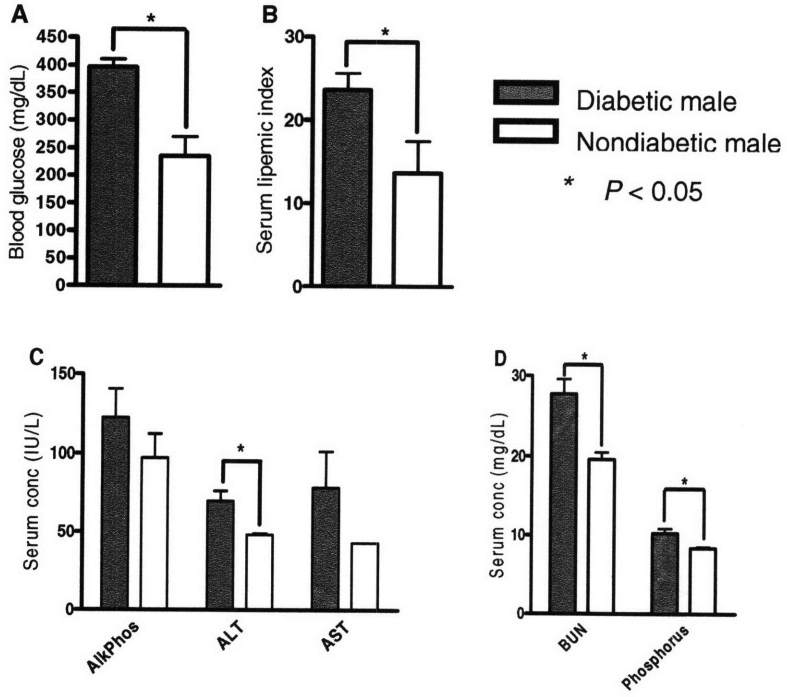

Figure 6: Biochemical characterization of diabetes in colony SW males. A) Diabetic male SW (n=9) mice showed significantly higher nonfasting, whole blood glucose concentration (**A**) and serum lipemic index (**B**) when compared with nondiabetic male SW (n=3). Serum alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were also significantly elevated (**C** and **D**, respectively) in diabetic male SW, but not serum creatinine (data not shown), suggesting prerenal or early renal azotemia. Mean values with standard error margin are displayed.

Gross pathology

Gross and histologic lesions in diabetic male mice, whether from the parent colony or dSW line, were similar in character. Diabetic male mice with advanced disease (blood glucose >300mg/dl, urine glucose positive) were distinguishable grossly from nondiabetic mice based on decreasing body condition. External gross findings included urine staining of the perineum and ventrum, unkempt hair coats, abdominal distension and decreased body condition scores (41). Body condition scores averaged 2 out of 5 as ascertained by palpation of the iliac bones and sacral prominences. Despite poor body conditions, diabetic mice maintained body weight due to abdominal distension (Figure 7) caused by enlarged, flaccid, ingesta-filled gastrointestinal tracts. The abdominal musculature of diabetic mice was thin and transparent, and the cecum markedly dilated and filled with ingesta, when compared with age- and gender-matched, nondiabetic controls (Figure 7). Dilation of the stomach and small intestine was evident less frequently. Diabetic males in the initial stages of disease showed increased body weight and abdominal fat deposition compared with nondiabetic males.

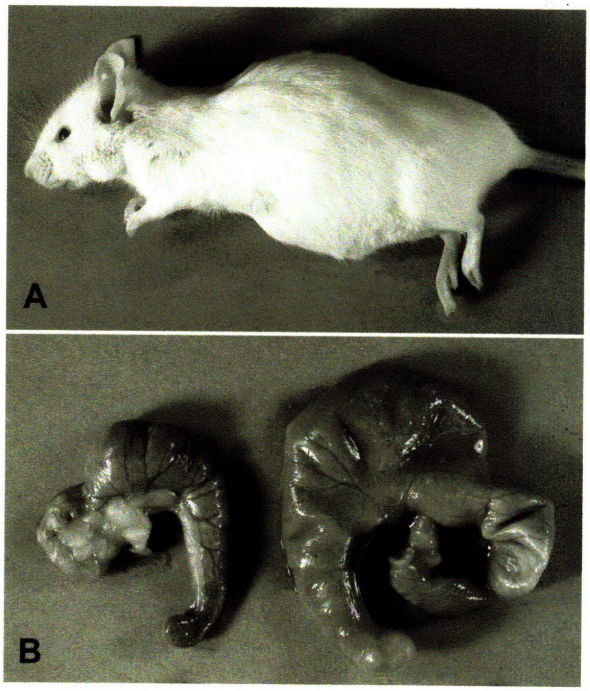

Figure 7: Gross pathology of diabetic colony SW males. A) Diabetic male colony SW mice with advanced disease were grossly distinguishable from nondiabetic males, showing decreased body condition scores, unkempt fur and a pot-bellied appearance due to enlarged, ingesta-filled gastrointestinal tracts. **B)** The ceca of diabetic males (right) were enlarged and flaccid compared with age-matched nondiabetic males (left). Gross findings suggested decreased gastrointestinal motility.

Kidneys of mice with ascending urinary tract infections were enlarged and irregularly disfigured by pelvic and, less frequently, cortical pale-to-yellow caseous abscesses up to 5 mm in diameter. Mice with marked pyelonephritis became moribund, exhibiting hunched posture, reluctance to move, poor body condition and an unkempt appearance. Ascending urinary tract infections were a significant cause of morbidity and mortality in the diabetic cohort. One mouse had uroabdomen and was suspected to have ruptured the ureter near the renal pelvis. Diabetic mice also showed mildly to moderately enlarged kidneys bilaterally with mild dilation of the renal pelvices and ureters (hydronephrosis and hydroureter).

Diabetic mouse livers appeared moderately enlarged and pale compared with nondiabetic mouse livers. 75% of diabetic males, 12-18 months of age (n=12) had grossly visible hepatic tumors. Liver tumors were not observed in age-matched nondiabetic males (n=4) or females (n=5) (Odds ratio = 24.43, P = 0.008). Tumors ranged from 0.1 x 0.1cm to 1.5 x 2.0cm. Affected mice frequently exhibited multiple tumors affecting multiple liver lobes, with only 3 out of 12 affected mice showing a single tumor. Interestingly, obese females but not males from the parent colony infrequently developed nonobstructive pigmented gallstones as determined by microscopic morphology and polarization pattern.

Histopathology

The pancreata of diabetic and nondiabetic mice were scored from 0 (none) to 4 (severe) for islet haphazard arrangement and vacuolation, and 0 (focal) to 4 (severe) for

histologic changes consistent with islet metaplasia (Figure 8). Virtually all male mice, whether clinically diabetic or not, displayed some degree of islet cell pleomorphism and atypia. Enlarged islets and mega-islets were occasionally seen in diabetic male mice (Figure 9). Islet metaplasia was defined by incomplete de novo parenchymal sublobulation, well removed from ductular regions, with a mixture of cells displaying islet, acinar, and/or intermediate morphology, (Figure 9). Because of the clinical history and characteristic histologic pattern, these regions were interpreted as zones of exocrine pancreatic acinar cells undergoing metaplasia to an islet phenotype. In other instances, cells with an islet phenotype were observed in small, poorly defined clusters adjacent to ducts. These foci were defined as islet neogenesis based on immature morphology and location near the normal site of islet development. In some mice, radiating cords of islet-type cells emanated from periductular regions and extended haphazardly into surrounding acinar regions (Figure 9). In many diabetic male mice, pancreatic ducts were ectatic and filled with secretory material, suggesting hypersecretion and/or outflow obstruction. Leakage from ectatic ducts may have resulted in a sclerosing pancreatitis observed in one aged, diabetic male (Figure 9). Although remarkable in presentation and variety, pancreatic islet histopathology did not correlate with glucosuria nor serum

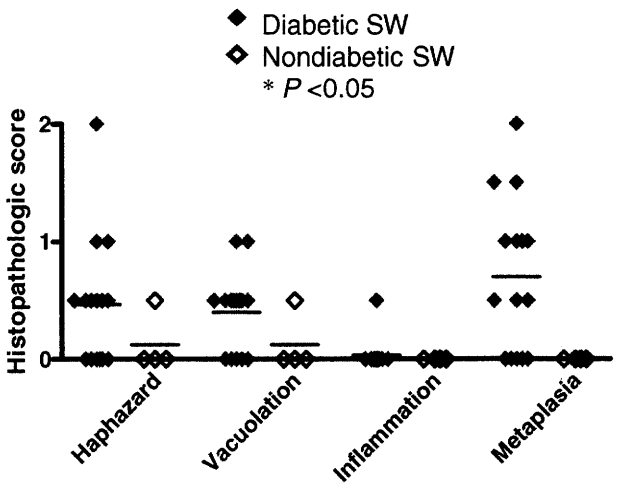

Figure 8: Pancreatic pathology scores in diabetic (n=15) and nondiabetic (n=4) colony SW mice. The pancreata of diabetic and nondiabetic mice were scored from 0 (none) to 4 (severe) for islet haphazard arrangement, vacuolation, inflammation and changes consistent with islet metaplasia. Islet metaplasia was only observed in diabetic males.

glucose or insulin levels in mice for which data were available. No significant pancreatic lesions were observed in female mice regardless of body weight or blood glucose level.

Diabetic mice were at increased risk for renal disease including increased numbers of mesangial cells, mesangial matrix expansion, and dilated renal pelvices as listed in Table 2. Kidney lesions in diabetic mice were more severe with advancing age, displaying membranous glomerulopathy characterized by decreased erythrocyte profiles and thickened endothelial basement membranes (Figure 9). A portion of diabetic mice developed ascending pyelonephritis as evidenced by kidney abscessation, pyuria and bactiuria. Intralesional bacterial colonies were readily apparent by both H&E and tissue Gram's stain.

Table 2: Kidney lesions in diabetic and nondiabetic colony SW mice at ~6mths

	Numbers of Mice (n=20)		
	Diabetic (n=16)	Nondiabetic (n=4)	
	Males (n=16)	Males (n=2)	Females (n=2)
Increased mesangial cells	10	1	0
Mesangial matrix expansion	9	1	0
Pyelonephritis	2	0	0
Glomerulosclerosis	2	0	0

Livers of aged (>1 year) diabetic males (n=12) and less frequently in mice less than one year of age exhibited moderate to severe centrilobular glycogen-associated hydropic degeneration superimposed over microvesicular and macrovesicular steatosis, and several had hepatocellular carcinoma. Steatosis was centrilobular in distribution and adjacent mid-zonal hepatocytes frequently demonstrated cellular atypia and an increased mitotic rate. Lipid-associated changes were histologically consistent with human non-alcoholic fatty liver disease (NAFLD). Liver tumors in diabetic males were consistent with HCC displaying cellular atypia and pleomorphism, local invasion into

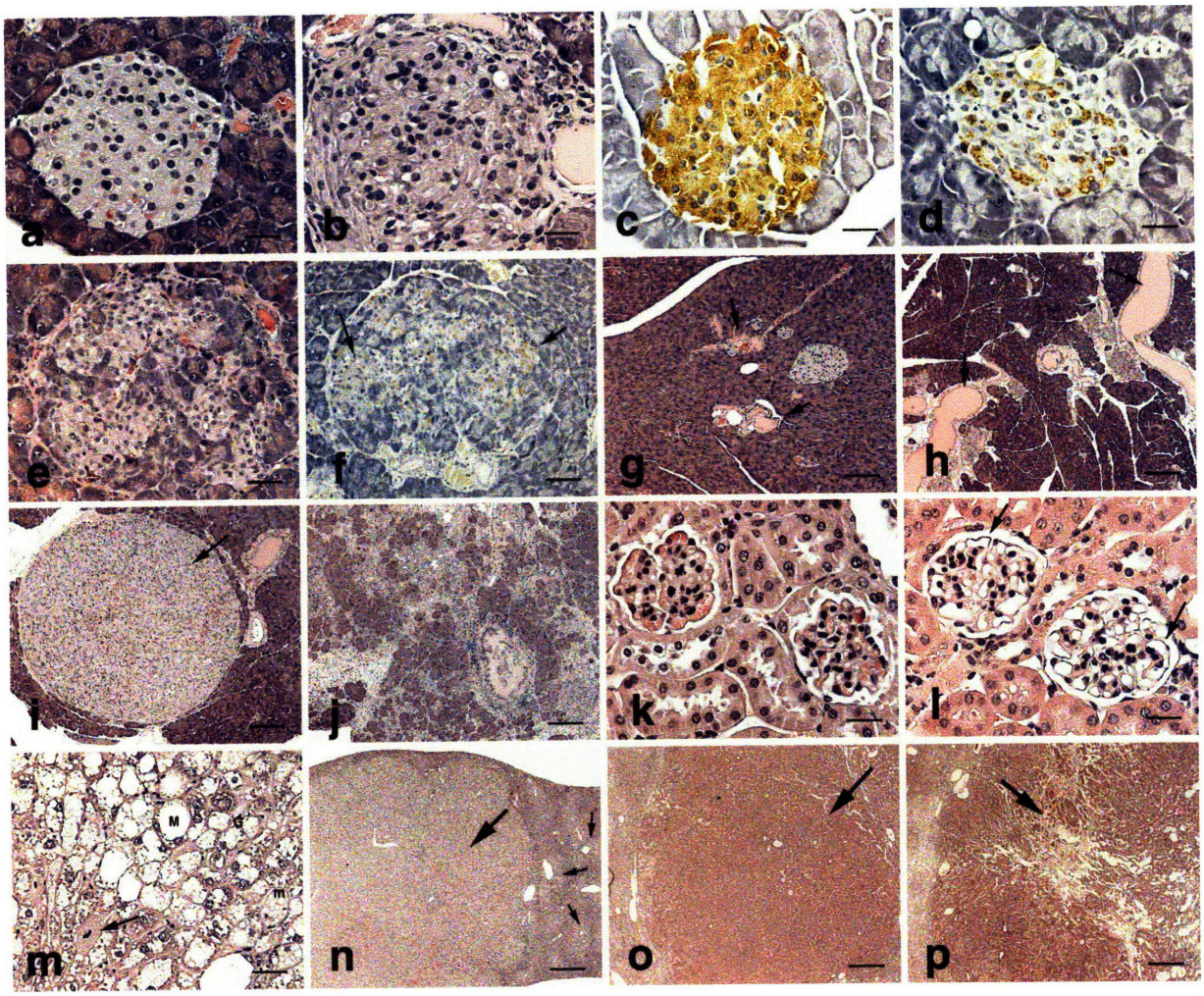

Figure 9: Histopathologic and immunohistochemical features of SW T2D. (a) Normal pancreatic island from obese but nondiabetic female mouse. **(b)** Islet from diabetic male demonstrating irregular cell arrangement and variable cytoplasmic swelling by granular to fibrillar pale eosinophilic material. **(c)** Insulin immunohistochemistry (IHC) demonstrating uniform distribution of insulin in female pancreatic islet. **(d)** Insulin IHC of diabetic male pancreatic islet shows patchy punctate staining in a subset of cells. **(e)** Islet metaplasia characterized by newly encapsulated sublobule containing a mixture of exocrine and endocrine pancreatic cell phenotypes. **(f)** Insulin IHC of islet metaplasia highlights irregular expression limited to endocrine cell phenotypes (arrows). **(g)** Female pancreas demonstrating normal ducts (arrows). **(h)** Diabetic male pancreas with ectatic ducts filled with secretory material (arrows) suggesting hypersecretion and/or outflow impairment. **(i)** Mega-islet in diabetic male. **(j)** Sclerosing pancreatitis in diabetic male characterized by replacement of acinar cells with wide bands of fibrous connective tissue, and mixed inflammatory cells surrounding ducts and infiltrating septa. **(k)** Normal glomeruli from female kidney. **(l)** Membranous glomerulopathy of kidney in diabetic male characterized by decreased erythrocyte profiles and thickened endothelial basement membranes (wireloop capillaries; arrows). **(m)** Liver of aged (>1 year) diabetic male demonstrating mixed fatty and hydropic hepatocyte degeneration with large lipid vacuoles (macrosteatosis; "M"), small lipid vacuoles (microsteatosis; "m"), and cloudy swelling characteristic of glycogen accumulation ("G"); adjacent hepatocytes had an increased proliferative index (mitotic figure at arrow). **(n)** Clear cell hepatocellular carcinoma (HCC; large arrow) arising from liver lobe of aged male mouse with preexisting centrilobular fatty and hydropic degeneration (small arrows). **(o)** Solid nodule (arrow) within larger mixed phenotype HCC. **(p)** Trabecular and pelioid tumor differentiation (arrow) within mixed HCC. Hematoxylin & eosin (a, b, e, g—p), insulin IHC (c, d, f); bar = 25 μm (a—d, k, l), 50 μm (e, f, m), 250 μm (g—j), 500 μm (n—p).

surrounding normal parenchyma, and a high mitotic index. HCCs were often the solid variant comprised primarily of clear cell hepatocytes. Mixed tumors with sublobules comprised of eosinophilic cells in a trabecular or pelioid pattern were also represented (Figure 9). Compared to diabetic males, no females ($P = 0.0079$) or nondiabetic males ($P = 0.0667$) developed liver tumors. Neither hepatitis nor fibrosis, were significant features in any of the mice, and no significant hepatic lesions were observed in SW <6 months of age.

No significant cardiovascular or neurologic lesions were observed in these mice by the age of 12 months.

Bacteriology

Positive cultures obtained from urine were primarily mixed infections, and included *Escherichia coli, Staphylococcus* spp, *Proteus* spp, and *Enterobacter aerogenes*. Occasionally, *Staphylococcus* spp were isolated from kidney, mandibular and musculoskeletal abscesses.

Immunohistochemistry

In diabetic male SW mice, degenerate islets showed patchy insulin positive cells and poor staining intensity compared with nondiabetic animals (Figure 9). Islet cells positive for insulin were generally larger than surrounding cells and had prominent, vesicular nuclei, suggestive of compensatory hypertrophy. Insulin IHC confirmed the presence of islet metaplasia by demonstrating isolated insulin-producing cells within the exocrine

pancreas and/or foci of insulin-producing cells admixed with exocrine-like cells in poorly defined islet-like sublobules as described previously (Figure 9). Small nests of insulin-positive cells located adjacent to pancreatic tubules were suspected to represent islet neogenesis (6), although specific cell lineage could not be determined morphologically. All female SW mice showed uniform, robust staining for insulin within islets. Isolated insulin-positive cells were extraordinarily rare in the exocrine pancreas of nondiabetic mice.

Discussion

We describe a cohort of outbred, male Swiss Webster mice and their offspring with clinical and biochemical signs consistent with the metabolic syndrome and type 2 diabetes mellitus (T2D) in humans, characterized by insulin resistance and abdominal obesity. Clinical signs of T2D in male SW mice, including polyuria, polydipsia and glucosuria were evident at an average age of 18 weeks. In these mice, a high incidence of diabetes was observed, approaching 60% in colony SW (n=70) and 80% in dSWs (n=20) by 30 weeks of age. The high prevalence of diabetes in the MIT SW breeding colony is suggestive of a founder effect—a loss of genetic variability initiated when the MIT colony was established from a relatively small number of animals from the original population of SW mice obtained from an outside source. Although mice from this colony are not typically assigned to research protocols, the affect of diabetes on the utility of SW mice for rodent health monitoring remains unknown. To limit the potential impact on

rodent health, the MIT breeding colony was depopulated and renewed with additional mice from a different commercial vendor.

Initially, diabetic males showed increased body weight and grossly observable fat stores within the abdominal cavity compared with nondiabetic males; however, advanced disease resulted in declining body condition and loss of mesenteric and epididymal fat pads. Fasting glucose tolerance tests performed in diabetic mice (nonfasting blood glucose > 300mg/dl, glucosuria positive) confirmed altered glucose homeostasis compared with nondiabetic mice showing elevated glucose response curves with a delayed return to baseline. Although mice were at variable stages of disease during glucose tolerance testing (10-13 months of age), previously determined nonfasting hyperglycemia and glucosuria were predictive of elevated glucose response curves, despite the presence of liver tumors.

Diabetic colony SW and dSW males showed variable insulin levels that displayed a nonlinear correlation with blood glucose concentration. In the diabetic males (blood glucose >300mg/dl), insulin levels were inversely proportional to the degree of hyperglycemia. Elevated serum insulin concentrations were seen at relatively mild stages of hyperglycemia. Mice with high insulin and mild hyperglycemia may reflect a compensated stage of diabetes with pancreatic beta cells producing increased insulin in response to insulin resistance. Increased beta cell mass has been described in numerous mouse models of spontaneous T2D, including the CBA/Ca, db/db mouse, and high-fat diet models(15, 27, 36), as well as humans (7). In our outbred cohort,

interindividual differences in pancreatic histopathologic presentation among diabetic males obscured any potential relationships between islet morphology and circulating insulin levels. Beta cell degeneration and subsequent 'burnout' could result from glucotoxicty, lipotoxicity or defective stimulation. This possibility is supported by the observation that beta cell degeneration was not seen in female mice. Importantly, beta cell degeneration with apoptosis and increasing levels of hyperglycemia have been described in diabetic rodents and humans and are important in the pathogenesis of T2D (3, 10, 25).

Although not directly correlated with circulating insulin or leptin levels, pancreata from male diabetic mice had mild to severe islet cell degeneration with islet metaplasia. Regions of islet metaplasia were presumed to represent the transdiffereniation of pancreatic cells from an exocrine to an endocrine phenotype. Islet neogenesis, defined by poorly arranged islet cells in the normal periductular location, is reported in rodent models of pancreatic injury (streptozotocin administration, ischemia, subtotal pancreatectomy), transgenic mice, and in spontaneously hyperglycemic rats (6, 28, 39). Additionally, neogenesis of human pancreatic islets has been described in obese and diabetic subjects (10). However, to our knowledge, it has not been previously reported in spontaneous outbred mouse models of diabetes mellitus. Islet neogenesis is thought to occur as a compensatory mechanism to increase beta cell mass and number, due to increased insulin demands.) The presence of histologic features such as the formation of megaislets and transdifferentiation/neogensis coupled with qualitatively inadequate production of insulin in from these islets (patchy, sparse insulin staining by IHC

compared with nondiabetic animals) is suggestive of a potential defect in islet production of insulin. However, the physiologic mechanisms underlying these compensatory responses are incompletely understood. Because this model shows a robust pancreatic islet cell response to the insulin resistant state, it should prove useful in exploring these mechanisms.

Similar to humans, the most notable features of the metabolic syndrome in the SW and dSW were abdominal obesity and insulin resistance. While a significantly higher lipemic index was observed in diabetic males compared with nondiabetic males, no grossly identifiable atherosclerotic plaques were seen in the proximal aorta, and systolic blood pressures were not measured. More extensive studies are required to fully ascertain the risk for cardiovascular disease in this model. Unlike widely used mouse models of the metabolic syndrome, the diabetic SW are maintained on a standard rodent diet, as opposed to experimental "westernized" or high fat diets. While environmental factors cannot be ignored, the SW and dSW may prove useful in exploring genetic mechanisms behind the development of the metabolic syndrome.

Diabetes mellitus, obesity and hepatosteatosis are known risk factors for development of HCC in human populations, predominantly in males (11, 13, 20). Liver tumors consistent with clear cell hepatocellular carcinoma and mixed HCC were seen in 75% of aged, diabetic male SW and dSW (n=12) associated with a background macrosteatosis, microsteatosis and hydropic change consistent with non-alcoholic fatty liver disease (NAFLD) in humans (14). Studies of NAFLD in obese ob/ob mice have shown evidence

of chronic liver injury with increased hepatocyte proliferation and decreased hepatocellular apoptosis that may explain an increased incidence of HCC in ob/ob mice. However, like the dSW mice, ob/ob mice do not develop evidence of hepatitis or cirrhosis (45). Questions remain as to the role of hyperinsulinemia in carcinogensis. High insulin levels, known hepatocyte mitogens and growth promotants in murine and human hepatocellular carcinoma (5, 34), may have accelerated the growth of tumors resulting from unrelated causes or background genetic mutations. Although limited numbers of aged, nondiabetic male SW were available for study (n=4), no gross tumors were seen in these mice or in aged nondiabetic females. Diabetic SW mice should provide a useful model to explore the mechanisms behind liver carcinogenesis associated with NAFLD in the diabetic state.

Additionally, in some aged mice with extensive HCC, a reversion to euglycemia or hypoglycemia was observed (data not shown). Hypoglycemia can occur in humans with HCC as a paraneoplastic syndrome associated with the production of insulin-like growth factors and/or through progressive malnutrition and increased glucose demands by neoplastic tissue (46). Generally, hypoglycemia associated with HCC is a poor prognostic indicator.

Selected diabetic male Swiss Websters (SW) also showed histologic evidence of glomerulopathy independent of bacterial infection at less than 6 months of age, including basement membrane thickening and expansion of the mesangial matrix. This is similar to other mouse models of spontaneous diabetic nephropathy, including the

db/db mouse (35); however, unlike the db/db mouse, the diabetic SW mice in this study lived beyond 12 months of age without food restriction and provide a useful model to study the natural progression of diabetic nephropathy. The diabetic SW mice also revealed more severe diabetic kidney disease including membranous glomerulopathy as the animals aged. In these mice, the degree and progression of renal insufficiency, a feature lacking from most spontaneous mouse models of diabetes (9), will require further studies.

Diabetic SW mice were also prone to bacterial infections of the urinary tract, in some cases resulting in severe renal disease. Mixed infections by opportunist bacteria were frequently cultured from kidney lesions and urine, including species of bacteria that are associated with urinary tract infection in human diabetics (8) and in mouse models of T2D (38).

Although diabetic mice had moderately to markedly dilated gastrointestinal tracts, most notably in the cecum, no degenerative lesions were observed within small intestinal or cecal myenteric plexi by light microscopy. However, electron microscopy and immunohistochemical staining may be required to demonstrate lesions of the autonomic nervous system consistent with diabetic neuropathy (26). It is also possible that functional perturbances to intestinal motility unassociated with morphologic changes (e.g. to interstitial cells of Cajal) may play a role in the flaccid paralysis of the bowel that was a consistent gross feature of the diabetic males we analyzed. Similar gross lesions have been observed in diabetic rats (22, 23), proposed as a model of gastrointestinal

autonomic neuropathy—a complication commonly seen in humans with type I diabetes (insulin-dependent) mellitus. Signs of digestive dysfunction and delayed gastric emptying (gastroparesis) have also been described in human diabetics and mouse models of T2D (4, 21). Gastroparesis without overt diabetes has also been noted by us in SW female mice (16).

Clinical signs of T2D were not observed in female SW mice. Male predilection for diabetes is commonly reported in mouse models of spontaneous T2D, and may be due to the lack of protective effects of estrogen. In humans, prevalence and progression of diabetes increases in patients with conditions that result in decreased estrogen levels such as polycystic ovarian syndrome and menopause. Studies in humans and mice have also shown that estrogen replacement therapy improves glycemic control and decreases the incidence of diabetes (24).

Female SW mice displayed increased abdominal fat deposition by 10 weeks of age (>30g BW) and marked obesity by 6 months (>50g BW). While overt signs of diabetes were not observed, obese females occasionally developed gallstones without a cholesterol component (assessed by light microscopy utilizing a polarized light filter) that were consistent with either black or brown pigment stones. Gallstones are also seen with increased frequency in human diabetics and are thought to result from gallbladder hypomobility and decreased emptying associated with reduced cholecystokinin-A receptor expression in biliary smooth muscle (12).

Although the female SW in our study consistently produced offspring between 2-4 months of age, no females became pregnant when bred after 8 months of age. This may be due to severe obesity rather than reproductive senescence. Dietary-induced obesity has been shown to reduce fertility by more than 60% in female DBA/2J mice with no effect on male fertility associated with hyperleptinemia and hypothalamic hypogonadism (40). In db/db and ob/ob females, fertility is disrupted by mutation-induced hypercytolipidemic utero-ovarian involution (17); however, histologic evidence of reproductive dysfunction was not found in the SW females. Also, no evidence of gestational diabetes was seen in pregnant SW females. The high successful pregnancy rate of the SW females less than 6 months of age, combined with their ability to successfully raise litters provides an advantage over infertile mouse models of T2D such as the db/db mouse.

The diabetic SW mice in this study share many features common to other mouse models of T2D including moderate obesity, maturity onset glucosuria and nephropathy. Importantly, additional, relevant features including high reproductive performance, a strong pancreatic islet cell response to the diabetic state as evidenced by islet metaplasia, and relatively early-onset kidney disease with biochemical evidence of renal insufficiency; all of which make this model particularly useful (Table 3).

Additionally, the reproductive ability of the female SW and variable alterations in the serum leptin concentrations of SW males, suggest that the leptin/leptin receptor axis is not primarily involved in the pathogenesis of T2D in this model—similar to human T2D,

Table 3: Features of type 2 diabetes in selected spontaneous mouse models

Feature	**diabetic SW**	**db/db**	**KKA^{y}**	**NONcNZO10/LtJ**
Outbred	Yes	No	No	No
Fertile	Yes	No	No	Yes
Obesity	Moderate	Moderate-severe	Severe	Moderate
Hyperglycemia	Moderate	Severe	Severe	Variable
Leptin	Variable	Increased	Variable	Variable
Nephropathy	Yes	Yes	Yes	?
Neuropathy	?	Yes	Yes	?
Polygenic	?	No	Yes	Yes

and unlike the widely used db/db and ob/ob mice. Other important characteristics include the SW mice's outbred background, and potentially the presence of autonomic neuropathy suggested by cecal and intestinal dilation, despite the lack of neuronal lesions visible by light microscopy. Importantly, the diabetic SW spontaneously develops late-onset HCC making them particularly useful for the study of diabetes-associated carcinogenesis.

Pathogenesis of diabetes in the SW follows a predictable course. At an early age, diabetic mice weigh significantly more than their nondiabetic counterparts and develop hyperglycemia at around 18 weeks of age. In mice less than six months of age, the pancreas attempts to compensate for disease by increasing the production of insulin, resulting in the presence of megaislets, pancreatic transdifferentiation and suspected islet neogenesis. As disease progresses, hyperinsulinemia is more prominent until the point of pancreatic decompensation, after which severe hyperglycemia may result (>400mg/dl) as insulin levels decrease. At approximately six months of age, diabetic comorbidities such as kidney and liver disease become apparent in diabetic males. By 12 months of age, most affected mice are entering end stage disease with progressive

cachexia, pancreatic disease and liver disease (hepatosteatosis with hepatocellular degeneration and hepatocellular carcinoma). In end stage diabetes, the pancreas may exhibit zymogen depletion and chronic exocrine pancreatitis due to suspected enzyme leakage from ectactic pancreatic ducts.

The genetic and physiologic mechanisms underlying the diabetic syndrome in this cohort of SW mice remain unknown and are the subjects of further investigation. SW mice retain the ability to breed, are normophagic and have variable serum levels of leptin. In the diabetic SW, serum leptin appears to be more dependent on body condition: as diabetes progresses, blood glucose increases and fat stores are depleted, serum leptin is also diminished. Other mechanisms may involve genes important in the transcription regulatory networks governing the liver and pancreatic islets such as the HNF transcription factors. HNF1α, HNF4α and HNF6 are all required for the normal function of the liver and pancreas, and HNF1α and HNF4α mutations are known to cause maturity-onset diabetes of the young (MODY) in humans (43). The dual involvement of the liver and pancreas in the dSW mice is suggestive of genetic defect affecting both liver and pancreas development; however, unlike some transgenic models of HNF dysfunction in mice that develop T2D and significant liver dysfunction early in life (44), the dSW mice develop liver pathology only after the development of late-stage diabetes.

In conclusion, while this cohort of outbred SW male mice shares some physiologic and biochemical similarities with other mouse models of T2D, differences in parental

background, reproductive capacity and pancreatic response to disease make it a promising new model of the metabolic syndrome, T2D and their comorbidities in humans. Further studies regarding the genetics and pathogenesis of the metabolic syndrome and T2D in this model are in progress.

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Part 4: Future Directions

Part 3 demonstrated unique and clinically relevant features of a novel mouse model of T2D and diabetic comorbidities including liver cancer in humans. While the study represents a thorough clinical description of diabetic features in the dSW mice, further study is required to completely characterize the model, physiologically and genetically. Although these studies were beyond the scope of this project, Part 4 proposes a series of experiments aimed at complete characterization of the dSW mice and is presented in a grant-based format.

Part A: Specific Aims

The overall goal of this project is to determine the pathogenesis of type II diabetes (T2D) in the diabetic Swiss Webster mouse (dSW) and thus validate it as a model of human T2D and its comorbidities, including liver disease. Additionally, the project will identify mechanisms in the pathogenesis of features unique to the model compared with other mouse models of T2D, including the development of hepatocellular carcinoma and pancreatic islet neogenesis/metaplasia.

Specific Aim 1: To determine what metabolic pathways are responsible for the development of murine T2D in the dSW

Aim 1.1: Measure parameters associated with the metabolic syndrome to determine risk factors associated with murine T2D

Metabolic syndrome is a known risk factor for the development of T2D in humans and involves perturbations in lipid and glucose homeostasis, and inflammatory pathways. Studies will be undertaken to measure parameters of the metabolic syndrome as defined by the National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) at various ages in diabetic SW (dSW) and nondiabetic SW (nSW) to determine if similar risk factors predict the development of T2D in this mouse model. Furthermore, biomarkers of T2D risk may also be identified.

***Aim 1.2*: Determine the influence of mitochondrial dysfunction on lipid accumulation in somatic cells**

Next, studies will be undertaken to determine the mechanisms behind lipid accumulation in dSW hepatocytes, adipocytes and skeletal muscle cells. Specifically, genetic mutations and mitochondrial dysfunction will be addressed.

Aim 1.3: Determine the mechanisms of insulin resistance (IR) in hepatocytes and skeletal muscle

Studies will be undertaken to elucidate the insulin signaling pathway from ligand-receptor interactions to intracellular signaling in both dSW and nSW in an effort to understand the mechanisms of IR in the model, utilizing novel techniques in mass spectrometry.

Aim 1.4: Determine the influence of environmental factors such as diet on the pathogenesis of IR and T2D

Excessive dietary fat has been associated with acceleration in the progression and severity of T2D in both human and rodent models. Mice will be fed a high fat

or western diet and compared with control and diet-restricted mice to determine the influence of non-hereditary factors in the progression of T2D and its comorbidities.

Specific Aim 2: To determine the mechanisms of liver carcinogenesis (hepatocellular carcinoma) in the dSW

Aim 2.1: Determine the frequency of genetic mutations in hepatocytes

The diabetic phenotype will be crossed onto a mutation reporter mouse line (BigBlue Assay) and liver cells will be collected to determine mutation frequency. If the transfer of the MIT-SW phenotype is unsuccessful, BigBlue mice will undergo diabetes induction through the administration of streptozotocin with subsequent analysis of hepatocytes

Aim 2.2: Determine the influence of DNA repair mechanisms on the development of diabetes-associated hepatocellular carcinoma in dSW

DNA repair mechanisms will be studied in neoplastic and normal hepatocytes of dSW and nSW.

Aim 2.3: Determine the influence of dietary and pathogen-mediated factors on the incidence of diabetes-associated hepatocellular carcinoma in dSW

dSW and nSW mice will be fed a high fat diet and compared to control and diet restricted animals to observe for differences in tumor incidence and morphology, as well as the progression/severity of nonalcoholic fatty liver disease (NAFLD). Inflammation, if present, will be assessed by qPCR analysis of mRNA for genes crucial in the inflammatory response.

Specific Aim 3: To determine the mechanisms of pancreatic response to IR and T2D in the MIT-SW

Pancreatic islet cells from normal, hyperplastic, neogenic and metaplastic islets from dSW and nSW will be isolated to determine the insulin production and response to exogenous glucose. Messenger RNA from these pancreatic islet cells will be isolated and subjected to microarray analysis to determine the up- and down-regulation of various genes important in the development and function of pancreatic beta cells.

Part B: Background and Significance

Disease Epidemiology and Significance

Despite increased awareness of the obesity epidemic and a higher incidence of the metabolic syndrome in humans, the incidence of obesity and its comorbidities—most notably, type II diabetes (T2D) and cardiovascular disease (CVD)—continues to increase in both adults and adolescents(Rosenbaum, Nonas et al. 2004). Metabolic syndrome is characterized by the presence of at least three of the following risk factors: abdominal obesity, dyslipidemia, hypertension, insulin resistance, a proinflammatory state (elevated C-reactive protein), and a prothrombotic state (elevated plasma plasminogen activator inhibitor) (Grundy, Brewer et al. 2004). A majority of affected humans are obese and insulin resistant, conferring an increased risk for T2D (Grundy, Brewer et al. 2004). Humans with T2D have a relative insulin deficiency characterized by fasting hyperglycemia due to peripheral insulin resistance combined with an insulin

secretory defect (i.e. inability to increase beta cell mass). An estimated 5-10% of the U.S. population suffers from T2D (Gregg, Cadwell et al. 2004; Sullivan, Morrato et al. 2005) and are at an increased risk for developing microvascular and neuropathic complications, macrovascular disease, microbial infections and neoplasia. Together with diabetic complications, T2D accounts for more than 92 billion dollars in direct health care costs in the U.S. alone with an additional 40 billion in indirect medical expenses (Yach, Stuckler et al. 2006).

Mouse Models of Type 2 Diabetes

Several spontaneous and induced genetic mutations resulting in varying degrees of obesity and diabetes have been described in mice, making them popular models in diabetes research (Accili 1995; Rees and Alcolado 2005). Mutation of the leptin or leptin receptor gene produces obesity and T2D in the ob/ob and db/db mouse respectively. The db/db mutation is available on a variety of mouse strain backgrounds, is well characterized, and displays many of the comorbidities of T2D found in humans, including cardiovascular disease, renal disease and neuropathies (Robertson and Sima 1980; Sharma, McCue et al. 2003; Ye, Donthi et al. 2005). However, these mice represent a monogenic model demonstrating features related to leptin resistance, including morbid obesity, hyperphagia, and alterations in thermoregulation and fertility. In contrast to popular mouse models of diabetes, common forms of T2D in humans are considered polygenic disorders, occurring through the simultaneous action of several genes and rarely involving alterations of the leptin/leptin receptor axis. Despite the wide availability of numerous rodent models of T2D, no mouse model completely

recapitulates human T2D or the metabolic syndrome and therefore, novel animal models are desired.

Much of the morbidity and mortality associated with diabetes is a result of various diabetic complications affecting numerous tissues including the liver, gastrointestinal tract, neuronal tissues and the kidney. However, mouse models of diabetic comorbidities are incomplete and no single mouse model consistently develops the full spectrum of diabetic complications. For example, while renal insufficiency is common in human cases of diabetic nephropathy, has not been shown to reliably occur in mice, despite the presence of kidney lesions (Sharma, McCue et al. 2003). Improved animal models in these realms could facilitate preclinical testing of diagnostic, preventive or therapeutic interventions.

Insulin Resistance and Mitochondrial Dysfunction

Insulin resistance has been recognized as the fundamental underlying metabolic defect in the pathogenesis of metabolic syndrome, a clustering of risk factors for heart disease and T2D that include central obesity, dyslipidemia and hypertension (Grundy, Brewer et al. 2004). Recent studies have linked mitochondrial dysfunction to the development of insulin resistance and obesity in humans, demonstrating abnormal mitochondrial structure, number and gene expression in diabetic patients and their offspring(Wiederkehr and Wollheim 2006). Mitochondrial DNA (mtDNA) mutations are also known to cause diabetes by affecting insulin secretion from pancreatic beta cells (Nomiyama, Tanaka et al. 2004). Furthermore, humans and animal models of obesity

and insulin resistance show improvement in insulin sensitivity and fat metabolism when treated with substances that enhance the function of mitochondria (Gumieniczek, Hopkala et al. 2006; Waugh, Keating et al. 2006) such as pioglitazone, a peroxisome proliferator-activated receptor gamma (PPAR γ) agonist.

Deciphering the role that mitochondria play in the development of insulin resistance is confounded by the influence of obesity on mitochondrial function. Obesity in rodent models has been shown to significantly reduce the numbers of mitochondria within adipocytes as a result of the expanding fat droplet displacing cellular organelles (Choo, Kim et al. 2006). Additionally, cellular lipid accumulation and changes in fatty acid metabolism that occur in the insulin resistant state leads to an increased cellular oxidative stress (Lin, Berg et al. 2005). The generation of free radicals further damages the highly sensitive mitochondria, resulting in further dysfunction and promotion of insulin resistance and thus, accumulation of cellular lipids. This leads to an augmentative cycle of increased cellular lipid accumulation, increased free radical damage and increased insulin resistance. Oxidative stress and the release of reactive oxygen species (ROS) promote inflammation through activation of NF-κB and have also been implicated in the progression of diabetes complications, including microvascular and macrovascular dysfunction and liver disease (Lin, Berg et al. 2005).

Liver Disease and Type 2 Diabetes

Diabetes mellitus, obesity and hepatosteatosis are known risk factors for development of liver disease in human populations (El-Serag 2004; Davila, Morgan et al. 2005;

Harrison 2006), including nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), glycogenic hepatopathy, cryptogenic cirrhosis and hepatocellular carcinoma (HCC). Despite the known association, studies regarding the mechanisms of liver carcinogenesis in the diabetic/insulin resistant state are lacking. Regardless of the mechanism, hyperinsulinemia and insulin resistance have been shown to be pro-inflammatory and profibrinogenic in the liver (Bugianesi, McCullough et al. 2005) and insulin is a known growth promotant for isolated hepatocellular carcinoma cells (Saito, Inoue et al. 2002; Boissan, Beurel et al. 2005). Further research into mechanisms of liver carcinogenesis associated with diabetes and the metabolic syndrome may uncover additional biomarkers and novel drug targets in human populations.

Part C: Preliminary Studies

Background:

Discovery of Diabetic Swiss Webster Mice at MIT

We previously identified a cohort of male, outbred Swiss Webster (SW) mice in an MIT breeding colony that required frequent cage changes due to urine-impregnated bedding, and more frequent water bottle changes (i.e. polyuria/polydipsia). Five males were isolated from the colony for further study. Metabolic comparisons with age-matched, control males showed markedly increased urine production and water consumption in the affected mice; however, there were no significant differences in food consumption or body weight (data not shown). Additionally, affected mice were glucosuric, and serum

chemistry analysis with insulin levels on one mouse revealed hyperglycemia, hypercholesterolemia, azotemia and hyperinsulinemia—signs consistent with both metabolic syndrome and T2D in humans. Histopathology of selected affected mice revealed no evidence of insulinitis or pancreatic pathology.

Part D: Research Design and Methods

Routinely used methods

The following methods will be routinely used and thus are described here and referred to throughout the proposal.

Blood collection and determination of blood glucose concentration

Whole blood is collected from unanesthetized mice following an overnight fasting period via puncture of the submandibular vascular bundle using an appropriately sized mouse lancet (Medipoint Inc, Mineola, NY). When necropsies are performed, whole blood is collected from fasted mice via cardiac puncture, immediately following euthanasia. Blood glucose concentration is determined from whole blood utilizing a bedside glucometer (LifeScan Inc, Miltapas, CA).

Husbandry, Monitoring of body weight and urinary glucose

Mice will be housed in polycarbonate microisolator cages on hardwood bedding (PharmaServ, Framingham, MA) under specific pathogen free (SPF) conditions (free of *Helicobacter* spp., *Citrobacter rodentium*, *Salmonella* spp., endoparasites, ectoparasites

and known murine viral pathogens) in an Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility. Mouse rooms will be kept at constant temperature and humidity on a 12:12 hour light to dark cycle. Mice will be regularly weighed and manually restrained to obtain free-catch urine samples. These samples will be directly applied to urinary glucose test strips (Diastix®) for determination of the presence or absence of urinary glucose according to the manufacturer's instructions.

Dietary manipulation

Mice receiving the high fat diet (HFD) will be weaned at three to four weeks of age and placed on a western-styled diet (TestDiet 5TJN: 40 kcal% fat) manufactured by Purina Mills (St. Louis, MO) until the termination of study. The "Western" diet for rodents is based on AIN-93G contains 4.49 kcal/g with 20% fat (providing 30% of fat from lard, 30% from butterfat, 30% from hydrogenated vegetable oils, and for esterified fatty acids, 7% from soybean oil and 3% from corn oil), 49.5% carbohydrates, and 18.3% protein. Approximate energy (kcal) is from fat 40%, carbohydrate 44%, protein 16% (protein:fat = 2:5) with 0.15% cholesterol added. Those animals receiving control diets are fed the standard mouse diet 5015 manufactured by Purina Mills (St. Louis, MO) until the termination of study. The standard diet contains 3.73 kcal/g, with10% fat, 53.5% carbohydrates, and 17.5% protein. Approximate energy (kcal) is from fat 25.8%, carbohydrates 55.9% and protein 18.3%. Mice receiving dietary restriction will be fed 70% of the consumption (30% dietary restriction) of the control diet fed mice according

to standard protocols. Diet restriction mice will not be allowed to fall below 75% of the expected body weight based on the control diet fed counterparts.

***Specific Aim 1*: To determine what metabolic pathways are responsible for the development of murine T2D in the dSW**

***Aim 1.1*: Measure parameters associated with the metabolic syndrome to determine risk factors associated with murine T2D**

Background

As demonstrated in our preliminary studies, the dSW mice develop evidence of the metabolic syndrome similar to humans, including obesity, insulin resistance and hyperlipidemia. To determine if dSW develop the full spectrum of criteria for the diagnosis of metabolic syndrome or subsets of risk factors that will predict the development of T2D, dSW and nSW will undergo rigorous metabolic characterization to include blood pressure analysis, serum lipid analysis and characterization of a whole body inflammatory and prothrombotic states.

Experimental Methods

Aim 1.1 will be executed in conjunction with Aim 1.4 to determine the influence of high fat diet on these risk factors. All mice tested will originate from the segregated diabetic colony at MIT housed under SPF conditions and will include animals from generation 5 and beyond. Animals will be divided into 2 experimental groups and 1 control group,

each containing 10 males and 10 females from the diabetic and 10 males and 10 females from the nondiabetic lines (n=120 mice total). The control group will be maintained on *ad libitum* standard rodent chow, and the HFD and RD groups will be fed as previously described. Individual animals will be subject to longitudinal studies with time points at 3 months (prediabetic) and 6 months (diabetic). The following parameters will be assessed: whole body composition (fat analysis), systolic blood pressure, and rodent multi-analyte serology (59 plasma biomarkers of inflammation and metabolic homeostasis, including C-reactive protein, apolipoprotein, fibrinogen, leptin and insulin). Particular attention will be paid to markers of inflammation to assist in the diagnosis of metabolic syndrome. Following the 6 month assessment, half of the animals will be humanely euthanatized using CO_2 overdose and tissues collected for histopathological analysis to monitor the development of T2D comorbidities such as liver, kidney and gastrointestinal disease. The remaining animals will be aged to 12 months, after which they will be euthanatized and tissues collected for histopathology.

Body composition analysis

Body composition will be measured in tribromoethanol-anesthetized mice using a dual energy X-ray absorptiometry (DEXA) scanner from Lunar/GE Medical Systems (PIXImus2, distributed by Faxitron X-Ray Corporation, Wheeling, IL), following an overnight fasting period. Values for whole body bone mineral density, bone mineral content, bone area, total tissue mass, total area, fat content, lean content and percent fat (fat content divided by total tissue mass) will be obtained. Additionally, regions of interest such as the abdominal cavity, may be isolated and analyzed separately by

DEXA software for determination of abdominal obesity. DEXA scanning methods have been validated in mice by commercial laboratories (Jackson Labs, Bar Harbor, ME) and individual investigators in obesity-related research (Nagy and Clair 2000). Discrepancies in percent fat content of mice due to variations in tissue hydration will be addressed by calculation of prediction equations for fat content and lean content based on chemical analysis of whole body composition (Pietrobelli, Wang et al. 1998).

Rodent multi-analyte testing

Table 4 Rodent MAP

1. Apolipoprotein A1	31. Insulin
2. CD40	32. IP-10
3. CD40L	33. KC/GROα
4. C-Reactive Protein	34. Leptin
5. EGF	35. LIF
6. Endothelin-1	36. Lymphotactin
7. Eotaxin	37. MCP-1
8. Factor VII	38. MCP-3
9. FGF-basic	39. MCP-5
10. FGF-9	40. M-CSF
11. Fibrinogen	41. MDC
12. GCP-2	42. MIP-1α
13. GM-CSF	43. MIP-1β
14. Growth Hormone	44. MIP-1γ
15. GST-α	45. MIP-2
16. Haptoglobin	46. MIP-3β
17. IFN-γ	47. MMP-9
18. IgA	48. Myoglobin
19. IL-1α	49. OSM
20.. IL-1β	51. RANTES
21. IL-2	51. SCF
22. IL-3	52. SGOT
23. IL-4	53. TIMP-1
24. IL-5	54. Tissue Factor
25. IL-6	55. TNF-α
26. IL-7	56. TPO
27. IL-10	57. VCAM-1
28. IL-11	58. VEGF
29. IL-12p70	59. von Willebrand Factor
30. IL-17	

Whole blood will be collected as previously described into EDTA plasma collection tubes. 50µL of plasma will be used to analyze 59 plasma biomarkers (Table 4) utilizing antigen-capture immunofluorescence Luminex technology (Luminex Corp, Austin, TX). Plasma lipid profile will also be assessed for triglycerides, cholesterol and chylomicrons (VLDL, LDL, HDL). Data mining will then be performed using a software program from OmniViz, Maynard, MA, that groups samples by their similarities in analyte expression patterns to generate a unique chemical signature using the concentration of the analytes measured in each sample. The relationship of each sample signature is then determined in a proximity map (Galaxy™ projection). Data mining will thus aid in identification of potential biomarkers of T2D and the metabolic syndrome.

Systolic blood pressure monitoring

Systolic blood pressure will be determined using the Visitech BP-2000-M4 Noninvasive Blood Pressure Analysis System (Visitech, Cary, NC). Following a 5-day training period to limit stress influences on blood pressure, mice will be tested over a three-day period at the same time every testing day. Blood pressure measurements will be averaged over the three days.

Glucose Tolerance Testing (Insulin Resistance)

After an overnight fasting period, a small amount of blood will be collected from the lateral saphenous vein (lateral tail vein will not be used due to sample site variations in biochemical parameters) to obtain a baseline blood glucose concentration as previously described. Following baseline measurements, each mouse is challenged with 1.5mg glucose/gram body weight (75mg/mL D-glucose stock solution) injected intraperitoneally. Twenty minutes following the glucose injection, blood is obtained from the previous bleeding site and a post-challenge blood glucose measurement is obtained. Insulin resistant mice are expected to have higher blood glucose values post-challenge and a longer time to return to baseline.

***Aim 1.2*: Determine the influence of mitochondrial function on lipid accumulation in somatic cells (adipose, liver, heart, skeletal muscle)**

As demonstrated in preliminary studies, dSW display increased body weight early in the disease process and body weight is a key determinant in onset of diabetes mellitus and glucosuria. Recent human studies suggest that mitochondrial dysfunction may lead to

lipid accumulation in cells, and subsequently, numerous metabolic disorders including obesity and insulin resistance. As lipid accumulation occurs, oxidative stress is increased resulting in deteriorating mitochondrial function. Mouse experiments have suggested decreased numbers of mitochondria in the db/db mouse adipocytes, but not the ob/ob mouse. This aim hopes to identify mitochondrial dysfunction as a key mechanistic etiology in the development of the obese and diabetic phenotypes observed in the dSW. If mitochondrial dysfunction is found to occur in the dSW, further identification of the pathways affected may identify additional genetic determinants of T2D. Mitochondrial number and function will be examined in mice from the dSW and nSW lines at 8-12 weeks of age (prior to onset of gross obesity) and at 6 months of age (obese and diabetic). Five males and 5 females from each line will be assessed.

Mitochondria Quantification in Tissues

Mice will be humanely euthanatized using CO_2 overdose and tissue from the liver, epididymal or ovarian fat pad, cardiac, and gastrocnemius muscle will be collected and fixed in 4% neutral buffered formalin. Tissues will be embedded in paraffin and sectioned at 4µm, deparrafinized in xylene and rehydrated using 100% and 95% ethanol. For mitochondrial staining, sections will be incubated with 250nmol/L MitoTracker (Molecular Probes) for 1hr at room temperature, then observed using fluorescent microscopy. For quantification of mitochondria, 10X fields will be analyzed using Openlab Image analysis software (Improvision Inc., Lexington, Massachusetts, USA) with the results are expressed as the percentage of the total area of each tissue section.

Mitochondrial Function Assays—Oxygen Consumption

Oxygen consumption is an indirect measure of metabolic activity in cells, dependent on mitochondrial oxidative respiration. To assess this activity, oxygen consumption of collected adipocytes, hepatocytes, myocardial and skeletal muscle cells will be assessed using the BD™ Oxygen Biosensor System (BD Biosciences, San Jose, CA) according the manufacturer's instructions. Briefly, isolated cells will be aliquotted into the Biosensor plate in duplicate, sealed and analyzed using a PowerWave Select multimode microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 1-minute intervals for 60 minutes at an excitation wavelength of 485 nm and emission wavelength of 630 nm.

Mitochondrial Function Assays—Fatty Acid Oxidation

Isolated adipocytes and hepatocytes will be aliquotted into 50-ml polystyrene tubes, and [14C]palmitic acid (Perkin Elmer Life Sciences, Wellesley, MA) will be added to a final concentration of 200 nCi/ml. Then, an uncapped Eppendorf tube containing a piece of No. 1 Whatman filter paper soaked in 300 µl benzethonium hydroxide (Sigma-Aldrich, St. Louis, MO) will placed inside each 50-ml tube and incubated 2 hours at 37°C, after which 12 M HCl will be added to the cells to release the $[14C]O_2$. Released $[14C]O_2$ will be allowed to absorb to the filter paper overnight, then quantified by scintillation counting of the filter paper.

***Aim 1.3*: Determine the mechanisms of insulin resistance (IR) in adipocytes, hepatocytes, pancreatic islet cells and skeletal muscle**

Recent advances in phosphoproteome analysis by mass spectrometry have allowed the identification of protein phosphorylation events regulating signal transduction cascades associated with insulin signaling. Numerous, previously unrecognized tyrosine phosphorylation sites have been identified in normal adipocytes following temporal insulin stimulation, implicating these proteins in insulin action. By utilizing similar techniques in the dSW mice, insight into the mechanisms of insulin resistance may be gained, and potentially novel defects may be discovered. This may identify novel drug targets and biomarkers of T2D in humans. Analysis at various time points within the pathogenesis of dSW T2D will be undertaken to identify early and late stage events in the pathogenesis of insulin resistance resulting in T2D occurring in multiple tissues. Additionally, pancreatic islets from dSW and nSW mice will be isolated to determine the insulin production and response to exogenous glucose that may influence the insulin resistance phenotype (rule in or rule out underlying defect in insulin secretion). Concurrent with measurements of blood insulin concentration and blood glucose concentration as assessed in previous subaims, prediabetic (normal insulin, normal blood glucose), compensated diabetic (high insulin, normal to mild hyperglycemia (<300mg/dL), and decompensated diabetic (variable insulin, hyperglycemia (>300mg/dL) mice will be assessed.

Identification of Experimental Groups

As demonstrated in preliminary studies, no diabetic dSW males are identified prior to 10 weeks of age; however, 80-100% of these mice go on to develop phenotypic T2D. Therefore, prediabetic mice will be selected at 10 weeks to include 10 male dSW, 5 female dSW (low standard deviation in dSW females). Age matched, gender matched nSW will also be selected. At around 3-5 months of age, prediabetic males are identified by rising insulin levels concurrent with glucose concentrations between 200-300mg/dL with peak insulin seen around 300mg/dL. Therefore, at 4 months of age, dSW males will be screened for blood glucose concentrations between 250-300mg/dL and 10 males will be selected for study with 5 age-matched dSW females, 5 nSW males and 5 nSW females. At 6-8 months of age, 80-100% of dSW males are diabetic with glucose concentrations >400mg/dL. These mice will be similarly screened and selected for study at blood glucose >400mg/dL.

Insulin Secretion from Isolated Pancreatic Islets

To separate pancreatic islets from mouse pancreata, mice will be humanely euthanatized by CO_2 overdose and pancreata collected. Collagenase digestion will be performed and islets will be hand picked under a stereomicroscope to avoid exocrine contamination. Islet cells will be cultured in a low-glucose Krebs solution containing (in mM) 2.8 glucose, 102 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.7 $CaCl_2$, 20 HEPES, 5 $NaHCO_3$, and 1 mg/ml BSA, pH 7.4 and incubated for 60 min to allow sufficient time for adjustments in metabolism to occur. After 60 min, samples will be collected at 2-min intervals for 10 min, and then the low-glucose solution will be replaced with a high-glucose solution containing (in mM) 11.1 glucose, 132 NaCl, 5 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 5

$NaHCO_3$, and 1 mg/ml BSA, pH 7.3. Samples will then be collected at 2-min intervals for the first 10 min and then at 5-min intervals for the next 20 min. Insulin concentration of collected samples will be measured using a Rat/Mouse Insulin ELISA kit (LINCO Research, St. Charles, MO) according to the manufacturer's instructions.

Identification of Insulin Signaling Pathways

Animals will be humanely euthanatized with CO_2 overdose and necropsied. Epididymal or ovarian adipocytes, hepatocytes and gastrocnemius skeletal muscle will be collected aseptically and individual cells isolated using standard methods. Cells will be stimulated with exogenous insulin for 0, 5, 15, and 45min and subjected to lysis, digestion, clean-up and analysis as previously described (Schmelzle, 2006). Briefly, samples will be labeled with isobaric tags (iTRAQ) and tyrosine phosophorylated peptides immunoprecipitated and enriched by immobilized metal affinity chromatography coupled to tandem mass spectrometry. This will result in identification and relative temporal quantification of tyrosine phosphorylation sites on proteins allowing assessment of defective pathways.

***Aim 1.4*: Determine the influence of environmental factors such as diet on the pathogenesis of IR and T2D**

High fat diet and dietary restriction are shown to influence the pathogenesis of T2D in genetically susceptible humans and rodents. Concurrent with Aim 1.1 animals will be fed a high fat diet or undergo dietary restriction as previously described. Changes in

progression and severity of T2D comorbidities will also be addressed by biochemical and histopathological analyses (see Aim 1.1).

***Specific Aim 2*: Determine the mechanisms of liver carcinogenesis (hepatocellular carcinoma) in the dSW mouse**

As shown in preliminary data, dSW develop hepatocellular carcinoma and varying degrees of macrosteatosis and microsteatosis, making the dSW a model for liver carcinogenesis in the diabetic state, and potentially, nonalcoholic fatty liver disease (NAFLD). Diabetic humans also show an increased risk of HCC; however, little is known regarding the mechanisms behind this increased risk. As a starting point for addressing the mechanism behind liver carcinogenesis in T2D, the frequency of genetic mutations and mechanisms of DNA repair in diabetic hepatocytes will be assessed. Additionally, to fulfill Aims 1.4 and 2.3, high fat diet and dietary restriction will be used to determine environmental influences on these mechanisms.

***Aim 2.1*: Determine the frequency of genetic mutations in hepatocytes**

Big Blue® Transgenic mice (Stratagene, La Jolla, CA) have the *LacZ* and *Lambda cII* genes incorporated into the genome, which allows researchers to efficiently package DNA from any tissue of interest and determine a quantitative level of mutagenesis. These mice will be used to asses the mutation frequency of hepatocytes in the dSW, HCC-prone mice by crossing the diabetic phenotype onto the mutation reporter mouse line. If the transfer of the dSW phenotype is unsuccessful, Big Blue® mice will undergo

diabetes induction through the administration of streptozotocin with subsequent analysis of hepatocytes, to address the general role of T2D in liver mutagenesis.

Induction of T2D in Big Blue® Mice

Ten week old dSW males will be crossed with age-matched Big Blue® C57BL/6 female homozygous mice and the F1 offspring monitored for the development of glucosuria for up to 6 months, concurrent with genotyping to ensure the continued incorporation of the *LacZ* and *Lambda cII* genes. Diabetic F1s will be used to develop a line of diabetic Big Blue®-Swiss Webster hybrids for future analysis. Alternatively, if difficulties arise with maintenance of the transgenes in hybrid mice, dSW female mice crossed to Big Blue® C57BL/6 males may be used. Concurrently, a line derived from the nSW mice will also be developed. If breeding strategies prove unsuccessful in producing diabetic Big Blue®-Swiss Webster hybrids, T2D will be induced in Big Blue® mice by the administration of streptozotocin utilizing standard methods (40mg STZ/kg body weight intraperitoneally for five consecutive days) at 10 weeks of age. Once diabetes is established in the Big Blue® mice (dSW hybrids or STZ), offspring will be divided into high fat diet, control diet and dietary restriction groups.

Quantification of Genetic Mutations in Hepatocytes of Diabetic Big Blue® Mice

Mice will be humanely euthanatized using CO_2 overdose at 6 months of age and the left lateral lobe of the liver will be collected for analysis at the lateral aspect. Genomic DNA will be isolated from liver tissue using the High Pure PCR Template kit (Roche Diagnostics, Pleasanton, CA). The LambdaSelect-cII™ mutation assay kit will be used

to quantitate mutation frequency based on the selection of mutations in the cII gene of the E. coli phage lambda.1. The cII gene encodes a protein that activates transcriptional promoters in lambda that are essential for lysogenization, and mutations in the cII region that lower the levels of cII protein result in a decreased ability of lambda to lysogenize. DNA will be packaged into the phage vector and grown under conditions that favor lysogeny under selective and nonselective media. Lambda prophages that carry mutations (cII-) will survive only by entering the lytic pathway, forming plaques (as opposed to wild-type DNA, which becomes incorporated into the DNA of the bacterial lawn). Therefore, only mutated phage can form plaques greatly reducing the odds of obtaining ex vivo mutations (from E. coli and not model system). Mutation frequency will be calculated by dividing the total lambda cII- plaque forming units (pfu) by the total number of pfu's screened.

***Aim 2.2*: Determine the influence of DNA repair mechanisms on the development of diabetes-associated hepatocellular carcinoma in dSW**

Alterations in DNA repair mechanisms have been implicated in the pathogenesis of HCC in humans with or without steatohepatitis; however, despite known oxidative damage that occurs within hepatocytes during progression of NAFLD, little work has addressed the role of DNA repair mechanisms in the development of HCC in diabetic patients. Certain DNA mismatch repair (MMR) genes are highly conserved amongst eukaryotes, including *Msh2* (required for all mismatch correction in nuclear DNA), *Msh3* (forms Msh2/ Msh3 heterodimers and plays the major role in recognition of mismatched DNA in eukaryotic MMR), *Msh6* (repair of distinct mismatched DNA during replication)

and *Mlh1*. These genes have also been implicated in various gastrointestinal, epithelial and other tumors of mice; therefore, gene expression of *Msh2, Msh3, Msh6,* and *Mlh1* will be studied in neoplastic and normal hepatocytes of dSW and nSW undergoing various dietary manipulations as described previously.

Gene Expression Anaylsis of Hepatocyte DNA Mismatch Repair (MMR) Genes

As shown in preliminary studies, dSW mice develop HCC at an increased frequency compared with nondiabetic mice by 12 months of age. If tumors are present in mice removed from study under Aim 1.1 at 12 months of age, mRNA will be collected from neoplastic and normal tissue using the Qiagen RNeasy Micro Kit and converted to cDNA utilizing Invitrogen SuperScript® III First-Strand Synthesis SuperMix. Purified cDNA will be analyzed via quantitative qPCR for the expression of murine *Msh2, Msh3, Msh6,* and *Mlh1,* utilizing commercially available primers and probes (Applied Biosystems). qPCR will be performed utilizing the Taqman® 7700 system and genes of interest normalized to *Gapdh*.

***Aim 2.3*: Determine the influence of dietary factors on the incidence of diabetes-associated hepatocellular carcinoma in dSW**

dSW and nSW mice fed a high fat diet or undergoing dietary restriction will be collected from studies under previous aims to observe for differences in tumor incidence and morphology, as well as the progression/severity of nonalcoholic fatty liver disease (NAFLD). Inflammation, if present, will be assessed by qPCR analysis of mRNA for

genes crucial in the inflammatory response using commercially available probes as described in Aim 2.2.

Specific Aim 3: To determine the mechanisms of pancreatic response to IR and T2D in the MIT-SW

As demonstrated in preliminary studies, the dSW mice show a robust pancreatic response to the diabetic state—most notably, areas of pancreatic islet metaplasia and suspected neogenesis were observed. While these phenomena have been described in diabetic humans and in rodent models of pancreatic injury, they have not been reported in spontaneous mouse models of T2D. Investigation of the mechanisms driving the pancreatic response to diabetes in the dSW mouse may identify novel pathways in the pathogenesis of pancreatic islet degeneration and regeneration, and provide potential biomarkers and drug targets for human T2D. To accomplish this aim, pancreatic islet cells from normal, hyperplastic (mega-islets), metaplastic (exocrine to endocrine transdifferentiation) and neogenic (endocrine cell clusters and tubular proliferation adjacent to pancreatic ductules) from dSW and normal islet cells from nSW will be isolated and messenger RNA from these pancreatic islet cells will be isolated and subjected to microarray analysis to determine the up- and down-regulation of various genes important in the development and function of pancreatic beta cells.

Identification of Pancreatic Islet and Exocrine Cells of Interest

Whole pancreata (to include the duodenal and gastric arms) will be collected from 6 month old dSW and nSW mice and flash-frozen in OCT media-filled cryomolds. Serial

sections at 4-8μm will be obtained. One section will be co-stained using standard fluorescent immunohistochemical techniques with guinea pig anti-swine insulin antibody (Riu, Ferre et al. 2002) (DakoCytomation, Carpinteria, CA) and rabbit anti-pancreatic amylase polyclonal antibody (Abcam, Cambridge, MA) at 1:150 on an automated immunostainer as previously described (Rogers, Taylor et al. 2005). This slide will be used to identify cells of interest for laser capture microdissection of the saved serial section.

Isolation of RNA from Cells of Interest

The second section will be stained using standard histology methods with hematoxylin and eosin (H&E). Cells of interest will be isolated using Laser Capture Microdissection using standard protocols. The cap post laser transfer is placed tightly onto an Eppendorf tube, and the tube is then inverted back and forth over the course of 2 minutes. It is then quick spun to collect all of the buffer. Roughly 1,000-5,000 cells are extracted for mRNA using the Qiagen RNeasy Micro Kit; however, if adequate samples cannot be obtained for cells types, RNA amplification strategies may be employed. cDNA will then be constructed utilizing Invitrogen SuperScript® III First-Strand Synthesis SuperMix. Purified cDNA will be analyzed via quantitative qPCR for the expression of murine Pdx-1 (pancreatic and duodenal homeobox gene-1: pancreatic patterning and development), EGF (epidermal growth factor), Ngn-3 (neurogenin-3: differentiation of endocrine cells), Ptf1a (pancreas transcription factor-1a: ventral pancreatic development), H1xb9 (homeobox transcription factor: dorsal pancreatic development), HNF1b (hepatocyte nuclear factor-1b: pancreatic ductular cell development) and Mist-1 (acinar exocrine cell

development), utilizing commercially available primers and probes (Applied Biosystems). qPCR will be performed utilizing the Taqman® 7700 system and genes of interest normalized to *Gapdh.*

Concluding Remarks and Future Directions

These studies are designed to further understand pathogenesis of T2D in the diabetic Swiss Webster mouse (dSW) and thus validate it as a model of human T2D and its comorbidities, including liver disease. Validation of the model will provide the research community with a novel model of the metabolic syndrome and T2D, and may provide additional mechanistic pathways resulting in the identification of novel biomarkers, diagnostic and therapeutic targets. Of particular interest is the development of HCC in the dSW, and although extensive genetic analyses are beyond the scope of this project, a thorough understanding of the pathogenesis of disease in the dSW mouse could identify genetic mutations that may play a role in human HCC.

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