Exploring the Function of G6PC2 in Pancreatic Islet Beta Cells

By

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Abbreviations

β-cell Pancreatic islet beta cell(s)
11-DHC 11-Dehydrocorticosterone
11β-HSD 11β- hydroxysteroid dehydrogenase
11β-HSD1 11β- hydroxysteroid dehydrogenase type 1
11β-HSD2 11β- hydroxysteroid dehydrogenase type 2
BMI Body mass index
CAM Cardiovascular associated mortality
CBG Corticosteroid Binding Globulin
DEX Dexamethasone
DIO Diet induced obesity
FBG Fasting blood glucose
FPI Fasting plasma insulin
G6P Glucose-6-phosphate
G6Pase Glucose-6-phosphatase
G6PC1 Glucose-6-phosphatase catalytic subunit
G6PC2 Glucose-6-phosphatase catalytic subunit, member 2 (formerly IGRP)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>G6PC3</td>
<td>Glucose-6-phosphatase catalytic subunit, member 3 (formerly UGRP)</td>
</tr>
<tr>
<td>G6PT</td>
<td>G6P translocase</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid(s)</td>
</tr>
<tr>
<td>GCK</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid receptor element</td>
</tr>
<tr>
<td>GREV</td>
<td>Glucocorticoid receptor expression vector</td>
</tr>
<tr>
<td>GSD1a</td>
<td>Glycogen storage disease type 1a</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association studies</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalmus-Pituitary-Adrenal Axis</td>
</tr>
<tr>
<td>IGRP</td>
<td>Islet specific glucose-6-phosphatase catalytic subunit related protein</td>
</tr>
<tr>
<td>IPGTT</td>
<td>Intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>ATP-sensitive potassium channel</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LCMV</td>
<td>The Lymphocytic Choriomeningitis Virus</td>
</tr>
<tr>
<td>MODY</td>
<td>Mature onset of diabetes of the young</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PR</td>
<td>Physical restraint</td>
</tr>
<tr>
<td>SD</td>
<td>Synthetic derivative</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>UGRP</td>
<td>Ubiquitously expressed G6Pase catalytic subunit related protein 3</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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I. INTRODUCTION

**Diabetes Mellitus**

According to the World Health Organization, as of 2014, approximately 347 million people worldwide have diabetes and this number is expected to steadily increase, as it has over the last 30 years. It is predicted that by 2030, diabetes will be the 7th leading cause of death worldwide. While diabetes typically results in a cardiovascular event, which will ultimately kill 50-80% of the diabetic population, diabetes mellitus is also the leading cause of amputation, blindness and kidney failure [1]. There is a pressing need for new and improved treatments and modes of preventing diabetes, as it has become an epidemic that continues to affect millions of humans while also costing the world billions of dollars annually. The next two sections will focus on the two major forms of diabetes: type 1 and type 2 diabetes (T1D and T2D).

**Type 1 Diabetes**

While there are similarities between T1D and T2D, they are fundamentally different diseases; this section will focus specifically on the etiology of T1D and models for studying its onset and progression. T1D is the less common form of diabetes, accounting for 10% of the 347 million diabetes diagnoses worldwide [1]. As T1D generally presents at an earlier age than T2D, with the average age of onset being 14, it is also referred to as juvenile or childhood onset diabetes. Patients will most often have symptoms of excessive urination, thirst, and hunger, which are all results of chronic hyperglycemia [2]. T1D is a chronic autoimmune disease that occurs as a result of genetic susceptibility paired with environmental factors. More specifically, T1D is an autoimmune disorder that results in specific destruction of the insulin producing islet beta cells (β-cells) and ultimately the ability to make and secrete insulin. Without insulin, the body is unable to maintain normal glycaemia and, untreated, this can lead to both hyper- and hypoglycemia [3]. Most often, progression of T1D begins with a genetically susceptible individual being exposed to a specific environmental trigger that promotes a pro-inflammatory state, which is associated
with the production of autoantibodies that results in T-cell mediated destruction of β-cells and ultimately T1D diagnosis. Patients with T1D are treated by exogenous administration of insulin either via injection or a subcutaneous pump. This treatment requires a great deal of discipline, as glucose levels must be monitored throughout the day for the remainder of the patient’s life in order to maintain normal glycaemia.

While the genetics of T1D have been extensively characterized, the environmental triggers leading to progression of the disease remain to be identified. The following section will focus on the implicated and characterized susceptibility genes in T1D.

T1D is a polygenic disorder with 40 currently known loci that affect disease susceptibility. Since the early 1970’s the Human Leukocyte Antigen (HLA) region on chromosome 6p21 has been implicated as a critical susceptibility locus for many autoimmune diseases, including T1D. Variation in this region accounts for the most significant association signal between T1D risk and development, accounting for about half of the genetic susceptibility of T1D, with an odds ratio of 6.8 [2, 3]. Despite many other susceptibility loci being identified, none have an association signal as significant as the signal between the HLA locus and T1D [3]. The other main identified loci that associate with T1D are in the insulin, PTPN22, CTLA-4 and IL2RA genes. The insulin gene has been established as a primary autoantigen in T1D and, as insulin is produced exclusively in the pancreatic islet β-cell, the mechanism by which these mutations result in T1D is obvious, directly relating to the body’s ability to produce insulin and death of the β-cell population. Of the remaining loci that have been implicated, the mechanisms by which variation at that location contribute to T1D relate to deficiencies in an individual’s immune response or aberrant signaling in immune response pathways [2]. Additionally, with the advent of genome wide association studies (GWAS), other loci have been identified that make a modest contribution to overall T1D risk. As previously mentioned, the specific environmental triggers that lead to T1D onset and progression are unclear and indirect. There are reports of certain viral infections and chemical exposures, the composition of an individual’s gut bacteria and vitamin D deficiency all associating with T1D diagnosis [3]. While environmental triggers and susceptibility loci have been identified, the relation between the two and T1D still remains elusive.
There are multiple models that are used to study T1D. The most commonly used genetic model is the non-obese diabetic (NOD) mouse, which is characterized by insulitis and leukocyte infiltration of the pancreas, resulting in decreased insulin content and β-cell mass as early as 12 weeks after birth [4]. Interestingly, by studying the progression of T1D in NOD mice, researchers discovered that a population of T-cells isolated from diabetic NOD mice target the β-cell specific protein glucose-6-phosphatase catalytic subunit, member 2 (G6pc2), formerly known as islet specific glucose-6-phosphatase catalytic subunit related protein (IGRP) [5]. It was further established that G6PC2 is also a T1D autoantibody in humanized mice and humans [6-8]. Further studies found that deletion of G6pc2 from NOD mice does not prevent or slow the progression of T1D. This finding suggests that T-cell recognition of G6pc2 autoantibodies is not necessary for T1D development and instead is a secondary, downstream event in the progression of T1D [9]. Another commonly used model to study the progression of T1D is the Akita mouse model. These mice have a spontaneous autosomal dominant deletion on chromosome 7 that results in defective protein folding and an inability to induce the unfolded protein response pathway. This results in endoplasmic reticulum (ER) stress and β-cell apoptosis, ultimately resulting in T1D [10]. A final method that is used to study T1D is the Lymphocytic Choriomeningitis Virus (LCMV) model. This involves injecting susceptible mouse strains with LCMV which results in a rapid T-cell mediated targeting of β-cells and thus the inability to produce and secrete insulin [11]. Using these models, the pathogenesis of T1D has successfully been studied; yet, despite the detailed characterization of T1D progression and multiple genetic susceptibility loci being identified, methods of preventing T1D onset or progression have not been discovered.

Another model that is commonly used to characterize the effects of β-cell destruction on glucose metabolism is a chemical model in which mice are treated with streptozotocin or alloxan, both of which are cytotoxic glucose analogs. Treatment with these toxins results in selective destruction of the islet β-cells and ultimately diabetes, however, the progression of disease in these models relative to that in T1D is different in that there is not an autoimmune response [12]. While treatment with these drugs does not result in an autoimmune response, they do ablate the β-cell such that insulin production, and therefore
secretion, does not occur, similar to T1D and therefore provides an alternative model for studying the effects of β-cell destruction on glucose metabolism.

**Type 2 Diabetes**

T2D is the most common form of diabetes, accounting for the remaining ~90% of diabetes diagnoses. It is characterized by hyperglycemia, insulin resistance, and comparative insulin deficiency [13]. Clinically, it is diagnosed with a glycated hemoglobin test, an indicator of average blood sugar levels over time, above 6.5% or a glucose level in an oral glucose tolerance test (OGTT) over 200mg/dl two hours post injection [14]. Importantly, T2D is almost entirely preventable with lifestyle factors being the major component in determining T2D risk [15]. It is well established that lack of physical activity, cigarette smoking, excessive alcohol consumption and obesity contribute to the development of T2D. Currently, it is predicted that ~55% of T2D cases are linked to obesity [15]. While there is a population of non-obese individuals diagnosed with T2D, this is presumably due to the loss of β-cells that naturally occurs with age. Treatment for T2D patients typically includes lifestyle interventions, oral medication and potentially exogenous insulin treatment. Two of the most common oral medications are glipizide and metformin. Glipizide, a sulfonyurea, acts to increase insulin secretion from the pancreas and metformin, a biguanide, decreases blood glucose levels by increasing muscle insulin sensitivity and limiting glucose production from the liver [16]. Despite the effects of metformin being clear and being the most common and frequently prescribed T2D drug, researchers have not been able to reach a consensus regarding its primary site of action. However, Buse et al. recently showed that the gut is the primary site of action [17]. Metformin accumulates at 300 times the concentration of plasma in the gut and this accumulation results in enhanced GLP-1 and peptide YY secretion. These hormones have glucose-lowering effects via reduction of hepatic glucose production through suppression of glucagon and enhanced glucose dependent insulin secretion, thereby providing a site of action and mechanism by which metformin lowers blood glucose levels [17].
Because obesity is the main risk factor for T2D, researchers have established multiple induced and genetic mouse models of obesity to study the disease. One of the most commonly used models to study the effect of obesity in mice is the diet induced obesity (DIO) model, which involves feeding mice high fat food in lieu of standard chow diet in order to induce obesity and, typically, impaired glucose tolerance. Male C57BL/6J mice fed 12+ weeks of high fat food mimics human metabolic syndrome, as defined as having hyperglycemia, increased blood pressure, excess body fat and increased cholesterol levels [18]. High fat feeding is a very common model for studying impaired glucose tolerance in the context of obesity; one observation in these studies is that there is inherent variability in responses between inbred mouse strains, resulting in differences in the degree of obesity, insulin resistance and impairment in glucose tolerance and alterations to plasma lipid composition among other parameters [19-22]. For example, AKR/J and C57BL/6J mice are hyper-responsive to DIO, becoming significantly obese, while SWR/J and, as will be shown in chapter V, 129SvEv mice are relatively resistant to DIO [19]. These results highlight the importance of genetic modifiers in different mouse genetic backgrounds.

In addition to the DIO model, there are multiple genetic models of T2D. The two most common genetic models are the leptin deficient (ob/ob) and leptin receptor deficient (db/db) knockout (KO) mouse models. The ob/ob mouse model, when bred on the KsJ genetic background, exhibits severe hyperphagia, which leads to obesity, insulin resistance and dyslipidemia, ultimately resulting in spontaneous development of impaired glucose tolerance [23-27]. Interestingly, when the ob/ob mutation is backcrossed on to the C57BL6/J genetic background, the same phenotype is not observed and T2D does not occur [28]. Similar to the ob/ob mouse model, the db/db mouse model also exhibits hyperphagia, insulin resistance, dyslipidemia and impaired glucose tolerance [23, 25-27].

A final model to study impaired glucose tolerance in mice involves administering the S961 insulin receptor antagonist. Mice treated with S961 are hyperinsulinemic, insulin resistant, glucose intolerant and hyperglycemic [29]. This is due to the inability of insulin to transduce its signal, and subsequently, lower
blood glucose levels. While there are other genetic models available to study impaired glucose tolerance in vitro in mice, these are the most commonly used and best characterized.

Although development of T2D is primarily attributed to lifestyle factors, there is also a genetic component. Individuals who have a relative with T2D have a ~25% chance of developing the disease [13]. Through advances in GWAS, many genetic loci have been identified that associate with altered risk of T2D development. Although a majority of the identified mutations occur in non-coding regions of the genomes, making it difficult to determine their function, these studies have also identified mutations in the GCK, IRS1, HNF1B, TCF7L2, PPARG, FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, IGF2BP2, SLC30A8, JAZF1 and HHEX genes, among others [13, 15]. The clinical relevance of some of these signals remains to be determined but the mechanism behind others, such as GCK and KCNJ11 are clearer, relating to β-cell dysfunction and/or improper insulin secretion [13, 15]. Specifically, mutations in KCNJ11, which encodes the Kir6.2 subunit of the ATP sensitive potassium channel (K\textsubscript{ATP}), result in diabetes [30]. These patients have impaired insulin secretion caused by a failure of the K\textsubscript{ATP} channel to close in response to increased cytoplasmic ATP [30]. These patients can be treated with sulfonylureas that specifically act to close the K\textsubscript{ATP} channel [30].

In addition to mutations that increase the risk of developing T2D, multiple genetic mutations have been identified that lead to monogenic forms of diabetes namely mature onset diabetes of the young (MODY). MODY is a third subset of diabetes that is 50% heritable and arises from a mutation in multiple genes, with the most common mutations being in HNF1A or GCK [31]. As of 2015, there are 28 distinct gene mutations that can result in monogenic diabetes, highlighting the fact that MODY is a multifactorial polygenic disease. Like T2D patients, MODY patients cannot produce adequate levels of insulin and subsequently become hyperglycemic and insulin resistant [31]. Overall, it is evident that the environment, genetics and the interactions between the two plays a significant role in diabetes risk. Only with better understanding of the underlying mechanisms governing normal glucose homeostasis and insulin secretion will more effective drugs be established to treat or prevent T2D.
**Discovery of Hepatic Glucose-6-phosphatase**

The liver plays a key role in regulating whole body glucose homeostasis by acting as a metabolic hub and providing key metabolites to other tissues. As the brain, under basal conditions, accounts for the vast majority (~60%) of glucose metabolism and skeletal muscle, following glucose stimulation, accounts for ~75% of glucose utilization, it is crucial that metabolite levels in the blood are tightly maintained by the liver so as to provide adequate energy in both basal and post-prandial states [32, 33]. In the post-prandial state, glucose is taken up by the liver and converted to glucose-6-phosphate (G6P), which is either metabolized through glycolytic or pentose phosphate pathways for immediate energy production or, in times of excess dietary glucose, can be stored as glycogen. When dietary glucose is not available, such as during a prolonged fast or in a pre-prandial state, the liver breaks down glycogen via glycogenolysis and/or produces glucose from the gluconeogenic precursors glycerol, amino acids and lactate. Because the terminal step in both gluconeogenesis and glycogenolysis generates G6P, it was postulated that there must be an enzyme that de-phosphorylates G6P to create glucose and a free phosphate, thereby driving this mechanism in the opposite direction to increase or modulate blood glucose levels [34]. While isolation and identification of this enzyme was difficult due to its localization in the ER membrane, Chou and colleagues successfully identified the gene that encodes the enzyme by irradiating mice to induce chromosomal deletions at the albino locus [35-37]. These mice were hypoglycemic and died shortly after birth due to reduced gluconeogenic activity caused specifically by decreased glucose-6-phosphatase (G6Pase) activity [35-37]. Using these mice, Chou et al. screened a murine cDNA library with cDNA probes to both wild type (WT) and mutant mice and were able to successfully isolate a glucose-6-phosphatase catalytic subunit (G6PC1) cDNA [36, 38]. Upon isolation, it was further confirmed that G6PC1 mRNA is expressed in the liver and kidney primarily, with low expression detected in the small intestine [36, 39, 40]. Further characterization of this enzyme has established that G6PC1 is a component of the G6Pase multi-component enzyme system located in the ER membrane with an active site facing the ER lumen (Fig. 1.1) [41]. The
liver G6Pase system is comprised of a glucose transporter, a G6P/Phosphate transporter (G6PT), encoded by the *SLC37A4* gene, and a catalytic subunit, G6PC1 (Fig. 1.1). The system functions by G6P entering the ER lumen through G6PT where, once in the ER lumen, the catalytic subunit catalyzes the hydrolysis of G6P to glucose and an inorganic phosphate (P\(_i\))(Fig. 1.1) [42]. The following sections will describe the G6Pase system in greater detail.

**The Glucose-6-Phosphatase Gene Family**

The G6Pase catalytic subunit gene family is composed of three members which mainly differ in their tissue expression pattern and activity: G6PC1 (formerly G6Pase), G6PC2 (formerly IGRP), and G6PC3 (formerly UGRP) (Fig. 1.1 and Table 1.1). Table 1.1 highlights key features of the G6Pase catalytic subunit gene family. Briefly, G6PC1 is primarily expressed in the liver, G6PC2 is exclusively expressed in islet β-cells and G6PC3 is almost ubiquitously expressed with the exception of in islet β-cells (Table 1.1) [42]. The *G6PC1* and *G6PC3* genes are located on chromosome 17 while the *G6PC2* gene is located on chromosome 2 (Table 1.1). All three genes encode transmembrane ER proteins that are predicted to have 9 transmembrane domains with similar topology [42] (Table 1.1). The other major differences between these isoforms are their enzymatic activity (Table 1.1) and roles in human health and disease, which will be discussed in greater detail in the following sections.

**G6PC1**

Liver G6PC1 functions as the terminal step in both glycogenolysis and gluconeogenesis. G6PC1 is the most catalytically active isoform of the G6Pase gene family (Table 1.1). Due to its high activity in the liver and role in glucose homeostasis, it is no surprise that mutations of *G6PC1* that decrease activity result in human disease, namely glycogen storage disease type 1a (GSD1a). Most frequently, GSD1a patients present with severe hypoglycemia, hyperlipidemia, hyperuricemia and lactic acidemia, although they are also prone to growth retardation, renal failure, hepatic steatosis, cirrhosis and adenomas [43]. Chou and Mansfield have extensively characterized *G6PC1* mutations identified in human Gsd1a patients [43, 44].
There are two other forms of glycogen storage disease, type 1b and type 1c, which occur as a result of mutations in the genes that encode G6PT, \textit{SLC37A4} (Fig 1.1). Gsd1b and Gsd1c, despite having mutations in the same gene, differ by whether the patients exhibit symptoms consistent with defective glucose transport or phosphate transport, respectively. Despite having a different defect in the G6Pase system, the patients have similar hepatic dysfunctions as GSD1a patients as well as dysfunctional neutrophils, which results in an increased susceptibility to bacterial infection [45]. Due to the observation that mutations in \textit{G6PC1} or G6PT result in reduced G6Pase activity, and leads to glycogen storage diseases, it is obvious that the G6Pase system is critical to maintaining glucose homeostasis.

Finally, increased hepatic G6PC1 activity and expression is also a characteristic of T1D and T2D. Multiple groups have detected a 2-4 fold increase in \textit{G6pc1} mRNA levels and a 2-3 fold increase in hepatic glucose-6-phosphatase activity from rodents and humans with diabetes [46-48]. The most likely explanation for this observation is, presumably, due to a reduction in insulin secretion, relative (T2D) or absolute (T1D). Further analysis of the mechanism causing decreased G6PC1 activity following insulin treatment revealed that insulin signaling regulates \textit{G6PC1} gene transcription through an HNF-1 and two FOXO1 transcription factor binding sites that make up an insulin response unit [49-52]. Insulin decreases \textit{G6PC1} transcription by targeting FOXO1, such that it is excluded from the nucleus and can’t bind the \textit{G6PC1} promoter to induce transcription. Thus, in the diabetic state where insulin signaling is altered and relatively (or absolutely as in T1D) decreased, there is an inappropriate elevation of \textit{G6PC1} transcription, which contributes to elevation of blood glucose and thus further contributes to the pathogenesis of T2D [49-52].
Figure 1.1. Model of the glucose-6-phosphatase multicomponent system [53]. The G6Pase system is composed of a glucose-6-phosphate transporter, a phosphate transporter and a catalytic subunit. The system functions by hydrolyzing G6P to glucose and a free phosphate.

| Table 1.1. The glucose-6-phosphatase catalytic subunit gene family |
|-------------------|-----------------|-----------------|-----------------|
| Gene              | G6PC1 (G6Pase)  | G6PC2 (IGRP)    | G6PC3 (UGRP)    |
| Tissue            | Liver           | Islet β-cells   | Ubiquitous      |
| Size              | 357 AA          | 355 AA          | 346 AA          |
| % Identity        | 100             | 50              | 36              |
| Chromosome        | 17q21           | 2               | 17q21           |
| Location          | ER              | ER              | ER              |
| # Transmembranes  | 9               | 9               | 9               |
| Substrate         | G6P             | G6P             | G6P             |
| Vmax (nmol/mg/min)| 666.7           | 32              | 108.7           |
| Km (mM)           | 2.5             | 0.45            | 2               |
These observations were further supported by studies in vitro that showed a 2.5 fold increase in G6PC1 activity from microsomes isolated from diabetic rat livers, which was accompanied by decreased hepatic glycogen stores. This effect was reversed with the administration of insulin [54]. In conclusion, G6PC1 expression and activity need to be tightly regulated in order to maintain normal whole body glucose homeostasis.

**G6PC3**

*G6PC3*, the third isoform of the glucose-6-phosphatase gene family, was discovered by a BLAST search against human *G6PC2*. G6PC3, formerly known as ubiquitously expressed G6Pase catalytic subunit related protein 3 (UGRP), as the name alludes, is ubiquitously expressed but at relatively higher levels in the heart, skeletal muscle, brain and kidney [55]. Further characterization of the cDNA and protein sequence showed that it has the same predicted topology and function as G6PC1 and G6PC2, however hydrolysis of G6P by G6PC3 in transient transfection assays has been unsuccessful [56]. Two groups were successful in demonstrating its hydrolase activity in COS7 cells via stable [57] or adenoviral transfections [58-60]. These groups estimated the V_MAX of G6PC3 to be one sixth of G6PC1, yet with similar Km values (Table 1.1) [58, 59, 61]. Analysis of G6pc3 KO mice showed that deletion of G6pc3 impairs G6P hydrolysis in brain and testis homogenates [61] and leads to defective neutrophil and macrophage function, resulting in neutropenia [60, 62]. These data support human studies that associate loss of *G6PC3* function with congenital neutropenia [60, 63]. One hypothesis explaining the dysfunctional neutrophil and macrophage phenotype is that in the absence of G6pc3, there is an inadequate supply of energy due to inadequate recycling of ER glucose back to the cytoplasm [64]. However, this hypothesis is not consistent with the previous observation that patients with mutations in *SLC37A4* (G6PT) have GSD1b and 1c. These mutations limit G6P entry into the ER, leaving glucose in the cytoplasm for energy usage, opposite of what is expected in G6pc3 KO mice, yet they still are diagnosed with GSD. Therefore, this contradiction regarding the
mutations in G6PT versus mutations in G6PC3 and the mechanisms linking G6PC3 to neutropenia remains to be determined.

**G6PC2**

Arden et al. successfully cloned G6pc2 from mouse insulinoma tissue [65]. G6PC2 is ~50% identical to G6PC1, having similar predicted topologies, conservation of catalytically important residues and an ER retention signal [65]. Historically, many groups struggled to demonstrate G6P hydrolysis following overexpression of either human or mouse G6pc2 [55, 58, 65, 66]. Only one group was successful; Petrolonis et al. demonstrated that mouse G6pc2 has 20-40 fold lower activity than G6PC1 when overexpressed in COS7 cells [67]. While determination of the specific activity of G6PC2 and its contribution to glucose cycling was controversial, recent data from Wall et al., obtained using a novel stable isotope method, has predicted the activity of mouse G6pc2 to be greater than previously appreciated [68]. They demonstrated that glucose cycling, as defined by the rate glucose is converted to G6P and back to glucose, occurs at ~16% of net glucose uptake at a submaximal concentration of 5mM glucose in islets isolated from chow fed mice [68]. Importantly, when G6pc2 KO islets were examined, glucose-6-phosphatase activity and glucose cycling was abolished (Fig. 1.2 and 1.3). These data confirm that G6pc2 is the major isoform in pancreatic β-cells acting to hydrolyze G6P [68, 69]. The role of G6pc2 function *in vivo* and in human disease will be discussed in greater detail in the following sections.

**The Identification of G6PC2 Single Nucleotide Polymorphisms (SNPs) that Regulate Fasting Blood Glucose in Humans**

Elevated fasting blood glucose (FBG) has been associated with increased risk for the development of T2D and cardiovascular associated mortality (CAM) [70-72]. Previous studies have shown that an increase in FBG of ~9-18 mg/dl is associated with a 30% increased risk of mortality [71], while a reduction in FBG of ~9 mg/dl is associated with a 25% reduction in mortality [72]. In an effort to identify genes associated with variations in human FBG, multiple groups performed GWAS. To date, these studies have
identified single nucleotide polymorphisms (SNPs) in over 50 loci that are associated with FBG variation, many of which are also associated with altered risk of T2D [73, 74]. Notably, the rs560887 SNP located in the intronic region of the \textit{G6PC2} locus has been identified as the strongest common genetic determinant of FBG levels in terms of significance and effect size, accounting for about one percent of total variance of FBG in humans [73, 75-78] (Table 1.2). GWAS also identified three additional common promoter SNPs in high linkage disequilibrium with rs560887 (rs13431652, rs2232316 and rs573225) as potentially causative with respect to variations in FBG [79-83]. \textit{In vivo} mouse models have further confirmed the function of G6pc2 in regulating FBG and GSIS, which will be discussed in later sections [69]. The goal of the studies described within this thesis is to provide further evidence of the role of G6pc2 in modulating FBG and GSIS.

\textbf{G6pc2 Function In Vivo}

While the liver, and to a lesser extent the kidney, are the main glucose producing organs, the presence of G6Pase catalytic subunit isoforms and glucose-6-phosphatase activity in other tissues and cell types, including the pancreatic \(\beta\)-cell, highlights the role of the G6pase system in modulating glucose metabolism in other tissues [78, 84]. As such, there has been growing appreciation for the contribution of other cell types to modulating glucose metabolism and whole body glucose homeostasis. With the finding that glucose-6-phosphatase activity is present in islet \(\beta\)-cells, it was hypothesized that glucose-6-phosphatase activity in the islet \(\beta\)-cell could act as a negative regulator of GSIS by opposing the actions of glucokinase (GCK) [69, 85].

Canonical GSIS is depicted in Figure 1.4. Briefly, GSIS occurs when blood glucose levels increase, such as after a meal. As glucose levels increase, glucose enters the \(\beta\)-cell through the bidirectional GLUT2 glucose transporter. GCK phosphorylates glucose to create G6P, which is metabolized by the glycolytic pathway and tricarboxylic acid cycle (TCA) in the mitochondrion (Fig. 1.4).
Figure 1.2. G6Pase activity is absent from G6pc2 KO islets [69]. Glucose-6-phosphatase activity was compared in two independent islet preparations isolated from G6pc2 WT and KO mice. The results show mean glucose-6-phosphatase activity ± SD [69].

![Glucose-6-Phosphatase Activity](chart1.png)

Figure 1.3. Glucose cycling activity is absent from G6pc2 KO islets [68]. Glucose cycling in WT or G6pc2 KO islets isolated from chow-fed mice and incubated in 11 mmol/L D7-glucose. WT islets n = 10; KO islets n = 8 incubations of 100 islets. *P < 0.05 vs. WT islets [68].

![Glucose Cycling](chart2.png)
Metabolism of G6P to pyruvate in the mitochondrion increases cytoplasmic ATP levels and consequently increases the ATP:ADP ratio, which causes a closure of K\textsubscript{ATP} channels. Decreased potassium influx into the β-cell results in depolarization of the β-cell membrane and opening of the voltage-gated calcium channels to promote calcium influx into the cytoplasm. As intracellular calcium increases, the exocytotic machinery is activated and promotes the fusion of insulin secretory vesicles with the cellular membrane, subsequently resulting with insulin secretion [86, 87]. However, the presence of G6PC2 in β-cells provides an alternate fate for G6P in addition to being metabolized by the mitochondrion (Fig. 1.4). Specifically, G6P can be hydrolyzed to glucose by G6PC2, creating a futile cycle with GCK, thereby modulating the FBG without affecting fasting plasma insulin (FPI) levels (Fig. 1.5). Therefore, as depicted in Figure 1.5, we predict that in the absence of \textit{G6pc2} there is a leftward shift of the dose response curve for GSIS. This shift in the dose response curve is predicted to decrease FBG in KO mice (Fig. 1.5). Moreover, activation of G6PC2 is expected to produce less ATP per glucose and, therefore, a relative decrease in the cytoplasmic ATP:ADP ratio. This decrease in the cytoplasmic ATP:ADP ratio is predicted to diminish GSIS (Fig. 1.6) [69]. Figure 1.6 predicts that islets from \textit{G6pc2} KO mice will secrete more insulin at a submaximal dose of glucose relative to WT. While a futile cycle is inefficient in terms of energy usage, this system allows for the set point of FBG to be modulated while also regulating GSIS at two points, GCK and G6PC2 [88, 89].

Before determining the mechanism by which G6PC2 modulates FBG and GSIS, groups wanted to determine if β-cell glucose cycling occurs at a sufficient rate to counteract GCK and ultimately affect GSIS. Khan et al. initially estimated glucose dephosphorylation to occur at a rate of 3-4.5% of phosphorylated glucose in healthy rat islets, 40% in \textit{ob/ob} islets and 15.7% in streptozotocin-induced diabetic islets [84, 90]. Another group found evidence of glucose-6-phosphatase activity in islets isolated from rats but they suggested that the level of glucose cycling occurred at too low of rate to significantly affect GSIS [91]. There are several important caveats to these rat studies.
Figure 1.4. Diagram depicting glucose-stimulated insulin secretion (GSIS). Glucose enters the cell through the Glut2 glucose transporter where once in the cell it is converted to glucose-6-phosphate by glucokinase. G6P is then metabolized via the glycolytic pathway and TCA cycle. This increases the ATP:ADP ratio, resulting in an opening of the ATP sensitive potassium channel and depolarization of the cell membrane and opening of the voltage dependent sensitive calcium channel, an influx of calcium into the cytoplasm and ultimately insulin secretion. G6PC2 acts to oppose glucokinase, creating a futile glucose cycle where glucose is converted to G6P by glucokinase and back to glucose by G6PC2. Adapted from [53].

Fig 1.5. A leftward shift in the dose response curve for GSIS results in decreased FBG in G6pc2 KO. Deletion of G6pc2 will result in a leftward shift of the dose response curve for GSIS and enhanced sensitivity to glucose. This will further result in decreased FBG in G6pc2 KO mice without a difference in FPI levels. The first is that G6pc2 is a pseudogene in rats [66]. Instead, as G6PC1 has been detected in rat islets, it is hypothesized that glucose cycling in rat islets is G6pc1 mediated [66]. A second caveat is that the rat islets
were cultured in low glucose. This is important because glucose stimulates \textit{G6pc1} gene transcription [92-94], indicating that in low glucose there would be low \textit{G6pc1} expression and thus activity would be expected to be relatively low. The final caveat relates to the use of radioisotopes to study glucose cycling. These studies yielded very low glucose cycling rates, which can be interpreted as minimal glucose cycling occurring, or alternatively, that there are technical issues regarding these radiotracer studies islets [90, 95]. These estimates may be inaccurate because G6PC2 could modulate GSIS independently of its ability to hydrolyze G6P, which would not be reflected in radiotracer assays. Moreover, because G6PC2 modestly affects glucose cycling in pancreatic islets [90, 95], has \(~40\)-fold lower G6Pase activity relative to G6PC1 [67, 69] and, finally, it possesses a phosphatidic acid phosphatase domain [55], a method that is more sensitive than radiotracer studies may need to be used in order to identify the contribution of G6pc2 to glucose cycling. To overcome the pitfalls of radiotracer studies, more recently, Wall et al. developed a stable isotope methodology to estimate the influence of G6PC2 on glucose cycling. Using this approach, they demonstrated higher levels of glucose cycling than has previously been reported. These studies showed that glucose cycling occurred at a rate of 16\% net glucose uptake when measured from islets incubated in 5mM glucose and up to 40\% when measured from islets incubated in 11mM glucose. Importantly, glucose cycling was abolished in \textit{G6pc2} KO mouse islets, suggesting that G6pc2 hydrolyzes G6P, thereby contributing to glucose cycling, and opposing the action of the \(\beta\)-cell glucose sensor GCK [68].

Consistent with the most recent glucose cycling data, previous data from the O'Brien lab further established the role of G6pc2 as a negative regulator of GSIS \textit{in vivo} [69]. Pound et al. hypothesized that deletion of \textit{G6pc2} would result in a leftward shift in the dose response curve for GSIS (Fig. 1.5). The implications for this shift in the dose response curve are two fold.
Fig 1.6. A leftward shift in the dose response curve for GSIS results in enhanced insulin secretion from $G6pc2$ KO. Deletion of $G6pc2$ will result in a leftward shift of the dose response curve for GSIS and enhanced sensitivity to glucose. This will further result in enhanced insulin secretion from islets isolated from $G6pc2$ KO at a submaximal glucose concentration.
The first is that \textit{G6pc2} KO mice will have decreased FBG (Fig. 1.5). The second is that there will be increased insulin secretion from \textit{G6pc2} KO mice due to enhanced sensitivity of GSIS to glucose (Fig. 1.6) [69, 96]. The following findings support these hypotheses and the role of G6PC2 as a negative regulator of GSIS. The first observation was that, as predicted in Fig. 1.6, there is significantly increased insulin secretion at submaximal glucose concentrations from islets isolated from \textit{G6pc2} KO mice relative to WT littermates (Fig 1.6 and 1.7) [69]. Secondly, in perfused pancreata, \textit{G6pc2} KO mice had enhanced insulin secretion at a submaximal glucose concentration, consistent with a leftward shift of the dose response curve for GSIS (Fig 1.6 and Fig 1.8) [69]. The final piece of data, as predicted in Fig. 1.5, is that in both male and female mice bred on either a C57BL/6J (Fig. 1.9) or mixed background (Fig. 1.10), male and female \textit{G6pc2} KO mice have significantly reduced FBG, consistent with the role of G6PC2 modulating the set point for FBG (Fig 1.5, 1.9 and 1.10) [69]. Additionally, these \textit{in vivo} data support the human GWAS data that identified \textit{G6PC2} SNPs associated with variation in FBG [70, 73, 74, 79, 96]. These data as a whole support the role of G6PC2 as a negative regulator of GSIS and as a key enzyme in modulating the set point for FBG.

The goal of the work described in this thesis was to further elucidate the mechanism and role of G6PC2 in modulating GSIS and the set point for FBG [69]. A preliminary study in the O'Brien lab showed that dexamethasone (Dex), a synthetic glucocorticoid (GC), stimulates \textit{G6pc2} promoter activity and expression in a fusion gene assay. We therefore hypothesized that under certain physiological conditions that activate \textit{G6pc2} gene expression or activity, such as stress, there will be a shift in the dose response curve of WT mice, resulting in an enhanced difference in FBG between WT and \textit{G6pc2} KO treated mice (Fig. 1.10). While we predict an enhancement in the difference of FBG between WT and KO glucocorticoid treated mice, there are two potential outcomes of these studies (Fig. 1.11 and 1.12). We predicted that if the whole body effect of glucocorticoids was to raise FBG levels, than the difference in FBG between WT and KO mice would be increased because of the effect of Dex on \textit{G6pc2} expression and deletion of \textit{G6pc2} would serve to limit the increase in FBG (Fig. 1.11).
**Fig. 1.7. Analysis of GSIS in islets isolated from G6pc2 KO mice [69].** GSIS from 13-week-old male WT and G6pc2 KO mouse islets were assayed *in vitro* as described in [69] following stimulation with 11 mmol/L glucose. The results show the mean insulin concentrations ± SEM determined using three independent islet preparations. *p<0.05 vs. WT.

**Fig 1.8. Analysis of GSIS from perfused pancreas experiments *in situ* in G6pc2 KO mice [69].** *In situ* perfused pancreas experiments demonstrate that G6pc2 deletion results in a leftward shift in the dose-response curve for GSIS. GSIS from perfused ~14-week-old male WT and G6pc2 KO mouse pancreata was assayed *in situ* as described in [69]. The results show the mean insulin concentrations ± SEM determined using three WT and five KO animals. *p < 0.05 vs. WT.
Fig. 1.9. *G6pc2* KO mice have significantly reduced FBG levels on a C57BL/6J genetic background [69]. FBG is reduced in *G6pc2* KO mice due to a leftward shift in the dose-response curve for GSIS relative to WT mice. At 17 weeks of age, mice were fasted for 6 h and then mice were anesthetized and blood was isolated. Blood glucose was measured as described in chapter II. Results are the mean ± SEM. *p*<0.05. Female WT N=12, KO N=11. Male WT N=31, KO=17.

Fig. 1.10. *G6pc2* KO mice have significantly reduced FBG levels on a mixed genetic background [96]. FBG is reduced in *G6pc2* KO mice due to a leftward shift in the dose-response curve for GSIS relative to WT mice. At 17 weeks of age, mice were fasted for 6 h and then mice were anesthetized and blood was isolated. Blood glucose was determined as described in chapter II. Results are the mean ± SEM. *p*<0.05. Female WT N=23, KO N=23. Male WT N=20, KO=28.
Fig. 1.11. Model predicting that the effect of glucocorticoid treatment is to increase FBG in WT and G6pc2 KO mice. This model predicts that following glucocorticoid treatment, there will be an increase in FBG of both WT and KO mice. This increase in FBG will increase the difference between WT and KO FBG relative to control mice. In this model, the presence of G6pc2 functions to limit the increase in FBG.

Fig. 1.12. Model predicting that the effect of glucocorticoid treatment is to decrease FBG in WT and G6pc2 KO mice. This model predicts that following glucocorticoid treatment, there will be a decrease in FBG of both WT and KO mice. This decrease in FBG will increase the difference between WT and KO FBG relative to control mice. In this model, the presence of G6pc2 functions to prevent hypoglycemia.
Alternatively, if the whole body effect of glucocorticoids was to repress FBG levels, than the difference in FBG between WT and KO mice would still be increased because of the effect of glucocorticoids on \(G6pc2\) expression and, in this case, the presence of \(G6pc2\) in WT mice would limit hypoglycemia (Fig. 1.12). The results of these studies will be outlined in chapters III, IV and VI.

**Characterization of Common \(G6PC2\) SNPs**

Because of the identification of \(G6PC2\) SNPs that strongly affect FBG levels in humans, labs further worked to characterize these signals and whether the SNPs were causative. Prior to discussing the characterization of \(G6PC2\) SNPs, the caveats relating to interpretation of GWAS data will be outlined. Primarily, one issue with interpretation of GWAS data, is that these studies typically identify common SNPs with low odds ratios [78]. This indicates that even the strongest “hits” individually may have a minimal or modest effect on disease manifestation or effect size [78]. Instead, it is more likely that the genetic cause of a disease or effect size of a parameter such as FBG is due to either rare, high impact variants or many, common, low impact variants being present. Moreover, there could also be an effect from the interaction of SNPs with other genes or an environmental cause [78]. Another critical caveat to GWAS is that, more often than not, SNPs are in non-coding regions and are therefore assumed to associate with the gene that is closest in proximity. This is important for two reasons. The first is that the identified SNP/SNPs may be causative but are altering the activity of a long-range enhancer, which in turn affects the expression of a distal gene, not the proximal one. An example of this was uncovered in studies done characterizing SNPs in the \(FTO\) gene [97]. Variation in an intronic region of the \(FTO\) locus has reproducibly been associated with obesity and T2D, yet years of research have not been able to identify the mechanism behind this association [98-100]. While studies in mice have demonstrated that \(FTO\) expression levels affect body mass and composition, studies have not been able to link these SNPs to mechanisms regulating obesity or T2D risk [101-106]. It wasn’t until more recently that Smemo et al. identified these SNPs to be located in a long-range enhancer that regulates the expression of the homeobox gene \(IRX3\) [97]. Further studies
characterizing IRX3 deficient mice revealed that these mice had a 25-30% reduction in body weight, strongly supporting the role of these SNPs in affecting IRX3 function, not FTO [97]. The second caveat to interpretation of GWAS data is that the SNP may not be causative for the observed phenotype but is instead in linkage disequilibrium with the causative SNP, as is demonstrated with the rs573225 G6PC2 SNP that is in linkage disequilibrium with the lead G6PC2 rs560887 SNP [107]. Because of the limitations of GWAS, it is crucial to design functional studies that identify the gene that the SNP is affecting as well as to establish that the SNP is in fact causative. The following section presents an overview of the characterization that has been done on the G6PC2 SNPs so as to determine which were most likely to be causative.

Table 1.2 highlights the main findings from studies which aimed to characterize the following common G6PC2 SNPs: rs560887, rs13431652, rs2232316 and rs573225 (Table 1.2). These studies were performed in order to determine the mechanism by which each SNP contributes to the association signal. Molecular studies from the O’Brien lab using minigene analyses showed that the rs560887 SNP is located at a branch site and affects G6PC2 protein expression by altering splicing efficiency [79] (Table 1.2). The rs560887-G allele enhances pre-mRNA splicing while the rs560887-A allele reduces pre-mRNA splicing. This is consistent with the genetic association between the rs560887-G allele, elevated FBG and the hypothesized function of G6PC2, suggesting that rs560887 is a potentially causative SNP [79]. It was further determined that with each additional A allele, the minor allele, there is an approximate 1mg/dl reduction in FBG levels [82]. It has been shown that the rs2232316, rs13431652 and rs573225 SNPs are in high linkage disequilibrium with rs560887 (>0.8) and are associated with FBG levels as part of the same association signal [80]. The O’Brien lab further characterized the effect of the rs2232316 SNP on G6PC2 expression and promoter activity (Table 1.2). They demonstrated that the rs2232316-A allele enhances G6PC2 transcription by promoting Foxa2 binding, which is predicted to increase expression and consequently increase FBG, however this SNP does not have an effect on FBG in the absence of the rs560887 SNP [79]. Another SNP that has been characterized is the rs13431652 promoter SNP, which was shown to alter binding of the NF-Y transcription factor to the G6PC2 promoter (Table 1.2). Specifically, the
rs13431652-A allele improved NF-Y binding and increased G6PC2 promoter activity, and is associated with elevated FBG and as such is potentially causative [80]. Finally, two groups performed studies to elucidate whether the rs573225 SNP, located in the promoter and within a Foxa2 binding site, affects G6PC2 promoter activity (Table 1.2). It was shown that the minor allele, rs573225-A, had a higher affinity for Foxa2 binding but decreased promoter activity, which would be expected to result in decreased FBG due to decreased G6PC2 protein expression. Interestingly though, the rs573225 SNP is associated with increased FBG in GWAS, which is inconsistent with the decreased promoter activity that was observed in these studies [80, 83]. There are two explanations for these findings. The first is that this is an artifact of experiments done in cell lines and not representative of human biology. The second is that the conclusions are correct but this SNP opposes the actions of other SNPs with which it is in linkage disequilibrium with.

Another important note in studying G6PC2 SNPs is that, despite being strongly associated with FBG, SNPs in G6PC2 are not reproducibly associated with T2D risk [108-111]. While there were two studies that successfully linked SNPs in G6PC2 to both FBG variation and T2D risk, these studies were performed with a small sample size in a Chinese population [112, 113]. Other studies done with individuals of a European descent and in much larger cohorts were not able to replicate these findings, despite replicating the association of G6PC2 with FBG [114, 115]. This is of interest because elevated FBG is reproducibly correlated with an increased risk of developing T2D as well as increased risk of cardiovascular associated mortality (CAM). As G6PC2 is associated with variation in FBG, it would be predicted to also associate with T2D risk and CAM why G6PC2 is not associated with T2D remains unclear [116, 117]. Studies in G6pc2 KO mice were consistent with GWAS data in that there were minimal differences in intraperitoneal or oral glucose tolerance relative to WT mice over a range of glucose concentrations [69]. Moreover, GWAS data showed no association of G6PC2 SNPs with alterations in insulin sensitivity or FPI, which was also confirmed in G6pc2 KO mice [69, 108-111].
Table 1.2. Characterization of common *G6PC2* single nucleotide polymorphisms associated with FBG (Adapted from Ref [107])

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location of SNP</th>
<th>Allele (Effect)*</th>
<th>Frequency of glucose raising allele</th>
<th>Mechanism</th>
<th>Effect (mmol/l/allele)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs560887</td>
<td>Intron 3</td>
<td>G</td>
<td>0.69</td>
<td>Altered splicing efficiency</td>
<td>0.071</td>
<td>[79]</td>
</tr>
<tr>
<td>rs573225</td>
<td>Proximal Promoter (-231)</td>
<td>A</td>
<td>0.66</td>
<td>Increased Foxa2 binding but decreased promoter activity</td>
<td>0.073</td>
<td>[80, 83]</td>
</tr>
<tr>
<td>rs13431652</td>
<td>Distal Promoter (-4,405)</td>
<td>A</td>
<td>0.68</td>
<td>Increased NF-Y binding, promoter activity</td>
<td>0.075</td>
<td>[80]</td>
</tr>
<tr>
<td>rs2232316</td>
<td>Upstream Variant (-238)</td>
<td>A</td>
<td>0.11</td>
<td>Increased Foxa2 binding, promoter activity</td>
<td>0.04</td>
<td>[79]</td>
</tr>
</tbody>
</table>

* Effect represents the allele associated with increased FBG.
These data further highlighted a complication in reconciling GWAS and in vivo mouse studies; namely that the rs560887-A allele, despite reducing G6PC2 expression and decreasing FBG, is actually associated with decreased insulin secretion during a glucose tolerance test, instead of the expected increase. One potential explanation for the inconsistency between GWAS and in vitro data is that G6pc2 affects insulin pulsatility by altering intracellular calcium levels, which have been shown to alter the magnitude of metabolic oscillations in islets [111, 118]. A change in insulin pulsatility would result in less efficient insulin signaling and could explain how decreased G6PC2 expression could reduce insulin secretion without a change in either glucose tolerance or insulin sensitivity to counterbalance those changes [108, 110, 111, 119]. In summary, rs560887 is the most predictive and causative G6PC2 SNP in relation to FBG but work still needs to be done reconciling the FBG and insulin secretion data.

Identification and Characterization of Rare SNP Variants

Because common SNPs tend to associate with mild phenotypes and only account for a small percentage of genetic heritability, it has been suggested that rare variants (defined as minor allele frequency (MAF) <0.5%) could account for the remaining heritability that cannot be explained by currently identified SNPs. It is hypothesized that this “missing heritability” may associate with more detrimental phenotypes and have more substantial effect sizes [78, 120]. These rare variants do not occur at a high enough frequency to be captured by current GWA genotyping techniques and do not carry an adequate effect size to be detected by familial linkage analysis studies [120]. An exception to this would be monogenic conditions in which the MAF is less than 0.5% but the effect size is large enough to be detected [120]. An example of this phenomenon is evident when comparing rare and common variants in G6PC2 and GCK. While the common rs560887 G6PC2 SNP accounts for approximately 1% of variation in human FBG levels and is the strongest common genetic determinant of FBG in terms of significance and effect size, G6pc2 KO mice have a mild metabolic phenotype showing ~14% decrease in FBG and, moreover, the rare variants identified to date do not associate with more severe phenotypes such as diabetes [69, 78, 96, 121].
In contrast, common variants in *GCK* have a low, but significant, association with FBG but when *Gck* is deleted in mice, it is lethal [122]. Importantly, in contrast to the currently identified common *G6PC2* SNPs, rare *GCK* variants, which result in heterozygous inactivation, cause MODY [123]. Similarly, homozygous inactivating *GCK* mutations lead to permanent neonatal diabetes mellitus, which is characterized by severe hyperglycemia. In contrast, rare activating mutations in *GCK* result in hypoglycemia due to hyperinsulinemia [123]. Because of these studies which highlighted the magnitude rare *GCK* variants have on a disease phenotype, many groups went on to look at the effect of *Gck* overexpression and tissue specific deletion to learn more about the function of the gene and protein [122, 124, 125]. These studies ultimately defined *GCK* as the pancreatic glucose sensor and established its role in glucose metabolism and GSIS. The work done to characterize *GCK* function exemplifies how rare variants, that have yet to be identified, might associate with more detrimental phenotypes. However, a more recent model has suggested that instead of rare variants accounting for the missing heritability, there are in fact numerous SNPs that have not yet been identified and, instead, these account for a large portion of the missing genetic heritability observed in current GWA studies [126-128]. Nevertheless, this new model does not rule out the importance of studying rare variants. Overall, these studies highlight an important caveat in analyzing GWAS data: the size of the effect of common genetic variants does not necessarily correlate with the importance of the gene in relation to the disease or phenotype being studied.

**Glucocorticoid Biology**

Glucocorticoids were aptly named due to their role in glucose metabolism (gluco-), secretion from the adrenal cortex (-cort-) and steroid structure (-coid). Cortisol and corticosterone are the main active endogenous glucocorticoids present in humans and mice, respectively, and play a variety of roles throughout the body [129]. These functions include, but are not limited to, regulating glucose [130-134] lipid [135-138] and protein metabolism [139, 140] to ultimately modulate energy homeostasis. Under stressful conditions, the canonical function of glucocorticoids is to inhibit glucose uptake, antagonize
insulin secretion and raise blood glucose levels so that vital organs are supplied with adequate energy [129, 141, 142]. In addition to regulation of nutrient metabolism in times of stress, glucocorticoids are potent anti-inflammatory, anti-allergic and immunosuppressive agents [143]. Synthetic glucocorticoids such as Dex and prednisolone are used mainly in medical practice for this purpose and are prescribed for chronic inflammatory diseases such as rheumatoid arthritis, asthma, eczema and allergic reactions [129, 144]. Side effects of glucocorticoid treatment depend on dose and length of treatment but can include peripheral insulin resistance, glucose intolerance, hyperglycemia, glucocorticoid induced diabetes, hepatic steatosis, dyslipidemia, decreased skeletal muscle mass and central adiposity [144-148]. These side effects highlight the importance of glucocorticoids in regulating whole body nutrient metabolism and glucose homeostasis. In the following sections, the regulation of glucocorticoid secretion as well as the mechanisms of action will be discussed.

Glucocorticoids are secreted from the cortex of the adrenal gland under the control of the hypothalmus-pituitary-adrenal (HPA) Axis. Briefly, the hypothalmus senses the emotional or physical stress and activates corticotrophin-releasing hormone (CRH) producing neurons, resulting in CRH secretion. CRH levels are sensed by the pituitary, which results in secretion of adrenocorticotropic hormone (ACTH), which is subsequently sensed by the adrenal cortex, resulting in glucocorticoid secretion (Fig. 1.13). An important characteristic of the HPA axis is the ability of glucocorticoids to exert feedback inhibition on CRH and ACTH, ultimately resulting in suppression of the HPA axis and reduced glucocorticoid secretion [149]. As such, adrenalectomy results in blunted HPA axis activity and decreased plasma corticosterone [150]. Circulating levels of cortisol range between ~50-100nM/L in humans, but this can vary from low nanomolar to low micromolar range depending on stress and time of day [151, 152]. Basal glucocorticoid secretion follows a circadian rhythm, with the peaks of glucocorticoid secretion, in humans, occurring on the onset of activity in the morning and the troughs occurring when activity ceases at night.
Fig. 1.13. Hypothalmic-pituitary-axis (HPA) signaling and regulation. In response to stress, the hypothalamus activates CRH producing neurons, which is sensed by the pituitary gland and results in ACTH secretion. ACTH levels are sensed by the adrenal gland, which then secretes glucocorticoids. Glucocorticoids exert negative feedback by inhibiting both CRH and ACTH secretion.
As rodents are nocturnal, this cycle is opposite; peaks occur in the evening and troughs in the morning [153]. Approximately 95% of all circulating glucocorticoids are bound tightly by corticosteroid binding globulins (CBGs) and albumin, with very little free glucocorticoids in the plasma. During times of stress, glucocorticoid levels exceed the capacity of CBGs, which results in increased circulating free glucocorticoids in the plasma [153]. The saturation point of CBGs occurs at glucocorticoid concentrations approximately equal to the peak glucocorticoid concentrations throughout the day (400-500nmol cortisol)[154]. This is important as only the fraction of glucocorticoids unbound to CBGs are able to diffuse across cellular membranes and exert their downstream effects.

Past the point of CBG saturation, free/unbound glucocorticoids diffuse across membranes where, once in the cell, local glucocorticoid concentrations are determined by the amount of 11β-hydroxysteroid dehydrogenases (11β-HSD) present in the specific tissue. There are two isoforms of 11β-HSD: 11β-HSD1 and 11β-HSD2. 11β-HSD1 is a reductase that converts inactive glucocorticoid to active glucocorticoid, specifically cortisol to cortisone in humans and 11-dehydrocorticosterone (11-DHC) to corticosterone in rodents [155, 156]. 11β-HSD2 functions as a dehydrogenase that catalyzes the reverse reaction [157]. The relative amounts of 11β-HSD1 and 11β-HSD2 present in a tissue act to control glucocorticoid levels and thereby determine local glucocorticoid concentrations and activity. 11β-HSD1 is predominantly expressed in liver and adipose tissue whereas 11β-HSD2 is mainly expressed in the kidney [144]. Importantly, 11β-HSD1 is also found in pancreatic islets but it remains unclear if this localization occurs specifically in α-cells [158], β-cells [159, 160] or both. Regardless of the cell type, the presence of 11β-HSD1 in pancreatic islets directly connects glucocorticoid metabolism with β-cell function, either in a direct or paracrine fashion [159, 160]. The role of glucocorticoids in modulating β-cell function will be discussed in greater detail in subsequent sections.

Glucocorticoids signal by binding to the glucocorticoid receptor (GR). The GR is part of the nuclear receptor superfamily and is a ligand-regulated transcription factor that is widely expressed throughout the body [161]. Once glucocorticoids are in the cytoplasm, they binds the GR, which is bound in an inactive
form by chaperone proteins [162]. Upon glucocorticoid binding, the GR undergoes a conformational change, which reveals a nuclear import signal on the receptor. As this occurs, the GC-GR complex enters the nucleus and, as a dimer, binds a glucocorticoid receptor element (GRE) in the promoter region of a target gene in order to activate or repress transcription [163]. It has been shown that approximately 2% of the human genome is regulated in this manner by glucocorticoids [164]. Specific examples of this type of induction are the phosphoenolpyruvate carboxykinase (PEPCK) and G6PC1 genes, which further highlight the role glucocorticoids play in activating gluconeogenesis and modulating glucose homeostasis in times of stress [144, 165-167]. Importantly, these GRE binding sites are critical for maximum promoter activity. It has been demonstrated in various glucocorticoids regulated genes that deletion of the GRE or mutation of key nucleotides in the GRE abolishes or drastically reduces promoter activity as shown in fusion gene assays [149]. While inducing transcriptional changes via GRE binding is the most common way glucocorticoids exert their effects, there are two other common mechanisms of glucocorticoid signaling. The first is that the GC-GR complex can interact, as a monomer, with other transcription factors via protein-protein interactions and trans-repress transcription of target genes. The final mechanism involves nongenomic modulation, both activation and inhibition, of protein activity by the GC-GR complex [144, 168]. Overall, it is clear that glucocorticoids have many pleiotropic effects throughout the body that are mediated through a variety of mechanisms and regulatory pathways.

**Glucocorticoids Effect Glucose Metabolism in the Liver, Skeletal Muscle and White Adipose Tissue**

The widely accepted dogma with respect to the effect of glucocorticoids in regulating glucose metabolism in both rodents and humans *in vivo* is that they inhibit glucose uptake by inducing insulin resistance [169], stimulating hepatic glucose production [170, 171] and inhibiting insulin secretion [147, 172-178], thereby inducing glucose intolerance and hyperglycemia [129, 179-185]. Fig. 1.14 gives a brief overview of how glucocorticoids are thought to exert their effects specifically on the liver, skeletal muscle, white adipose tissue (WAT) and pancreas. As mentioned in the previous section, glucocorticoids have
pleiotropic effects and regulate nutrient metabolism in complex ways. The next section will highlight the central mechanisms by which glucocorticoids modulate glucose homeostasis (Fig. 1.14).

It is well established that treatment with glucocorticoids activates hepatic gluconeogenesis (Fig. 1.14). In both humans and rodents, injection with Dex results in increased hepatic glucose output by transcriptional activation of many genes involved in the gluconeogenic pathway, as mentioned in the previous section [165-167, 184, 186]. These genes include pyruvate carboxylase, PEPCK, fructose-1,6-bisphosphatase 1, phosphofructokinase 2/fructose bisphosphatase 2, G6PT and G6PC1 [169]. While the specific mechanisms by which glucocorticoids induce transcription of PEPCK, phosphofructokinase 2/fructose bisphosphatase 2 and G6PC1 have been abundantly characterized, more work needs to be done to understand how glucocorticoids alter transcription of the other mentioned genes. Despite the mechanisms not being entirely established, it is clear that induction of these genes by glucocorticoids ultimately results in increased hepatic glucose output, subsequently resulting in increased blood glucose levels [187, 188]. Paradoxically, glucocorticoids also increase liver glycogen storage following glucocorticoid treatment by inducing glycogen synthase activity [187, 188]. Glucocorticoid treatment in various species, including humans, results in rapid deposition of liver glycogen that correlates with increased activity of glycogen synthase in liver homogenates following glucocorticoid treatment [188-190]. This is thought to occur by glucocorticoid activation of glycogen synthase phosphatase, which activates glycogen synthase through dephosphorylation [191]. Overall, in the liver, glucocorticoids act to increase glucose output, which results in increased blood glucose levels. However the reasons behind the observed concomitant inactivation of glycogen phosphorylase and activation of glycogen synthase, resulting in a net increase in liver glycogen content, remains elusive [191, 192].

Glucocorticoids further modulate glucose metabolism by mediating their effects on skeletal muscle and WAT (Fig. 1.14). Primarily, glucocorticoids inhibit glucose uptake and glucose oxidation by interfering with insulin stimulated glucose uptake in both tissues [169]. This occurs in skeletal muscle, in part, by decreased GLUT4 translocation to the cell membrane in myocytes, thereby diminishing the availability of
glucose present in the cytoplasm for glycolysis [193, 194]. The specific mechanism whereby glucocorticoids are able to influence GLUT4 translocation and interfere with insulin signaling in myotubes is unclear at this time. Glucocorticoids also affect glucose metabolism by reducing glycogen storage in skeletal muscle, in contrast to the increased glycogen storage observed in liver [169]. This occurs by glucocorticoids interfering with insulin’s ability to dephosphorylate and therefore activate glycogen synthase, thereby inhibiting insulin stimulated glycogen synthase activity and glycogen synthesis [195-197]. The different effects that glucocorticoids have on glycogen metabolism in the liver and skeletal muscle highlight the important point that there are tissue specific differences in glucocorticoid signaling and its downstream effects. The final way glucocorticoids affect glucose homeostasis in skeletal muscle and WAT is by breaking down either amino acids or lipids, respectively, to provide gluconeogenic precursors for the liver. Specifically in WAT, glucocorticoids enhance lipolysis, which results in increased plasma glycerol and fatty acids, the former which can be used as a gluconeogenic precursor and the latter providing energy for gluconeogenesis [198, 199]. Similarly, in skeletal muscle, glucocorticoids increase protein degradation to provide amino acid precursors [169]. Unsurprisingly, chronic glucocorticoid treatment or chronic glucocorticoid secretion, as is observed in Cushing’s syndrome, results in decreased muscle mass and muscle atrophy due to prolonged protein degradation in skeletal muscle [200]. As skeletal muscle accounts for approximately 75% of glucose utilization following glucose stimulation, the advantages of glucocorticoid action on skeletal muscle are clear; they inhibit muscle glucose uptake in order to increase blood glucose levels so as to supply vital organs with adequate energy. This is just a brief overview of primary mechanisms thought to contribute to metabolic regulation of glucose homeostasis by glucocorticoids in skeletal muscle and WAT. The final tissue affected by glucocorticoids that affects glucose metabolism are pancreatic islets, which will be discussed in greater depth in the next section.
Fig. 1.14. Overview of the effects of glucocorticoids on liver, white adipose tissue, skeletal muscle and the pancreas. Adapted from [169].
Importantly, from an evolutionary standpoint, these mechanisms described in the liver, skeletal muscle and WAT are meant to be activated as an acute and temporary reaction to perceived stress and, as such, it has been shown that this transient increase in blood glucose is beneficial over short periods of time [201]. However, it is not surprising, given the major role that glucocorticoids play in glucose homeostasis and nutrient metabolism, that prolonged elevation of glucocorticoids, as occurs in Cushing’s disease or chronic glucocorticoid treatment [202, 203], can lead to a metabolic syndrome like phenotype associated with insulin resistance, increased central adiposity, and hyperglycemia, which can result in an increased risk of glucocorticoid induced diabetes and/or a cardiovascular event [179]. Because of the similarities between metabolic syndrome and chronic glucocorticoid treatment, there has been increasing interest in determining if targeting steps in glucocorticoid signaling pathways could ablate or prevent these negative health outcomes.

**Glucocorticoids Effect Glucose Metabolism in Pancreatic Islets**

This section will highlight the effects of glucocorticoid treatment on islet function, specifically in α and β-cells. The studies performed, which characterize the mechanisms related to the effects of glucocorticoids on islet function, are performed in either islets isolated from glucocorticoid treated animals or, islets that are treated with glucocorticoids following isolation. The literature on isolated rodent islets is contradictory with multiple studies reporting that glucocorticoids inhibit [160, 204-208] or stimulate [209-213] GSIS *in vitro*. However, studies that were performed in dispersed β-cells and insulin secreting cell lines showed a decrease in GSIS following glucocorticoid treatment [204, 208, 214, 215]. These discrepancies *in vitro* cannot simply be explained by variations in the duration of glucocorticoid exposure or differences in the steroids used [206]. Instead, the discrepancies almost certainly relate to variations in experimental conditions coupled with the kinetic complexity of the transcriptional actions of glucocorticoids [216] and the ability of the GR to signal through non-genomic mechanisms [168]. Moreover, given the observation that marked differences exist between the regulation of gene expression
by glucocorticoids in isolated cells and \textit{in vivo} [217], the relevance of these isolated islet studies to \textit{in vivo} glucocorticoid action is questionable.

Unfortunately, the \textit{in vivo} studies on glucocorticoid regulation of islet function are just as contradictory as the \textit{in vitro} data. Human studies show that individuals who are obese or older respond differently to glucocorticoid treatments. These individuals exhibit hyperinsulinemia, insulin resistance and glucose intolerance [144]. Conversely, glucocorticoid treatment in healthy, non-obese individuals showed that a single dose of glucocorticoids inhibits insulin secretion during a meal or OGTT [184, 218]. Similarly in rodents and primates it has been shown, contradictorily, that glucocorticoids enhance insulin secretion, beyond the level required to counteract insulin resistance. This ultimately resulted in improved glucose tolerance and enhanced glycogen deposition [219-223]. Moreover, glucocorticoids have been shown to increase insulin secretion during an intraperitoneal glucose tolerance test (IPGTT) or OGTT in healthy men and adult rats and that glucocorticoids actually enhance β-cell function in response to glucocorticoid induced insulin resistance [224, 225]. This \textit{in vivo} hyperinsulinemia is consistent with \textit{in vitro} data that shows enhanced GSIS from pancreatic islets isolated from glucocorticoid treated rodents [209-213]. Hyperinsulinemia is thought to be a compensatory mechanism to counterbalance the insulin resistance in skeletal muscle, liver and WAT and can result, in healthy individuals, in normal glycaemia [129]. It remains unclear whether the observed hyperinsulinemia is a glucocorticoid dependent or independent effect. It has been hypothesized that the hyperinsulinemia is a compensatory response to the insulin resistance and not a direct action of glucocorticoids on β-cells to increase insulin secretion. This hypothesis is supported by studies that show that treatment of isolated islets with glucocorticoids, in the absence of whole body insulin resistance, directly inhibits insulin secretion [204]. Whether hyperinsulinemia is an effect directly mediated by glucocorticoid action on β-cells or indirectly mediated by glucocorticoid induced insulin resistance, hyperinsulinemia is one of the few consistent findings in experiments on healthy volunteers [131, 141, 142, 184, 218, 226] and in healthy rats [147, 210, 227]. A final observation that confounds these studies is that there is a glucocorticoid dependent increase in β-cell mass, which has been demonstrated to
occur in a time and dose dependent manner relative to the degree of insulin resistance [147, 210]. This increase in β-cell mass could also explain the hyperinsulinemia. It should be noted that a majority of studies characterizing the effects of glucocorticoid on insulin secretion and islet function have been performed in rats and humans. This is important because G6pc2, as previously mentioned, is a pseudogene in rats, indicating that the results from these studied were obtained in the absence of G6pc2 mediated glucose cycling and therefore may not be representative of the human or mouse condition [66].

Lastly, a few in vivo studies have been performed to look at the effect of glucocorticoid treatment in mice. Unfortunately though, the in vivo mouse data is sparse, with the majority of work being performed in transgenic or ob/ob mice. Studies performed with obese ob/ob mice have not measured insulin sensitivity or glucose tolerance but they did observe decreased insulin secretion and unaltered fasting insulinemia, in contrast to human and in vitro rat studies [228, 229]. Notably, Khan and colleagues found that glucose cycling is markedly enhanced in isolated islets treated with glucocorticoids from ob/ob mice and this enhancement was accompanied by a reduction in GSIS [90, 228-230]. Interestingly, these data were published prior to the identification of G6pc2 and G6pt and, at the time it was published, there was not a mechanism that explained the observed increase in glucose cycling following glucocorticoid treatment in islets. Now that the role of G6pc2 in glucose cycling in islets has been discovered, G6pc2’s function could explain the previously published findings from Khan et al [90, 228, 229]. Finally, the effect of glucocorticoid treatment in transgenic models that overexpress the GR specifically in β-cells showed glucose intolerance, increased or unaltered fasting glycaemia and decreased fasting insulinemia [160, 173, 231]. As with the previously discussed in vitro isolated islet data, the in vivo mouse data is contradictory, making interpretation of the data difficult in the context of determining what the direct contributions of glucocorticoids are on the mouse islet.

While the main goal of this section has been to outline the effects of glucocorticoids on β-cell function, glucocorticoids also affect other pancreatic cell types and secretion of other islet hormones. Similar to the data on glucocorticoids and β-cell function, studies on α-cell function and glucagon secretion
are just as conflicting. Studies show no change, an increase and a decrease in glucagon secretion following glucocorticoid treatment. For example, in vivo, one study showed that treatment of healthy subjects with Dex does not change glucagon secretion [232]. Similarly, treatment of isolated rat or mouse islets with glucocorticoids was shown to have no effect on glucagon secretion [233, 234]. In contrast to these findings, treatment of mouse islets with glucocorticoids for 2 hours reduced glucagon secretion relative to control islets in the presence of low glucose and was reversed by antagonizing the GR, implicating glucocorticoid signaling directly [158]. Finally, in vivo there is fasting hyperglucagonemia observed in glucocorticoid treated rhesus macaques [219] and healthy, non-obese men [141, 235, 236] and rats [234, 237]. Consistent with this finding, there is enhanced α-cell mass from Dex treated rats, indicating most likely that there is islet hyperplasia in glucocorticoid treated subjects [234]. As with the data on β-cell function, there are different paradigms for studying glucocorticoid biology and these vary in the type of glucocorticoids used and the length of treatment and dose, which may explain the inconclusive results. In conclusion, the effects of glucocorticoids on pancreatic β-cell function are complicated in part because of the pleiotropic effects of glucocorticoids throughout the whole body but also because of technical consideration regarding the design and interpretation of results. The studies described in Chapter III, IV and VI will examine the effect that endogenous and synthetic glucocorticoids have on G6pc2 expression and whole body glucose metabolism, namely fasting blood glucose.

**Hypothesis: Glucocorticoids Modulate G6pc2 Expression and Glucose Metabolism**

Because of the findings that Dex stimulates human G6PC2 promoter activity and that glucocorticoids have widespread effects throughout the body, we hypothesize that stressing mice or treating them with glucocorticoids will result in increased G6pc2 gene expression and modulation of FBG. The studies performed in Chapter III examine the effects of endogenous glucocorticoids in the physical restraint paradigm, Chapter IV outlines the effects of Dex treatment and Chapter VI examines the effect of 11-DHC
water supplementation. The data described in these chapters support the role of G6pc2 in modulating the set point for FBG under conditions of physiological stress.

Hypothesis: Rare $G6PC2$ SNPs Exist and Affect Protein Expression and Enzyme Activity

Due to the successful studies on rare $GCK$ variants, we hypothesized that there are rare variants in $G6PC2$ that could account for the missing heritability observed in GWA studies examining the genetics of FBG. We predict that these rare variants would have a significant effect on either protein expression or enzyme activity, which would be expected to modulate the set point for FBG. Since the identified SNP’s in the $G6PC2$ locus have a minor effect on FBG (~1mg/dl) yet $in$ $vivo$ deletion of $G6pc2$ has a relatively major effect on FBG, decreasing it by ~14 mg/dl; we hypothesize that a rare variant, which disables G6PC2, would have the same effect on FBG as seen in $G6pc2$ KO mice [69]. The studies described in Chapter VII outline a systematic functional analysis of 23 non-synonymous $G6PC2$ SNPs using a novel $in$ $situ$ functional assay for G6Pase activity.
II. MATERIALS AND METHODS

Generation of G6pc2 KO Mice

The G6pc2 targeting vector and G6pc2 mutant mice were generated as previously described [96, 238]. Both the 129SvEv and C57BL/6J G6pc2 KO congenic strains were developed via a speed congenic breeding strategy [239, 240]. A male G6pc2 heterozygous mouse on a mixed 129SvEv X C57BL/6J background was bred with female 129SvEv or C57BL/6J mice. The male offspring with the mutated G6pc2 allele and with the highest content of C57BL/6J or 129SvEv genome, respectively, as determined by microsatellite DNA, was used for the next round of breeding. Further rounds of backcrossing were performed using the same approach. Backcrossing was complete after 6 generations in C57BL/6J mice and 129SvEv mice. In order to fix the Y chromosome, a male mouse with 100% C57BL/6J or 129SvEv genome based on marker analysis was bred with female C57BL/6J or 129SvEv, respectively. Female offspring were crossed with male C57BL/6J or 129SvEv mice in order to ensure that all subsequent offspring carried the correct C57BL/6J or 129SvEv Y chromosome.

Speed congenic breeding was achieved by using a panel of 61 microsatellite markers that were equally spaced throughout the genome (~30cM intervals). These markers were used to differentiate the genetic background or the original/donor and target/recipient mouse strains. The markers selected to distinguish between strains were chosen using a “panel generator” at http://www.cidr.jhmi.edu/mouse/mmsset.html. Genomic DNA was isolated by standard proteinase K digestion protocols, mixed with True Allele PCR Premix (Applied Biosystems, Foster City, CA) and dispensed into a panel of Mouse Mapping Primers (Applied Biosystems). The multiplexing reaction and amplification parameters followed manufacturer directions. After PCR, 4 mls of multiplexed product, 0.6 mls of GS500-ROX size standard and 6 mls of Hi-Di formamide were mixed (Applied Biosystems). Denaturing was done at 94°C for 3 minutes and loaded onto the ABI 3100 Avant Genetic Analyzed. Chromatogram data was analyzed using GeneMapper 3.5 software (Applied Biosystems). Once mice were
confirmed to only have C57BL/6J or 129SvEv markers, heterozygous mice were bred to generate WT, KO and heterozygous mice.

**PCR Genotyping of G6pc2 KO Mice**

Mice were tailed and their DNA was genotyped by PCR and primers, which distinguish between WT and target alleles. Specific details of G6pc2 genotyping are found in reference [96].

**Animal Care**

All animal housing and mouse facilities met the American Association for the Accreditation of Laboratory Animal Care standards. The Vanderbilt University Medical Center Animal Care and Use Committee approved all protocols used. Mice were maintained on a standard rodent chow diet (LabDiet 5001; 23% protein and 4.5% fat; PMI Nutrition International) with food and water provided *ad libitum*. Where specified, mice were placed on a high fat (60% fat calories; Mouse diet F3282; BioServ) diet at 8 weeks of age and maintained on the diet for 12-14 weeks. Where specified, mice were maintained on a standard chow diet and water supplemented with 5mg/ml 11-dehydrocorticosterone (Steraloids, Inc) as described in ref [241].

**Phenotypic Analysis of Fasted WT and G6pc2 KO Mice**

Mice were fasted for 5 hours and then weighed. After an additional hour of fasting, mice were anesthetized using isoflurane and blood samples were isolated from the retro-orbital venous plexus. Glucose concentrations were measured in whole blood using a glucose monitor (Accu-Check Advantage; Roche, Indianapolis, USA). EDTA (5 μl; 0.5 M) was then added to blood samples prior to isolation of plasma by centrifugation. Insulin samples were assayed using RIA by the Vanderbilt Diabetes Center Hormone Assay Core [242].
Intraperitoneal Glucose Tolerance Tests

Intraperitoneal glucose tolerance tests (IPGTTs) on 6-24 hour fasted conscious mice were performed as previously described [243]. Mouse age and duration of the fast are indicated in the respective experiments. In all of the described IPGTTs, mice were fasted for the respective time and weighed an hour prior to the experiment. After an hour of recovery, the mice were either given an injection or oral gavage of 0.75 or 2.0 mg/kg body weight glucose in sterile PBS. Tail vein blood was used to obtain glucose measurements after the injection at the following time points: 0, 15, 30, 60, 90 and 120 using a Freestyle glucose meter (Abbott). In the physical restraint paradigm, IPGTTs were performed 4 hours after a final hour of physical restraint.

Analysis of Glucose-Stimulated Insulin Secretion In Vivo

Insulin secretion during IPGTTs was assessed in Male WT, Het and KO mice following a 6 or 18 hours fast as previously described [69]. Mice were anesthetized with isoflurane and blood samples were collected from the retro-orbital venous plexus in order to obtain basal glucose and insulin levels. Mice were given 15-30 minutes to recover from anesthesia and were then injected or orally administered a 0.75 or 2.0 mg/kg body weight bolus of glucose. Basal insulin and glucose were assessed after a specific amount of time that is indicated in the respective chapters. Blood glucose was measured using a glucose monitor (Accu-Check Aviva, Roche, Indianapolis, IN, USA). EDTA (5 μl; 0.5 M) was added to blood samples prior to isolation of plasma by centrifugation. Insulin samples were assayed using RIA by the Vanderbilt Diabetes Center Hormone Assay Core [242].

Dexamethasone Injection Paradigm

Mice were injected at 8 am with 13 μg/g dexamethasone (Dex) phosphate (2 mg/ml dissolved in PBS) for 4-5 days prior to IPGTTs. This dose of Dex was based on the results of a previous publication [244]. Alternate doses were used as stated in figure legends.
Physical Restraint Paradigm

The repeated physical restraint experimental paradigm has been previously described [245]. Briefly, mice were immobilized in a 50 ml conical tube at 10 am each day for 1 hour for 10 consecutive days.

Mouse and Human Islet Isolation

Mouse islets were isolated by the Vanderbilt Islet Procurement and Analysis Core as previously described [246]. Human islets were obtained by A.C.P. through the NIDDK-funded Integrated Islet Distribution Program (https://iidp.coh.org/).

Analysis of G6pc2 Gene Expression in Mouse Pancreata by Quantitative RT-PCR

Gene expression was quantitated using real time PCR. Pancreatic and tissue culture cell gene expression were quantitated after RNA isolation by using the Turbo DNA-free DNase Treatment Kit (Ambion, Carlsbad, CA) to remove trace genomic DNA followed by cDNA generation using the iScript DNA Synthesis Kit (Bio-Rad, Hercules, CA) and then PCR using the dUTP-containing FastStart SYBR Green Master Mix in conjunction with Uracil-Glycosylase (Roche, Nutley, NJ). Islet gene expression was quantitated using the primer-probe TaqMan® approach from Life Technologies (Carlsbad, CA) as described [247]. Fold induction of gene expression was calculated using the $2^{(-\Delta\Delta C(T))}$ method [248].

The following primer pairs were used for the analysis of pancreatic RNA expression:

Mouse G6pc2 Forward  5′-GTCTGTGGTGAGCAGGAC-3′
Mouse G6pc2 Reverse  5′-CCCTGATGGTGCTTA-3′
Mouse Slc37a4 Forward 5′-GCCAGTAAGGCTGAGTTG-3′
Mouse Slc37a4 Reverse 5′-TCTGGCTTACCCTTGA-3′
Mouse Fkbp5 Forward  5′-AGGCCGTGATCAGTGACAGG-3′
Mouse Fkbp5 Reverse  5′-GAACGACTCTGGAGGCTTTGG-3′

For the analysis of mouse and human islet gene expression TaqMan® primers were purchased from
Life Technologies (Carlsbad, CA). The catalogue numbers are as follows:

Mouse $G6pc2$  Mm00491176_m1
Human $G6PC2$  Hs01549773_m1
Mouse $Slc37a4$  Mm00484574_m1
Human $SLC37A4$  Hs00259865_m1

The genes used as internal controls in islet gene expression analyses were as previously reported [247].

**Electronic Health Record (EHR)-Based Phenotyping of Human Research Subjects**

EHR-based phenotyping was conducted using data on human subjects in the Vanderbilt University Medical Center (VUMC) BioVU DNA databank. Genotyping data in BioVU is linked to the Synthetic Derivative (SD), a de-identified version of the VUMC EHR repository. Detailed descriptions of program operations, ethical considerations, and continuing oversight and patient engagement have been published [249, 250]. For these studies we used a previously genotyped cohort of 29,722 European descendants from VUMC with longitudinal medical care. Genotyping was performed on the Illumina Human Exome BeadChip platform. For this study, we specifically analyzed the intronic $G6PC2$ SNP rs560887. Lipid measurements utilized routine clinical laboratory testing values present in the EHR.

**Site Directed Mutagenesis and Plasmid Preparation**

Human $G6PC2$, mouse $G6pc2$ or mouse $G6pc1$ in the pcDNA3.1D v5-His-TOPO Vector with a C-terminal V5-His Tag was used to generate non-synonymous variants in the coding sequence using Quikchange II Site-Directed Mutagenesis (Agilent). V5-His constructs are terminally extended versions of the plasmids, which incorporate a V5 epitope and His6 tag generated by deletion of the stop codon [56]. Sanger Sequencing was used to verify all mutations. Two to three independent preps were made for each mutant described. All mutant plasmid constructs were purified by centrifugation through cesium chloride gradients [51].
Cell Culture

Rat islet-derived 832/13 cells and monkey kidney-derived COS 7 cells were passaged as subconfluent cultures in RPMI medium supplemented with 10% (vol/vol) fetal bovine serum, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin.

SNP Databases

Human G6PC2 SNPs were identified using the UCSC Genome Browser (https://genome.ucsc.edu/), HumSAVR (http://omictools.com/humsavar-tool) or dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) databases.

G6PC1 Expression Vector Construction

The construction of plasmids encoding human G6PC2 (accession number NM_021176), mouse G6pc2 (accession number NM_021331), human G6PC1 (accession number NM_000151) and mouse G6pc1 (accession number NM_008061) in the pcDNA3.1D v5-His-TOPO vector with a C-terminal V5-His Tag has been previously described [55, 56]. Our human G6PC2 cDNA contains a leucine at amino acid (AA) 219 [66]. Alternate alleles of SNP rs492594 switch a valine for a leucine at AA 219.

Human G6PC2:mouse G6pc2 and mouse G6pc2:human G6PC2 chimeras in the pcDNA3.1D v5-His-TOPO vector were constructed by ligating fragments of the respective open reading frames. This was achieved by sub-cloning using a combination of restriction enzyme sites in the pcDNA3.1D v5-His-TOPO vector and the Dra I, Stu I and BamH I restriction enzyme sites, which are common to both human G6PC2 and mouse G6pc2. These three sites truncate G6PC2 at AAs 72, 192 and 249, respectively, from the N-terminus.

Site-directed mutagenesis was used to change specific codons in human G6PC2 and mouse G6pc1. This was achieved either by using the Quikchange II kit (Agilent) or a three-step PCR protocol [51]. DNA sequencing was used to verify all codon changes and the absence of secondary mutations. Two to three
independent plasmid preparations were analyzed for each mutant described. Plasmids were purified by centrifugation through cesium chloride gradients [51].

**G6pc1 and Pklr Fusion Gene Construction**

A bacterial artificial chromosome (BAC) clone (CH230-220)5 containing the entire rat *G6pc1* gene (Accession number AC123346) was purchased from BACPAC Resources, Children’s Hospital Oakland Research Institute, Oakland, CA. This clone was digested with *Kpn* I to isolate a 7319 bp fragment, representing the rat *G6pc1* promoter region between -7253 and +66 [251], that was then ligated into a *Kpn* I digested pGEM7 vector (Promega). This fragment was then re-isolated, blunted ended using Klenow and ligated into the *Xba* I - *Bgl* II, digested and Klenow treated pCAT(An) vector, a gift from Dr. Howard Towle [252]. Fragments of the rat *G6pc1* promoter, representing promoter sequence between -7248 and +62 and -1640 and +62, were then re-isolated from the pCAT(An) plasmid by digestion with *Hind* III and *Xho* I and ligated into the *Hind* III and *Xho* I digested pGL3 MOD luciferase vector [55].

Two fragments of the rat liver pyruvate kinase (*Pklr*) promoter representing sequences from -206 to +1 and -100 to +1 [253], were generated by PCR reaction using rat genomic DNA as the template in conjunction with the following primers:

(5'-**CCCCAGCT**(-206)TCTGCAGACAGGCAAAGGGATCC-3'),

(5'-**CCCCAGCT**(-100)TGCTAGCTGGTTATACTTTAAC-3'), and

(5'-**CCGCTCGAGA**(+1)CCTGCTGTGTCTGTGGGTCTGCT-3'); *Hind* III and *Xho* I cloning sites underlined. The PCR fragments generated were digested with *Hind* III and *Xho* I, ligated into the *Hind* III and *Xho* I digested pGEM7 vector (Promega) and then sequenced to ensure the absence of polymerase errors. The fragments were then re-isolated from the pGEM7 plasmid and ligated into the *Hind* III and *Xho* I digested pGL3 MOD luciferase vector [55].

**RNA Isolation and Quantification**

To compare wild type human *G6PC2* and mouse *G6pc2* RNA expression plasmids encoding human
G6PC2 and mouse G6pc2 (3 μg) were expressed by transient transfection of semi-confluent COS 7 cells in 10 cm diameter dishes using the lipofectamine reagent (Invitrogen, Waltham, MA) as previously described [254]. Following transfection, cells were incubated for 18-20 hours in serum-containing medium. RNA was then harvested and purified using the RNAqueous® kit (Ambion, Carlsbad, CA). Gene expression was then quantitated by using the Turbo DNA-free DNase Treatment Kit (Ambion, Carlsbad, CA) to remove trace genomic DNA followed by cDNA generation using the iScript DNA Synthesis Kit (Bio-Rad, Hercules, CA) and then PCR using the dUTP-containing FastStart SYBR Green Master Mix in conjunction with Uracil Glycosylase (Roche, Nutley, NJ). PCR products were analyzed by electrophoresis on 1% agarose gels.

The following primer pairs, that recognize non-coding sequences in the pcDNA3.1D v5-His-TOPO vector, were used for the analysis of both human G6PC2 and mouse G6pc2 expression:

pcDNA Forward 5’ CCAAGCTTGGTACCGAGCTCGGATCCAGT
pcDNA Reverse 3’ CCCGGTTTAATCGCTGATGGTGATGTCGGTGATGACCCT

The pcDNA forward primer recognizes 5’ untranslated leader sequence in the mRNA. This sequence represents part of the polylinker 3’ of the CMV transcription start site. The pcDNA reverse primer recognizes 3’ untranslated sequence in the mRNA. This sequence represents the region immediately 3’ of the V5 His tag.

Monkey cyclophilin A (PPIA) expression was quantitated as an internal control using the following primers:

Monkey PPIA Forward 5’-AATGGCACTGGTGGAAGTC-3’
Monkey PPIA Reverse 5’-GCTCCATGGCCTCCACAATA-3’
Ins2 Forward 5’-CACAGGAGGTGTTGCTCAAGC-3’
Ins2 Reverse 5’-CCAGTGCAAGGAATTGAAGG-3’
Protein Expression Analysis by Transient Transfection, Western blotting and Luciferase Assays

Semi-confluent 832/13 or Cos7 cell lines were used for all transfection experiments. For luciferase assays, cells were cultured and co-transfected with plasmids encoding WT or mutated hG6PC2, mG6pc2 or mG6pc in the pcDNA3.1D v5-His-TOPO vector (2ug) and the Renilla luciferase expression vector (0.5ug) using lipofectamine as previously described [66, 254-256]. After the transfection, cells were incubated for 18-20 hours in serum-containing medium supplemented with 2 or 30 mM glucose. As previously described, the cells were harvested and Renilla and firefly luciferase activity were assayed using the Promega Dual-Luciferase Reporter Assay System according to the manufacturer's instructions [255]. To correct for variations in transfection efficiency, the results were calculated as a ratio of firefly to Renilla luciferase activity. Results were presented either as this ratio or relative to the ratio obtained with 30 mM glucose or relative to the ratio obtained at 30 mM glucose with either catalytically dead G6pc1 or WT G6pc1. The data was analyzed using the principles described in the novel 832/12 transcription assay described in chapter VII. Each construct was analyzed in duplicate across multiple transfections using at least two independent plasmid preparations.

For protein expression analysis, as just described, semi-confluent cells were cultured and transfected with V5-his tagged WT or mutated hG6PC2, mG6pc2 or mG6pc (2ug) using lipofectamine [61, 254]. After the transfection, cells were incubated for 18-20 hours in serum-containing medium. Cells were harvested using passive lysis buffer and protein was isolated. Protein samples were quantified using the BCA Protein Assay Kit according to manufacturer’s instructions as previously described [257]. Western blots were performed using a 10% SDS-PAGE gel and transferred to a PVDF membrane. Protein expression was determined by immunoblotting with a mouse monoclonal Anti-V5-HRP antibody (1:100-1:5000, Invitrogen). A primary Anti-Beta Actin monoclonal antibody (1:10,000, Sigma) with an Anti-Mouse HRP secondary antibody was used as a loading control (1:10,000, Promega). Protein bands were detected using ECL reagent (Pierce Thermo Fisher Scientific). Protein expression data were normalized by scanning both
V5 and actin signals on Western blots. The ratio of V5 to actin expression obtained with the variants shown was expressed as a percentage relative to the ratio obtained with WT human G6PC2 or mouse G6pc1.

The expected sizes of human G6PC2, mouse G6pc2, human G6PC1 and mouse G6pc1 with V5 His tags are 45.60, 45.71, 45.54 and 45.51 kDa. As previously observed, both the human G6PC1 [55] and mouse G6pc1 [56] expression plasmids generate doublets, possibly though the use of alternate methionine start codons (Fig. 7.6).

**Fusion Gene Analyses**

The construction of wild type human *G6PC2*-luciferase fusion genes have been previously described [255], as have *G6PC2* SNP promoter variants [79, 80]. Site directed mutations of the Maf and GR binding sites in the *G6PC2* promoter and 5’ truncations were constructed using PCR as described [79, 80]. The mouse *G6pc2* promoter was isolated using PCR and 129SvEv or C57BL/6J genomic DNA as the template.

Cells were transfected using lipofectamine as described [255] and incubated for 18-20 hrs in the indicated concentration of Dex prior to harvesting.

**Gel Retardation Assays**

Gel retardation assays were used to assess MafA binding exactly as previously described [255].

**Statistical Analyses**

Mouse data were analyzed using a two-way ANOVA assuming normal distribution and equal variance. A post hoc analysis was performed using the Bonferroni correction for multiple comparisons. The level of significance was as indicated. Other data including analysis of time zero basal glucose and singular time point measurements were analyzed using Student’s t-test: two sample assuming equal variance or one-way ANOVA’s. The level of significance was as indicated (two-sided test).

To analyze genetic associations with lipids in BioVU, we used the median value for each individual. The associations between the genotypes and the aggregated laboratory values (as continuous variables) were performed on R with linear model, adjusted for age, sex, and body mass index (BMI). We report beta
values, 95% confidence intervals (CI), and p values. P < 0.05 was considered to be significant. All tests assumed a two-tailed distribution.
III. G6PC2 MODULATES FASTING BLOOD GLUCOSE IN MALE MICE IN RESPONSE TO STRESS

Introduction

The role of G6PC2 in islet β-cells has been extensively covered in the introduction of this dissertation. Figure 1.1 highlights the main function of G6PC2 to hydrolyze G6P to glucose and a free phosphate, thereby creating a futile cycle that opposes the actions of glucokinase. Previous data supports the role of G6PC2 as a negative regulator of GSIS and as contributing to glucose cycling in β-cells [69].

Because chronically elevated FBG has been associated with both increased risk for cardiovascular-associated mortality [72] and the development of T2D [70], this implies that G6PC2 is actually detrimental to health in the modern Western environment, which is associated with nutrient excess and longevity. In contrast, during evolution the ability of G6PC2 to regulate FBG must have conferred a specific advantage. We hypothesized that factors that induce G6PC2 expression would confer a transient beneficial change in FBG. We therefore screened for factors that regulate G6PC2 promoter activity. In this study we show that glucocorticoids stimulate G6PC2 expression and we present in vivo data suggesting that G6PC2 may have evolved to modulate FBG in response to stress.

Results

Dexamethasone Stimulates Human G6PC2 Expression

Although chronically elevated G6PC2 expression increases FBG and is detrimental over the long term to human health we hypothesized that the ability of G6PC2 to transiently regulate FBG must nonetheless confer an evolutionarily conserved benefit. We therefore screened factors known to regulate FBG to determine whether they could also modulate G6PC2 promoter activity. Figure 3.1A shows that the synthetic glucocorticoid Dex stimulates a marked, concentration dependent activation of the human G6PC2 promoter, as assessed by fusion gene transient transfection assays in the TC-3 islet-derived cell line. The effect of Dex was enhanced by co-transfection with a plasmid encoding the glucocorticoid receptor (GR) (Fig. 3.1B), consistent with previous studies that observed a limiting intracellular concentration of this
receptor [258]. A similar marked stimulation of human G6PC2–luciferase fusion gene expression by Dex was observed in primary mouse islet cells (Fig. 3.1C). Furthermore Dex stimulated endogenous G6PC2 and SLC37A4 gene expression in primary human islets (Fig. 3.1D). Human G6PC2–luciferase fusion gene expression was also induced by the endogenous glucocorticoid corticosterone (Fig. 3.1E).

To localize the G6PC2 glucocorticoid response element (GRE) a series of 5’ truncated G6PC2-luciferase fusion genes were analyzed by transient transfection. Figure 3.1F shows that Dex-stimulated fusion gene expression was abolished when the region of the promoter between -171 to -131 was deleted. Visual inspection of this region identified the presence of a putative GRE [259] that is conserved across multiple species (Fig. 3.1G). A point mutation of a nucleotide in this putative GRE known to be required for GR binding (Fig. 3.1G; [259]), in the context of an otherwise intact promoter, had a limited effect on basal fusion gene expression but abolished the Dex response (Fig. 3.1H).

**Dexamethasone Stimulates 129SvEv but not C57BL/6J Mouse G6pc2 Promoter Activity**

We next sought to confirm that Dex also regulates mouse G6pc2 gene expression in vivo. Surprisingly, 3 hours following a single Dex injection we observed that endogenous pancreatic G6pc2 expression was induced in 129SvEv (Fig. 3.2A) but not C57BL/6J (Fig. 3.2B) mice, whereas Slc37a4 gene expression was induced in both (Figs. 3.2A & B). Consistent with these in vivo gene expression data, Dex only stimulated endogenous G6pc2 expression in isolated 129SvEv islets (Fig. 3.2C) but not C57BL/6J islets (Fig. 3.2D), whereas Slc37a4 gene expression was induced in both 129SvEv (Fig. 3.2E) and C57BL/6J (Fig. 3.2D) islets.

An explanation for this unexpected result became apparent from sequence analyses, which revealed that the alternate alleles of a mouse SNP, rs32980497, affect a key nucleotide in the G6pc2 GRE, that is required for GR binding [259]. 129SvEv mice, but not C57BL/6J mice, harbor the allele that supports GR binding (Fig. 3.1G).
Figure 3.1. Dexamethasone Stimulates Human G6PC2 Expression.
A: Induction of G6PC2-luciferase fusion gene expression in TC-3 cells by Dex.
B: Co-transfection of an expression vector encoding the glucocorticoid receptor (GREV) enhances the induction of G6PC2-luciferase fusion gene expression in TC-3 cells by Dex (250 nM).
C: Induction of G6PC2-luciferase fusion gene expression in dispersed primary mouse islet cells by Dex (250 nM). These primary cells represent a mixed population of islet cell types.
D: Induction of endogenous G6PC2 and SLC37A4 gene expression in human islets by Dex. Human islets were treated for 3 hrs with 250 nM Dex.
E: Induction of G6PC2-luciferase fusion gene expression in 832/13 cells by corticosterone (250 nM).
F: Localization of the G6PC2 GRE through the analysis of the effect of Dex (250 nM) on truncated G6PC2-luciferase fusion gene expression in TC-3 cells.
G: Cross-species sequence alignment of the G6PC2 GRE and comparison with the consensus [259].
H: Point mutation of the G6PC2 GRE reduces basal G6PC2-luciferase fusion gene expression in TC-3 cells and abolishes the effect of Dex (250 nM).
Results show the mean ± S.E.M. of 3-4 experiments. *p < 0.05 versus control.
Figure 3.2. Dexamethasone Stimulates 129SvEv but not C57BL/6J Mouse G6pc2 Promoter Activity.

A-B: Selective induction of G6pc2 gene expression in 129SvEv (A) but not C57BL/6J (B) mice by short term Dex treatment. Pancreatic RNA was isolated from mice (129SvEv, n=6; C57BL/6J n=6) 3 hrs following injection with PBS or Dex phosphate (13 μg/g). Results show the mean ± S.E.M. *p < 0.05 versus control.

C-E: Induction of G6pc2 and Slc37a4 gene expression in 129SvEv (C; E) and C57BL/6J (D) mouse islets by Dex. RNA was isolated from islets incubated for 3 hrs in the presence or absence of variable concentrations (C; E) or 250 nM (D) Dex. Results show the mean ± S.E.M. of 3-9 experiments. *p < 0.05 versus control.

F-G: Dex-stimulates 129SvEv (F) but not C57BL/6J (G) G6pc2-luciferase fusion gene expression in 832/13 cells. Results show the mean ± S.E.M. of 3 experiments. *p < 0.05 versus control.

H: The stimulation of C57BL/6J G6pc2-luciferase fusion gene expression in 832/13 cells by Dex is modulated by the alternate alleles of rs32980497. Results show the mean ± S.E.M. of 3 experiments. *p < 0.05 versus control. Different vectors (F, G: pGL4) and (H: pGL3) were used in these experiments, explaining the differences in basal expression.
As a result, Dex markedly activates the 129SvEv G6pc2 promoter (Fig. 3.2F) but not the C57BL/6J G6pc2 promoter (Fig. 3.2G), as assessed by fusion gene transient transfection assays. Four other nucleotides differ between the proximal C57BL/6J and 129SvEv G6pc2 promoters (data not shown) but substitution of the alternate allele of rs32980497 in the C57BL/6J promoter is sufficient to restore responsiveness to Dex (Fig. 3.2H). The reverse experiment, mutating the equivalent nucleotide in the human G6PC2 promoter, abolishes the Dex response (Fig. 3.1H).

**The Induction of G6pc2 in Response to Physical Restraint Modulates FBG in 129SvEv Mice**

Previous studies have shown that G6Pase activity and glucose cycling are increased in islets isolated from Dex-injected mice [229, 231]. These observations, which were published before the G6PC2 gene was identified [260], can now be explained by the induction of G6PC2 gene expression by glucocorticoids and are entirely consistent with our model of G6PC2 function. To extend these *in vitro* isolated islet studies, we next sought to study the physiological consequences of glucocorticoid-stimulated 129SvEv G6pc2 expression *in vivo*.

For these studies the G6pc2 KO allele was backcrossed onto the 129SvEv genetic background using a speed congenic approach [69]. Before analyzing the effect of G6pc2 deletion on the response to glucocorticoids in 129SvEv mice, we first sought to compare the effect of G6pc2 deletion on FBG and glucose tolerance in control 129SvEv mice. As seen in mixed genetic background [96] and C57BL/6J mice [69] FBG was reduced in 129SvEv G6pc2 KO mice relative to WT mice whereas fasting insulin was unchanged (Figs. 3.3A & B). As in C57BL/6J mice [69], IPGTTs showed no difference in glucose tolerance between WT and G6pc2 KO 129SvEv mice (Fig. 3.3C). These data suggest that G6pc2 regulates FBG rather than glucose tolerance in control 129SvEv mice as previously observed in C57BL/6J mice [69].

To study the physiological consequences of glucocorticoid-stimulated 129SvEv G6pc2 expression *in vivo*, we examined the effect of a 10 day, repeated physical restraint experimental paradigm [245] on FBG in 129SvEv G6pc2 WT and KO mice. Figure 3.3D shows that this treatment stimulated endogenous
pancreatic G6pc2 and Slc37a4 gene expression in 129SvEv mice with little change in body weight (Fig. 3.3E). This paradigm is associated with elevated endogenous glucocorticoid levels (Fig. 3.3F) but also the activation of many other autonomic and neuroendocrine axes, beside the hypothalamic-pituitary-adrenal axis, resulting in the release of epinephrine and norepinephrine [261]. The observed induction of G6pc2 expression could therefore be mediated by factors in addition to glucocorticoids. This presumably explains why G6pc2 expression is also induced to a lesser degree in C57BL/6J mice (Fig. 3.3G), despite the GRE mutation (Fig. 3.1G).

We have previously shown that, under fasting conditions, insulin levels are identical in WT and G6pc2 KO mice, but FBG is lower in G6pc2 KO mice due to a leftward shift in the dose response curve for GSIS [69]. We hypothesized that the induction of G6pc2 expression by physical restraint would enhance this existing difference in FBG between WT and KO mice (Fig. 3.3H & I). However, we reasoned that the physiological benefit conferred by G6pc2 induction would depend on the overall combined effects of physical restraint on whole body glucose metabolism in vivo because FBG is determined by multiple factors other than G6PC2. We predicted that if physical restraint results in an elevation in FBG in WT mice then the induction of G6pc2 expression would contribute to that elevation and it would be prevented or reduced in G6pc2 KO mice (Fig. 3.3H). On the other hand we predicted that if physical restraint results in hypoglycemia in G6pc2 KO mice then the induction of G6pc2 expression in WT mice would limit or prevent hypoglycemia (Fig. 3.3I). Following a 6 hr fast, FBG was unchanged in physically restrained 129SvEv WT mice but decreased in physically restrained 129SvEv G6pc2 KO mice (Fig. 3.3J), indicating that the difference in FBG between 129SvEv WT and KO mice was enhanced by physical restraint and that the induction of G6pc2 expression protects against low blood glucose, consistent with the model shown in Figure 3.3I. Fasting insulin levels were unchanged in 129SvEv mice following physical restraint (Fig. 3.3K), again consistent with the model shown in Figure 3I. Physical restraint had no effect on FBG in C57BL/6J mice (Fig. 3.3L) consistent with the small change in G6pc2 expression (Fig. 3.3G). In these studies no difference in FBG was observed between control 129SvEv (Fig. 3.3J) or C57BL/6J (Fig. 3.3L) WT and KO
mice because the difference in FBG is small such that it is only consistently observed with much higher n values (Fig. 3.3A; Ref. [69]). In contrast a clear difference in FBG was observed in physically restrained 129SvEv WT and KO mice (Fig. 3.3).

Discussion

We show here that the synthetic glucocorticoid Dex induces human G6PC2 promoter activity (Fig. 3.1). Dex also induces 129SvEv but not C57BL/6J mouse G6pc2 promoter activity, the difference being due to a mutation in the C57BL/6J promoter GR binding site (Fig. 3.2). In response to stress generated by physical restraint, G6pc2 expression is induced, enhancing the difference in FBG between 129SvEv WT and KO mice and protecting against low blood glucose (Fig. 3.3). Thus while in modern society the chronic influence of G6PC2 on FBG affects the risks of cardiovascular-associated mortality and T2D, our data suggest that G6PC2 may have initially evolved to transiently modulate FBG under conditions of glucocorticoid-related stress. Interestingly, G6PC2 expression is decreased in islets isolated from individuals with T2D [262] but it is unclear whether this contributes to islet dysfunction, given that the resulting enhanced glycolytic flux would promote the cytotoxic effects of glucose [263], or whether, because this would also lead to enhanced insulin secretion, this represents a compensatory change in unhealthy islets designed to maintain glucose homeostasis.

The widely accepted dogma with respect to the effect of glucocorticoids on glucose metabolism in both rodents and humans in vivo is that they inhibit glucose uptake by inducing insulin resistance, stimulate hepatic glucose production and inhibit insulin secretion, thereby inducing glucose intolerance [129, 181]. While the resulting increase in blood glucose is considered beneficial during periods of transient stress [201], prolonged elevation of glucocorticoids, as occurs in Cushing's disease [264], can lead to diabetes. In contrast our studies (Fig. 3.3) and others [219] demonstrate the complexity of glucocorticoid physiology by suggesting that, in some experimental paradigms, glucocorticoid action leads to improved FBG and/or glucose tolerance.
Figure 3.3. The Induction of G6pc2 in Response to Physical Restraint Modulates FBG and Glucose Tolerance in 129SvEv Mice.

A & B: Blood glucose (Panel A) and plasma insulin (Panel B) levels in control 6 hr fasted 129SvEv male WT (n=16) and G6pc2 KO (n=14) mice. Results show the mean ± S.E.M. *p < 0.05 versus WT.

C: IPGTTs using 2.0 g/kg glucose performed on 6 hr fasted conscious male WT (n=13) and G6pc2 KO (n=11) mice.

D: Induction of G6pc2 and Slc37a4 gene expression in 129SvEv mice by physical restraint (PR). Pancreatic RNA was isolated following a 6 hr fast from control (C) mice and mice that had been physically restrained (PR) (n=10-11). G6pc2 and Slc37a4 expression were quantitated using real-time PCR. *p < 0.05 versus control.

E: Weight change in 129SvEv (WT, n=21; KO, n=22) mice following physical restraint (PR). Results show the mean ± S.E.M. *p < 0.05 versus initial body weight. The difference in weight loss between WT and KO was significant (**p < 0.05).

F: Induction of plasma corticosterone by physical restraint (PR). Blood was isolated following a 3 hr fast from control (C) mice and mice that had been physically restrained (PR) (n=7-10). *p < 0.0001, one-way ANOVA.

G: Induction of G6pc2 and Slc37a4 gene expression in C57BL/6J mice by physical restraint. Pancreatic RNA was isolated following a 6 hr fast from control (C) mice and mice that had been physically restrained (PR) (n=8). G6pc2 and Slc37a4 expression were quantitated using real-time PCR. *p < 0.05 versus control.
H & I: Diagrams proposing that the induction of G6pc2 expression by physical restraint (PR) will increase the difference in FBG between fasted WT and KO mice. The diagrams indicated that the actual values of the X axis (glucose) may be shifted to the right (Panel G) or left (Panel H), relative to those in control mice, depending on the effects of PR on other aspects of metabolism. In either case, FPI levels are predicted to not differ between WT and G6pc2 KO mice, as observed in control mice [69].

J: Blood glucose levels in 6 hr fasted conscious control (C) (WT, n=13; KO, n=11) or 6 hr fasted conscious physically restrained (PR) (WT, n=8; KO, n=10) 129SvEv male mice. Results show the mean ± S.E.M. *p < 0.0001, one-way ANOVA; NS, not significant.

K: Plasma levels in 6 hr fasted conscious control (C) (WT, n=16; KO, n=14) or 6 hr fasted conscious physically restrained (PR) (WT, n=21; KO, n=24) 129SvEv male mice. Results show the mean ± S.E.M.

L: Glucose levels in 6 hr fasted conscious control (C) (WT, n=12; KO, n=15) or physically restrained (PR) (WT, n=6; KO, n=7) C57BL/6J male mice. Results show the mean ± S.E.M.
Thus in 6 hr fasted mice this induction of \textit{G6pc2} by glucocorticoids limits the decrease in FBG (Fig. 3.3I) which increases circulating glucose levels that could provide energy under stressful circumstances relative to KO mice. While in the physical restraint paradigm the induction of \textit{G6pc2} expression protects against low blood glucose (Fig. 3.3H) we predict that in other stress-associated paradigms this induction may contribute to a transient increase in FBG (Fig. 3.3G), which could be a survival mechanism that is valuable for keeping the brain supplied with glucose is times of stress [201].

In summary, our data suggest that \textit{G6PC2} may have initially evolved to transiently modulate the sensitivity of GSIS to glucose under conditions of glucocorticoid-related stress. \textit{G6PC2} is thought to be expressed exclusively in pancreatic islet \( \beta \)-cells [78, 82] but future studies on beta-cell specific KO mice will be required to prove that low \textit{G6pc2} expression in rare cell types is not regulating FBG and FPI through other mechanisms.
IV. G6PC2 MODULATES THE EFFECTS OF DEXAMETHASONE ON FASTING BLOOD GLUCOSE AND GLUCOSE TOLERANCE

Introduction

Experiments using perfused pancreata and isolated islets demonstrate that deletion of G6pc2 enhances the sensitivity of GSIS to glucose, by shifting the dose response curve for GSIS to the left, rather than affecting maximal GSIS [69]. This has two consequences. First, GSIS is enhanced in G6pc2 KO islets at sub-maximal but not maximal glucose levels [69]. Second, this results in a counterintuitive reduction in FBG levels in G6pc2 KO mice that is associated with no change in FPI levels [69, 96, 265]. These observations are consistent with data from GWAS showing that common SNPs in the G6PC2 locus are associated with variations in FBG but not FPI [82, 266].

Since chronically elevated FBG is associated with increased risk for the development of both T2D [70] and cardiovascular-associated mortality [72] it implies that high G6PC2 expression is actually detrimental to health over the long term. However, presumably during evolution the ability of G6PC2 to regulate FBG must have conferred a specific advantage. We recently showed that glucocorticoids induce G6PC2 expression and speculated that G6PC2 evolved to transiently modulate FBG in response to stress [265]. We extend that observation here by exploring the mechanism by which the GR regulates G6PC2 promoter activity and how G6pc2 deletion modulates the effects of the synthetic glucocorticoid Dex on FBG and glucose tolerance.

Results

The Glucocorticoid Receptor Stimulates G6PC2 Promoter Activity by Displacing MafA

We have previously shown that corticosterone and Dex markedly activate the human G6PC2 promoter through a GRE located between -171 and -157 relative to the transcription start site [265]. Sequence analyses suggest that the G6PC2 GRE overlaps a binding site for the islet-enriched transcription factor MafA (Fig. 4.1A) [267]. This putative human G6PC2 MafA binding site shows conservation at 13/14 nucleotides with a previously identified MafA binding site in the mouse G6pc2 promoter that contributes
to basal $G6pc2$ promoter activity [255]. Gel supershift assays confirmed that this human $G6PC2$ promoter region also binds MafA (Fig. 4.1B). This raised the mechanistic question as to whether MafA and GR compete for binding to this promoter region or whether MafA is acting as an accessory factor to stabilize GR binding and promote glucocorticoid signaling. Thus, while the $G6PC2$ GRE closely matches the endogenous consensus sequence (Fig. 4.1A), this is not the optimal sequence for high affinity GR binding [259] such that accessory factor involvement is likely required for glucocorticoid signaling [268]. To address this question, two $G6PC2$ promoter site-directed mutants (SDMs) were generated, designated Maf SDM 1 and 2, that were predicted to reduce or increase MafA binding, respectively, without affecting GR binding (Fig. 4.1A). The Maf SDM 1 mutation disrupts nucleotides that are required for MafA binding whereas the Maf SDM 2 mutation enhances the $G6PC2$ MafA binding site such that it matches the consensus sequence (Fig. 4.1A). Neither mutation disrupts nucleotides required for GR binding (Fig. 4.1A).

Gel retardation competition assays demonstrated that these mutations have the anticipated effect on MafA binding (Fig. 4.1C; Fig. 4.2). When analyzed by transient transfection in TC-3 cells, the Maf SDM 1 mutation, which reduced MafA binding (Fig. 4.1C), led to reduced basal fusion gene expression (Fig. 4.1D), whereas the Maf SDM 2 mutation, which increased MafA binding (Fig. 4.1C), led to increased basal fusion gene expression (Fig. 4.1E). These data confirm the importance of MafA for basal $G6PC2$ promoter activity as observed for the mouse $G6pc2$ promoter [255]. These mutations altered $G6PC2$ promoter activation by Dex (Fig. 4.1F-I). When expressing data as fold induction (Fig. 4.1F & G), the Maf SDM 1 mutation enhanced the Dex response (Fig. 4.1F) whereas the Maf SDM 2 mutation impaired the Dex response (Fig. 4.1G) leading to the conclusion that MafA and GR compete for binding rather than MafA acting as an accessory factor supporting GR binding. In contrast, when expressing data as maximal induction (Fig. 4.1H & I), both the Maf SDM 1 (Fig. 4.1H) and Maf SDM 2 (Fig. 4.1I) mutations impaired the Dex response such that only the SDM2 data support a competition model. However, co-transfection experiments demonstrated that overexpression of MafA (Fig. 4.1J) but not NeuroD (Fig. 4.1K), which binds an E-Box in the $G6pc2$ promoter
[269], impairs the Dex response again leading to the conclusion that MafA and GR compete for binding rather than MafA acting as an accessory factor supporting GR binding.

With a view to further exploring this model through the use of chromatin immunoprecipitation (ChIP) assays, we also examined Dex-regulated endogenous gene expression in several islet-derived cell lines. Dex induced human G6PC2-luciferase fusion gene expression in the mouse TC-3 [265], NIT-1 (Fig. 4.3A), TC-tet (Fig. 4.3B) and hamster HIT (Fig. 4.3C) islet-derived cell lines, with the effect of Dex being enhanced in each case by co-transfection with a plasmid encoding the glucocorticoid receptor, consistent with previous studies that observed a limiting intracellular concentration of this receptor [258]. However, it failed to induce endogenous G6pc2 gene expression (data not shown). In the TC-3 and NIT-1, but not the TC-tet and HIT cell lines, the lack of Dex-induced endogenous G6pc2 expression is likely explained by the absence of a consensus GRE in the G6pc2 promoter (Fig. 4.3D). However, Dex stimulates endogenous Fkbp5 and Sgk expression (Fig. 4.4), but not Slc37a4 gene expression (data not shown), in the three mouse cell lines suggesting that G6pc2 and Slc37a4 gene expression are regulated by Dex through mechanisms that are not recapitulated in these islet-derived cell lines. We also considered ChIP assays in primary islets but the effect of Dex on G6pc2 (Fig. 4.1E), and Slc37a4 (Fig. 4.1F), gene expression were transient and reduced in magnitude relative to effects on the endogenous genes in mouse pancreas in vivo [265].

The rs2232316 G6PC2 SNP has Opposite Effects on Basal and Dex-Stimulated Promoter Activity

The transcription factor Foxa2 is required for endogenous G6pc2 expression in mouse islets [270] and high G6pc2 promoter activity [255].
Figure 4.1. The Glucocorticoid Receptor Stimulates G6PC2 Promoter Activity by Displacing MafA.
A: The G6PC2 GRE overlaps a binding site for MafA [267].
B: Gel retardation supershift assays demonstrate that MafA binds the -182/-154 G6PC2 promoter region. The c-Maf antiserum cross-reacts with MafA [255]. A representative gel is shown.
C: Gel retardation competition experiments demonstrate that the order of MafA binding affinity to the sequences shown in Panel A is Maf SDM 2 > WT > Maf SDM 1. A representative gel is shown.
D-I: Effect of promoter mutations on basal and Dex-stimulated G6PC2-luciferase fusion gene expression in TC-3 cells. A reduction in MafA binding (Maf SDM 1) decreases basal expression but has little effect on the Dex response. An increase in MafA binding (Maf SDM 2) increases basal expression and reduces the Dex response. Results show the mean ± S.E.M. of 3 experiments. *p < 0.05 versus control.
J-K: Effect of MafA (J) or NeuroD (K) overexpression on Dex-stimulated G6PC2-luciferase fusion gene expression in 832/13 cells. Overexpression of MafA but not NeuroD inhibits G6PC2-luciferase fusion gene expression. Results show the mean ± S.E.M. of 3 experiments. *p < 0.05 versus control.

Figure 4.2. Gel Retardation Competition Assay with Maf Mutants. Experiment was performed as described in Materials and Methods.
Figure 4.3. Dexamethasone Stimulates G6PC2 Promoter Activity in Multiple Islet-Derived Cell Lines. A-C: Co-transfection of an expression vector encoding the glucocorticoid receptor (GREV) enhances the induction of G6PC2-luciferase fusion gene expression in NIT-1, βTC-Tet and HIT cells by Dex (250 nM). Results show the mean ± S.E.M. of 3 experiments. *p < 0.05 versus control. D: Cross-species sequence alignment of the G6pc2 GRE in various cell lines and comparison with the consensus. E-F: Induction of G6pc2 (E) and Slc37a4 (F) gene expression in 129SvEv mouse islets by Dex. RNA was isolated from islets incubated in the presence or absence of 250 nM Dex for the times indicated. Results show the mean ± S.E.M. of 3-9 experiments. *p < 0.05 versus control.

Figure 4.4. Dexamethasone Does Not Stimulate Fkbp or Sgk Gene Expression in Multiple Islet-Derived Cell Lines. A-B: Endogenous Fkbp (A) or Sgk (B) is not induced in NIT-1, βTC-Tet or HIT cells by Dex (250 nM). Results show the mean ± S.E.M. of 3 experiments.
Because in the related \(G6pc1\) gene Foxa2 can act as an accessory factor to enhance glucocorticoid-stimulated gene expression [165] and since the \(G6PC2\) promoter contains two Foxa2 binding sites, located between \(-261\) and \(-250\) and between \(-246\) and \(-235\), we investigated whether Foxa2 also acts as an accessory factor to enhance glucocorticoid-stimulated \(G6PC2\) gene expression. Specifically we investigated the effect of two common SNPs in each of these Foxa2 binding sites, rs573225, located at \(-259\), and rs2232316, located at \(-238\), that we have previously shown affect Foxa2 binding and basal \(G6PC2\) fusion gene expression [79, 80]. The ‘G’ allele of rs573225 is associated with altered kinetics of Foxa2 binding and increased basal promoter activity (Fig. 4.5A; [80]). However, Figure 4.5B shows that this SNP has no effect on Dex-induced \(G6PC2\)-luciferase fusion gene expression. In contrast, while the ‘A’ allele of rs2232316 is associated with enhanced Foxa2 binding affinity and increased basal promoter activity (Fig. 4.5C; [79]), Figure 4.5D shows that it is also associated with reduced Dex-induced \(G6PC2\)-luciferase fusion gene expression. These data suggest that it is unlikely Foxa2 acts as an accessory factor to enhance Dex-stimulated \(G6PC2\) gene expression through binding to these sites. Moreover, while rs573225 and rs2232316 are both genetically associated with variations in FBG [79, 80], the data in Figure 4.3D suggest that whether rs2232316 has a positive or negative influence on FBG may be dependent on glucocorticoid levels.

**\(G6pc2\) Modulates the Effect of Dexamethasone on FBG and Glucose Tolerance in 129SvEv Mice**

We have previously studied the physiological consequences of glucocorticoid-stimulated 129SvEv \(G6pc2\) expression on FBG in 129SvEv \(G6pc2\) WT and KO mice using a repeated physical restraint experimental paradigm [265]. This treatment stimulated endogenous pancreatic \(G6pc2\) and \(Slc37a4\) gene expression in 129SvEv mice but is associated not only with activation of the hypothalamic-pituitary-adrenal axis and elevated endogenous glucocorticoid levels [265], but also with activation of many other autonomic and neuroendocrine axes resulting in the release of epinephrine and norepinephrine [261]. The
observed induction of \textit{G6pc2} expression could therefore have been mediated, in part, by factors in addition to glucocorticoids.

Given this caveat, we examined the impact of \textit{G6pc2} deletion on the action of glucocorticoids using an alternate experimental paradigm involving 5 days of once daily Dex injections [244]. Figure 4.6 shows that a dose of 13 \textmu g/g Dex induced both pancreatic \textit{G6pc2} and \textit{Slc37a4} expression. This is consistent with our previous studies showing that Dex markedly activates the 129SvEv \textit{G6pc2} promoter as well as acutely stimulating endogenous \textit{G6pc2} gene expression in primary 129SvEv mouse islets and mouse pancreas \textit{in vivo} [265]. Surprisingly, Dex had little effect on hepatic \textit{G6pc1} and \textit{Slc37a4} expression in 129SvEv mice \textit{in vivo}, despite the fact that both gene promoters are activated by Dex in hepatoma cells [165, 271]. This probably is because plasma insulin is elevated in Dex treated 129SvEv mice (see below) and insulin suppresses both \textit{G6pc1} and \textit{Slc37a4} gene expression [251, 272].

We have previously shown in mixed genetic background [96], C57BL/6J [69] and 129SvEv [265] mice that FPI levels are identical in WT and \textit{G6pc2} KO mice, but FBG is lower in \textit{G6pc2} KO mice. This counterintuitive change in glucose without a change in insulin occurs because deletion of \textit{G6pc2} results in a leftward shift in the dose response curve for GSIS rather than a change in maximal GSIS [69]. We hypothesized that the induction of \textit{G6pc2} expression by Dex would enhance this existing difference in FBG between WT and KO mice (Fig. 4.7A & B). However, we reasoned that the physiological benefit conferred by \textit{G6pc2} induction would depend on the overall combined effects of Dex on whole body glucose metabolism \textit{in vivo} because FBG is determined by multiple factors other than G6PC2. We predicted that if Dex results in an elevation in FBG in WT mice, then the induction of \textit{G6pc2} expression would contribute to that elevation and it would be prevented or reduced in \textit{G6pc2} KO mice (Fig. 4.7A). On the other hand, we predicted that if Dex results in hypoglycemia in \textit{G6pc2} KO mice, then the induction of \textit{G6pc2} expression in WT mice would limit or prevent hypoglycemia (Fig. 4.7B).
Figure 4.5. The rs2232316 G6PC2 SNP has Opposite Effects on Basal and Dex-Stimulated Promoter Activity. The influence of the alternate alleles of the rs573225 and rs2232316 SNPs on basal (A, C) and Dex-stimulated (B, D) G6PC2-luciferase fusion gene expression was analyzed by transient transfection in TC-3 cells. The rs573225-G and rs2232316-A alleles both increase basal expression but only the rs2232316-A allele affects the Dex response. Results show the mean ± S.E.M. of 3 experiments. *p < 0.05 versus control.

Figure 4.6. Chronic Dexamethasone Treatment Stimulates Pancreatic G6pc2 Gene Expression in 129SvEv Mice. Effect of chronic Dex treatment on pancreatic G6pc2 and Slc37a4 (A-C) and hepatic G6pc1 and Slc37a4 (D-F) gene expression in 129SvEv mice. Pancreatic RNA was isolated following a 6 hr fast from control mice and mice that had received daily injections of the indicated amount of Dex phosphate for 5 days. Results show the mean ± S.E.M. of 3 experiments. *p < 0.05 versus control.
Unexpectedly 5/17 WT (Fig. 4.7C) and 5/15 G6pc2 KO (Fig. 4.7D) mice developed diabetes following Dex treatment, defined here as a blood glucose level > 200 mg/dl at the 60 min time point in an intraperitoneal glucose tolerance test (IPGTT). The molecular basis for the development of diabetes in a sub-set of mice is unknown, though this variable effect of Dex treatment on diabetes incidence resembles that in a previous study by Ogawa et al. [175] who observed a 16% incidence of diabetes in Dex treated Wistar rats. Considering only the non-diabetic 129SvEv WT and G6pc2 KO mice, prolonged Dex treatment was associated with modest ~3% weight loss (Fig. 4.7E). Following a 6 hr fast, the difference in FBG between control 129SvEv WT and KO mice was similar to the difference in FBG between Dex treated 129SvEv WT and KO mice, however, only the latter difference was statistically significant (Fig. 4.7F). This likely reflects the fact that large n values are usually required to detect the difference in FBG between control WT and G6pc2 KO mice [69, 96, 265]. Figure 4.7F also shows that FBG was decreased in Dex treated 129SvEv G6pc2 KO relative to control KO mice. While FBG was statistically unchanged in Dex treated 129SvEv WT relative to control WT mice, there was a clear trend towards reduced FBG in WT mice. Overall these data suggest that the induction of G6pc2 expression by Dex did not markedly enhance the difference in FBG between WT and KO mice. Nevertheless, the presence of G6pc2 in WT mice is clearly protecting against low blood glucose, resembling the model shown in Figure 4.7B. As in control mice, FPI levels did not differ between Dex treated 129SvEv WT and G6pc2 KO mice (Fig. 4.7G). However, FPI levels were higher in both WT and KO mice following Dex treatment presumably due to the well characterized induction of insulin resistance by Dex [169]. Figure 4.7B therefore represents an oversimplification that does not account for the effect of Dex on insulin signaling.

Interestingly, Dex treatment improved glucose tolerance in both 129SvEv WT and KO mice relative to untreated mice and a clear trend (p<0.06) towards a difference in glucose tolerance was apparent between Dex treated 129SvEv WT and G6pc2 KO mice in contrast to no difference in control mice (Fig. 4.7H). Similarly, following 10 days of repeated physical restraint glucose tolerance was improved in both 129SvEv WT and KO mice relative to untreated mice and in this case a difference in glucose tolerance was
apparent between physically restrained 129SvEv \( G6pc2 \) KO mice and WT mice in contrast to no difference in control mice (Fig. 4.7I).

**\( G6pc2 \) Also Modulates the Effect of Dexamethasone on FBG and Glucose Tolerance in C57BL/6J Mice**

We have previously shown that Dex fails to activate the C57BL/6J \( G6pc2 \) promoter or acutely stimulate endogenous \( G6pc2 \) gene expression in primary C57BL/6J mouse islets and mouse pancreas *in vivo* due to a SNP that inactivates the GRE in the C57BL/6J \( G6pc2 \) promoter [265]. Surprisingly, Figure 4.8 shows that daily injections of a dose of 13 \( \mu \)g/g Dex induced both pancreatic \( G6pc2 \) and \( Slc37a4 \) expression in C57BL/6J mice, though not as markedly as in 129SvEv mice (Fig. 4.8). This observation suggests that the regulation of \( G6pc2 \) expression by Dex *in vivo* is mediated through overlapping but distinct acute and chronic mechanisms in 129SvEv and C57BL/6J mice (Fig. 4.9). In contrast to 129SvEv mice (Fig. 4.6), Dex treatment induced hepatic expression of both \( G6pc1 \) and \( Slc37a4 \) in C57BL/6J mice (Fig. 4.8), though because plasma insulin is elevated in Dex treated C57BL/6J mice (see below) and insulin suppresses both \( G6pc1 \) and \( Slc37a4 \) gene expression [251, 272], this probably limited the magnitude of the induction.

Also in contrast to 129SvEv mice (Fig. 4.10), no Dex treated C57BL/6J mice developed diabetes (Fig. 4.10A). Interestingly, as with 129SvEv mice (Fig. 4.7), Dex treatment improved glucose tolerance in both C57BL/6J WT and KO mice relative to untreated mice (Fig. 4.10A). In addition, a difference in glucose tolerance was apparent between Dex treated C57BL/6J WT and \( G6pc2 \) KO mice in contrast to no difference in control mice (Fig. 4.10A).

As in 129SvEv mice, prolonged Dex treatment was associated with modest ~4% weight loss in C57BL/6J mice (Fig. 4.10B). Following a 6 hr fast, the difference in FBG between control C57BL/6J WT and KO mice was similar to the difference in FBG between Dex treated C57BL/6J WT and KO mice (Fig. 4.10C). Figure 4.10C also shows that FBG was decreased in Dex treated C57BL/6J \( G6pc2 \) KO relative to control KO mice.
**Figure 4.7. G6pc2 Modulates the Effect of Dexamethasone on FBG and Glucose Tolerance in 129SvEv Mice.**

A-B: Diagrams proposing that the induction of G6pc2 expression by Dex will increase the difference in FBG between fasted WT and KO mice. The diagrams indicated that the actual values of the X axis (glucose) may be shifted to the right (Panel A) or left (Panel B), relative to those in control mice, depending on the effects of Dex on other aspects of metabolism. In either case, FPI levels are predicted to not differ between WT and G6pc2 KO mice, as observed in control mice.

C: Analysis of glucose tolerance using IPGTTs in 6 hr fasted conscious Dex treated WT 129SvEv mice. Glucose tolerance was significantly different between non-diabetic Dex-treated (n=12) and diabetic Dex-treated (n=5) WT mice based on ANOVA and at the indicated time points based on post-hoc analyses (*p < 0.05).

D: Analysis of glucose tolerance using IPGTTs in 6 hr fasted conscious Dex treated KO 129SvEv mice. Results show the mean ± S.E.M. Glucose tolerance was significantly different between non-diabetic Dex-treated (n=10) and diabetic Dex-treated (n=5) KO mice based on ANOVA and at the indicated time points based on post-hoc analyses (*p < 0.05).

E: Weight change following 4 days of Dex (D) injections in non-diabetic 129SvEv mice (WT, n=12; KO, n=10). Results show the mean ± S.E.M. *p < 0.05 versus initial body weight.
F: Glucose levels in 6 hr fasted conscious control (C) (WT, n=14; KO, n=12) or Dex treated non-diabetic (D) (WT, n=12; KO, n=10) 129SvEv male mice. Results show the mean ± S.E.M. *p < 0.05 versus control KO; **p < 0.05 versus matching WT.

G: Insulin levels in 6 hr fasted conscious control (C) (WT, n=11; KO, n=10) or Dex treated non-diabetic (D) (WT, n=7; KO, n=6) 129SvEv male mice. Results show the mean ± S.E.M. *p < 0.05 versus control WT or KO.

H: Analysis of glucose tolerance using IPGTTs in 6 hr fasted conscious control (WT, n=14; KO, n=12) or Dex treated (WT, n=12; KO, n=10) 129SvEV mice. Results show the mean ± S.E.M. Glucose tolerance was significantly different between control and non-diabetic Dex-treated WT mice and control and non-diabetic Dex-treated KO mice based on ANOVA and at the indicated time points based on post-hoc analyses (*p < 0.05).

I: Analysis of glucose tolerance using IPGTTs in 6 hr fasted conscious control (WT, n=14; KO, n=12) or physically restrained (PR) (WT, n=8; KO, n=10) 129SvEV mice. Results show the mean ± S.E.M. Glucose tolerance was significantly different between control and physically restrained KO mice and between physically restrained WT and KO mice based on ANOVA and at the indicated time points based on post-hoc analyses (*p < 0.05).
While FBG was statistically unchanged in Dex treated C57BL/6J WT relative to control WT mice, there was a clear trend towards reduced FBG in WT mice. Overall these data suggest that the induction of G6pc2 expression by Dex did not markedly enhance the difference in FBG between WT and KO mice. Nevertheless, as in 129SvEv mice (Fig. 4.7F) the presence of G6pc2 in WT mice is clearly protecting against low blood glucose, resembling the model shown in Figure 4.5B. As in control mice, FPI levels did not differ between Dex treated C57BL/6J WT and G6pc2 KO mice (Fig. 4.10D), though FPI levels were higher in both WT and KO mice following Dex treatment, again presumably due to the well characterized induction of insulin resistance by Dex [169].

Strikingly different results were observed in 24 hr fasted mice. Dex treatment increased FBG in both C57BL/6J WT and KO mice and a difference in FBG was observed between Dex treated, but not control, C57BL/6J WT and KO mice (Fig. 4.10E). These results indicate that, in this context, G6pc2 deletion is limiting the rise in blood glucose induced by Dex treatment, consistent with the model shown in Figure 4.7A. FPI levels did not differ between 24 hr fasted control C57BL/6J WT and KO mice or between Dex treated C57BL/6J WT and KO mice (Fig. 4.10F), though FPI levels were again higher in both WT and KO mice following Dex treatment, again presumably due to the well characterized induction of insulin resistance by Dex [169]. Figure 4.7A, like Figure 4.7B, therefore also represents an oversimplification that does not account for the effect of Dex on insulin signaling.

The reversal in the observed effect of Dex on FBG may be due to the marked decrease in FPI after 24 hr fasting (Fig. 4.10F) compared to 6 hr fasting (Fig. 4.10D). Presumably the low FPI in 24 hr fasted Dex treated WT and KO mice is insufficient to counteract Dex-induced insulin resistance explaining why FBG is now greater in Dex treated than control mice. Despite this reversal in the effect of Dex on FBG in 24 hr versus 6 hr fasted mice, glucose tolerance was still improved in 24 hr fasted Dex treated G6pc2 KO mice with a similar trend in WT mice (Fig. 4.10G), as was observed in 6 hr fasted mice (Fig. 4.10A). However, in contrast to 6 hr fasted mice (Fig. 4.10A), glucose tolerance was not improved in Dex treated G6pc2 KO relative to WT mice (Fig. 4.10G).
Figure 4.8. Chronic Dexamethasone Treatment Stimulates Pancreatic G6pc2 Gene Expression in C57BL/6J Mice. Effect of chronic Dex treatment on pancreatic G6pc2 and Slc37a4 (A-C) and hepatic G6pc1 and Slc37a4 (D-F) gene expression in C57BL/6J mice. Pancreatic RNA was isolated following a 6 hr fast from control mice and mice that had received daily injections of the indicated amount of Dex phosphate for 5 days. Results show the mean ± S.E.M. of 3 experiments. *p < 0.05 versus control.

Figure 4.9. Mechanisms of G6pc2 Gene Regulation in C57BL/6J and 129SvEv Mice. Regulation of G6pc2 expression by Dex in vivo is mediated through overlapping but distinct acute and chronic mechanisms in 129SvEv and C57BL/6J mice.
Figure 4.10. The Induction of \textit{G6pc2} by Dexamethasone Modulates FBG and Glucose Tolerance in C57BL/6J Mice.

\textbf{A}: Analysis of glucose tolerance using IPGTTs in 6 hr fasted conscious control (WT, \textit{n}=14; KO, \textit{n}=12) or Dex treated (WT, \textit{n}=13; KO, \textit{n}=11) C57BL/6J male mice. Results show the mean ± S.E.M. Glucose tolerance was significantly different between control and Dex treated mice and between Dex treated WT and KO mice based on ANOVA (*\textit{p} < 0.05).

\textbf{B}: Weight change following 5 days of Dex (D) injections in WT (\textit{n}=20) and KO (\textit{n}=20) C57BL/6J mice. Results show the mean ± S.E.M. *\textit{p} < 0.05 \textit{versus} initial body weight.

\textbf{C}: Glucose levels in 6 hr fasted conscious control (C) (WT, \textit{n}=12; KO, \textit{n}=22) or Dex treated (D) (WT, \textit{n}=36; KO, \textit{n}=46) C57BL/6J male mice. Results show the mean ± S.E.M. *\textit{p} < 0.05 \textit{versus} control KO; **\textit{p} < 0.05 \textit{versus} matching WT.

\textbf{D}: Insulin levels in 6 hr fasted conscious control (C) (WT, \textit{n}=12; KO, \textit{n}=22) or Dex treated (D) (WT, \textit{n}=33; KO, \textit{n}=42) C57BL/6J male mice. Results show the mean ± S.E.M. *\textit{p} < 0.05 \textit{versus} control WT or KO.

\textbf{E}: Glucose levels in 24 hr fasted conscious control (C) (WT, \textit{n}=9; KO, \textit{n}=8) or 24 hr fasted conscious Dex treated (D) (WT, \textit{n}=6; KO, \textit{n}=8) C57BL/6J male mice. Results show the mean ± S.E.M. *\textit{p} < 0.05 \textit{versus} control WT or KO; **\textit{p} < 0.05 \textit{versus} matching WT.

\textbf{F}: Insulin levels in 24 hr fasted conscious control (C) (WT, \textit{n}=9; KO, \textit{n}=9) or 24 hr fasted conscious Dex treated (D) (WT, \textit{n}=15; KO, \textit{n}=12) C57BL/6J male mice. Results show the mean ± S.E.M. *\textit{p} < 0.05 \textit{versus} control WT or KO.

\textbf{G}: Analysis of glucose tolerance using IPGTTs in 24 hr fasted conscious control (WT, \textit{n}=9; KO, \textit{n}=8) or Dex treated (WT, \textit{n}=6; KO, \textit{n}=8) C57BL/6J male mice. Results show the mean ± S.E.M. Glucose tolerance was significantly different between control and Dex treated KO mice based on ANOVA and at the indicated time points based on post-hoc analyses (*\textit{p} < 0.05).
Discussion

We show here that the synthetic glucocorticoid Dex induces human *G6PC2* expression through a mechanism that, competition and mutagenesis studies suggest, involves displacement of the islet-enriched transcription factor MafA by the glucocorticoid receptor (Fig. 4.1). The effect of Dex on *G6PC2* promoter activity is influenced by the rs2232316 SNP, which affects Foxa2 binding and has been linked to variations in FBG (Fig. 4.5; Ref. [79]). Chronic 5 day treatment with Dex induces *G6pc2* expression in both 129SvEv (Fig. 4.6) and C57BL/6J (Fig. 4.8) mouse pancreata *in vivo*. This contrasts with a selective acute effect of Dex on *G6pc2* expression in 129SvEv mice [265]. In both 6 hr fasted 129SvEv (Fig. 4.7) and C57BL/6J (Fig. 4.10) mice, the induction of *G6pc2* expression by Dex was insufficient to markedly enhance the difference in FBG between WT and KO mice but the presence of G6pc2 in WT mice served to limit the repression of FBG by Dex. In contrast, in 24 hr fasted C57BL/6J mice (Fig. 4.10) the induction of *G6pc2* expression by Dex enhanced the difference in FBG between WT and KO mice and *G6pc2* deletion served to limit the elevation of FBG induced by Dex. The latter observation is consistent with the hypothesis that the induction of *G6pc2* gene expression by Dex reduces the sensitivity of GSIS to glucose (Figs. 4.7A & B) [69, 265]. These data suggest that G6PC2 initially evolved to transiently modulate the sensitivity of GSIS to glucose under conditions of glucocorticoid-related stress, in contrast to modern society where the chronic influence of G6PC2 on FBG affects the risks of cardiovascular-associated mortality and T2D. Interestingly, this conclusion is consistent with pathway analyses that demonstrate connectivity between *G6PC2* and genes influencing steroid action [273].

In both rodents and humans *in vivo* the widely accepted dogma with respect to the effect of Dex/glucocorticoids on glucose metabolism is that they inhibit glucose uptake by inducing insulin resistance [169], stimulate hepatic glucose production [171] and inhibit insulin secretion [173, 175], thereby inducing glucose intolerance [129]. These effects result in a transient increase in blood glucose that is considered beneficial during periods of stress. In contrast, prolonged elevation of glucocorticoids, as occurs in Cushing’s disease [202], can lead to diabetes. This dogma contrasts with our studies (Figs. 4.7
& 4.10) and others [219, 221] that demonstrate the complexity of glucocorticoid physiology by showing that, in some experimental paradigms, Dex/glucocorticoids can enhance insulin secretion in vivo, beyond the level required to counteract insulin resistance, thereby leading to improved glucose tolerance. This improved glucose tolerance was observed in 6 hr fasted mice following chronic Dex treatment (Fig 4.5H & 4.7A) and following the elevation of endogenous glucocorticoids by physical restraint (Fig 4.7I). In addition, the contrasting effects of G6pc2 deletion in 6 hr versus 24 hr fasted Dex treated mice demonstrate that G6pc2 modulates the sensitivity of GSIS to glucose regardless of whether the overall whole body effect of Dex results in reduced (Fig. 4.10D) or elevated (Fig. 4.10E) FBG. Similarly, in 6 hr fasted mice, the elevation of endogenous glucocorticoids by physical restraint induces G6pc2 expression thereby modulating the sensitivity of GSIS to glucose and increasing the difference in FBG between WT and KO mice [265].

The literature on the effects of Dex/glucocorticoids on isolated rodent islets is equally complex with multiple studies reporting that they inhibit [204] or stimulate [209] GSIS in vitro. The explanation for these conflicting observations is unclear; they cannot simply be explained by variations in the duration of Dex/glucocorticoid exposure. Instead it almost certainly relates to variations in experimental conditions coupled with the kinetic complexity of the transcriptional actions of glucocorticoids [216] and the ability of the glucocorticoid receptor to also signal through non-genomic mechanisms [168], with the initial health of individuals studied [274] and strain background also being factors in human and mouse studies, respectively.

Human GWAS data have linked common SNPs in G6PC2 to variations in FBG [82, 266] but not altered glucose tolerance [108-111]. In contrast, we observed that glucose tolerance was improved in 129SvEv G6pc2 KO mice relative to WT mice following Dex treatment (Fig. 4.7H) or physical restraint (Fig. 4.7I). Similarly, we observed that glucose tolerance was improved in C57BL/6] G6pc2 KO mice relative to WT mice following Dex treatment (Fig. 4.10A). We hypothesize that the explanation for this difference between the human and mouse data relates to the fact that G6pc2 deletion affects the sensitivity of GSIS
to glucose rather than maximal GSIS [69]. This means that G6pc2 will only influence glucose tolerance if a low amount of glucose is injected in the experiment or if insulin-stimulated glucose disposal is enhanced. In both cases, the peak blood glucose will be in a sub-maximal region of the dose response curve for GSIS, which would be optimal for detecting an effect of G6pc2 deletion (Fig. 4.11). Consistent with this concept, in those experiments where G6pc2 deletion improved glucose tolerance, the peak glucose concentration in the IPGTT was ~150 mg/dl in 129SvEv mice (Figs. 4.7H & 4.5I) and ~250 mg/dl in C57BL/6J mice (Fig. 4.10A), which are known to have lower insulin sensitivity [275]. This contrasts with peak glucose concentrations of ~230 mg/dl (Figs. 4.7H & I) and ~350 mg/dl (Fig. 4.10A) in control 129SvEv and C57BL/6J mice, respectively. Furthermore, in the 24 hr fasted mice where G6pc2 deletion did not improve glucose tolerance, the peak glucose concentration in the IPGTT was almost 400 mg/dl (Fig. 4.10G). This concept is also consistent with previous studies in control, non-Dex treated mice in which G6pc2 deletion was associated with improved glucose tolerance when a dose of 0.4 g/kg glucose was injected but not higher doses of 0.75 and 2 g/kg [69].

In summary, our data suggest that G6PC2 initially evolved to modulate the sensitivity of GSIS to glucose under conditions of glucocorticoid-induced stress. Future studies will examine whether other hormones/metabolites also regulate G6PC2 gene expression.
Figure 4.11. Diagram of Changes in Glucose Levels Following Injection. G6pc2 only influences glucose tolerance if a low amount of glucose is or if insulin-stimulated glucose disposal is enhanced. In both cases, the peak blood glucose will be in a sub-maximal region of the dose response curve for GSIS, which would be optimal for detecting an effect of G6pc2 deletion.
V. THE EFFECT OF G6PC2 DELETION ON BODY WEIGHT AND FAT MASS IN MICE IS DEPENDENT ON DIET, GENETIC BACKGROUND AND GENDER

Introduction

The role of G6PC2 in islet β-cells has been extensively covered in the introduction of this dissertation. Figure 1.1 highlights the main function of G6PC2 to hydrolyze G6P to glucose and a free phosphate, thereby creating a futile cycle that opposes the actions of glucokinase Fig. 1.4. Previous data supports the role of G6PC2 as a negative regulator of GSIS and as contributing to glucose cycling in β-cells [69] [276, 277]. Deletion of G6pc2 results in leftward shift in the dose response curve for GSIS [69]. At submaximal glucose levels this shift results in enhanced GSIS from G6pc2 KO relative to WT mouse islets [69]. Under fasting conditions, where insulin levels are the same in WT and G6pc2 KO mice, this shift results in reduced FBG in KO mice [69, 96]. Consistent with these mouse studies, GWAS have linked SNPs in the G6PC2 gene to variations in FBG [82, 266]. The rs560887 SNP located in the third intron of the G6PC2 gene has been identified as the strongest common genetic determinant of FBG levels in human in terms of significance and effect size, accounting for about one percent of total variance in FBG [82, 266]. The association between G6PC2 and FBG has been confirmed in multiple GWAS and in different populations [76, 81, 112-115, 278, 279].

Numerous GWAS have also examined the genes that are associated with variations in body mass index (BMI), fat mass and fat distribution and have shown that greater than 160 loci are linked to these parameters [280]. While these analyses did not identify G6PC2 as one of the loci linked to these parameters [280], a GWAS performed in a relatively small cohort of Mexican Americans linked the G6PC2 rs560887-A allele with decreased BMI and adiposity in this population [111]. This observation prompted us to examine whether G6pc2 KO mice are protected from diet-induced obesity (DIO). We previously showed that female, but not male, G6pc2 KO mice on a pure C57BL/6J genetic background had reduced body weight and body fat on both a chow and high fat diet relative to WT mice, indicating that the effect of G6pc2 on these parameters is influenced by gender [69]. This current study extends those analyses by examining the
influence of genetic background on the impact of \textit{G6pc2} deletion on body weight and fat mass in mice. Specifically, we look at the effect of chow and high fat feeding in \textit{G6pc2} KO mice bred on a pure 129SvEv or mixed C57BL/6J X 129SvEv genetic background. The results confirm striking differences in the response of C57BL/6J and 129SvEv mice to high fat feeding [281] and show that the effect of \textit{G6pc2} deletion on FBG is largely independent of gender, genetic background and diet whereas the effects of \textit{G6pc2} deletion on body weight and fat mass are highly dependent on these variables. We also show that \textit{G6pc2} deletion affects plasma triglyceride and cholesterol levels in a manner dependent on gender, genetic background and diet and demonstrate an association between \textit{G6PC2} and plasma cholesterol in humans through electronic health record-derived phenotype analyses. These observations suggest that, in both mice and humans, the action of \textit{G6PC2} on FBG is independent of modifier genes whereas the action of \textit{G6PC2} on BMI and body fat are influenced by other genes.

**Results**

**Analysis of the Effect of \textit{G6pc2} Deletion on Body Weight and Composition in Chow Fed 129SvEv Mice**

We have previously shown that 16 week old chow fed female, but not male C57BL/6J \textit{G6pc2} KO mice are slightly lighter than WT littermates and have reduced body fat [69]. When this analysis was repeated using 16 week old chow fed 129SvEv \textit{G6pc2} mice no differences in either body weight or body fat were observed between female WT or KO mice (Table 5.1). However, male chow fed 129SvEv \textit{G6pc2} KO mice were slightly lighter than WT littermates (Table 5.2). This suggests that the effect of \textit{G6pc2} deletion on body weight varies with both gender and genetic background.

A comparison of body weight and composition between 16 week old WT chow fed C57BL/6J [69] and 129SvEv mice (Table 5.2) reveals little difference in body weight or composition between male mice. However, female 129SvEv mice were \textasciitilde 2g heavier than female C57BL/6J mice and had approximately double the fat mass [69] (Table 5.1).
Table 5.1. NMR Analysis of Female Chow Fed 129SvEv *G6pc2* KO Mouse Body Composition. Body composition of 6 hour fasted, 16 week old animals was assessed using a mq10 NMR analyzer. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; KO=knockout.

<table>
<thead>
<tr>
<th>Gender &amp; Genotype</th>
<th>Body Weight (g)</th>
<th>Fat (g)</th>
<th>Muscle (g)</th>
<th>Free Fluid (g)</th>
<th>Fat (%)</th>
<th>Muscle (%)</th>
<th>Free Fluid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female WT</td>
<td>22.23 ± 0.26 (15)</td>
<td>2.76 ± 0.26 (11)</td>
<td>13.94 ± 0.27 (11)</td>
<td>0.58 ± 0.05 (11)</td>
<td>13.04 ± 1.15 (11)</td>
<td>66.32 ± 0.94 (11)</td>
<td>2.78 ± 0.26 (11)</td>
</tr>
<tr>
<td>Female KO</td>
<td>22.11 ± 0.54 (14)</td>
<td>3.09 ± 0.46 (9)</td>
<td>14.69 ± 0.25 (9), *</td>
<td>0.60 ± 0.03 (9)</td>
<td>13.48 ± 1.62 (9)</td>
<td>66.05 ± 1.34 (9)</td>
<td>2.71 ± 0.15 (9)</td>
</tr>
</tbody>
</table>

1. Muscle: *p < 0.04

Table 5.2. NMR Analysis of Male Chow Fed 129SvEv *G6pc2* KO Mouse Body Composition. Body composition of 6 hour fasted, 16 week old animals was assessed using a mq10 NMR analyzer. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; KO=knockout.

<table>
<thead>
<tr>
<th>Gender &amp; Genotype</th>
<th>Body Weight (g)</th>
<th>Fat (g)</th>
<th>Muscle (g)</th>
<th>Free Fluid (g)</th>
<th>Fat (%)</th>
<th>Muscle (%)</th>
<th>Free Fluid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male WT</td>
<td>28.47 ± 0.34 (16)</td>
<td>2.02 ± 0.35 (11)</td>
<td>19.23 ± 0.37 (11)</td>
<td>0.82 ± 0.10 (11)</td>
<td>7.40 ± 1.29 (11)</td>
<td>69.90 ± 0.91 (11)</td>
<td>2.96 ± 0.25 (11)</td>
</tr>
<tr>
<td>Male KO</td>
<td>26.93 ± 0.54 (12), *</td>
<td>1.51 ± 0.25 (9)</td>
<td>18.21 ± 0.38 (9)</td>
<td>0.75 ± 0.12 (9)</td>
<td>6.03 ± 1.00 (9)</td>
<td>72.45 ± 0.96 (9)</td>
<td>3.01 ± 0.51 (9)</td>
</tr>
</tbody>
</table>

1. Weight: *p < 0.009
Analysis of the Effect of G6pc2 Deletion on FBG and FPI in Chow Fed 129SvEv Mice

We have previously shown that 16 week old chow fed female and male G6pc2 KO mice on either a mixed [96] or C57BL/6 [69] genetic background have reduced FBG but no change in FPI relative to WT littermates. When this analysis was repeated using 16 week old female and male chow fed WT and G6pc2 KO mice on the 129SvEv genetic background the same reduction in FBG was observed in female G6pc2 KO mice (Fig. 5.1A) with a similar trend toward reduced FBG in male KO mice relative to WT mice (Fig. 5.1B).

Consistent with previous results with mixed genetic background [96] and C57BL/6 G6pc2 KO mice [69], FPI was unchanged in female G6pc2 KO relative to WT mice (Fig. 5.1C). However, in contrast to previous results with mixed genetic background [96] and C57BL/6 G6pc2 KO mice [69], FPI was reduced in male G6pc2 KO mice relative to WT mice (Fig. 5.1D). This reduction in FPI in chow fed 129SvEv G6pc2 KO mice may explain the lack of a statistically significant difference in FBG. Indeed, in a slightly older cohort of chow fed 129SvEv mice we recently reported that FBG was reduced in male G6pc2 KO mice relative to WT with no change in FPI [265].

The reduction in FPI in this cohort of chow fed 129SvEv G6pc2 KO mice (Fig. 5.1D) is unlikely to be explained by an effect of G6pc2 deletion to enhance insulin sensitivity since glucose tolerance is not altered in these mice [265]. Rather, because we are not looking at a steady state, we think that this result may simply be explained by a difference in the kinetics of the fall in blood glucose during fasting. Two hypothesized relationships between glucose and insulin levels in WT and G6pc2 KO mice when measured following a 6 hr fast are shown diagrammatically in Figures 5.1E and 5.1F. These schematics illustrate how a difference in FBG could exist between WT and G6pc2 KO mice without (Fig. 5.1E) or with (Fig. 5.1F) a difference in FPI. Specifically, Figures 5.1E and 5.1F show how absolute glucose concentrations can be different while absolute insulin levels at the two glucose levels may or may not be different depending on where these values are on the curve. Despite these subtle differences, overall these data suggest that the effect of G6pc2 on FBG is independent of both gender and genetic background.
Analysis of the Effect of \textit{G6pc2} Deletion on Body Weight and Composition in High Fat Fed 129SvEv Mice

We next investigated the effect of high-fat feeding, a standard nutritional challenge in the field of obesity and diabetes research that induces insulin resistance and is considered to model human disease [282]. After starting high fat feeding at 8 weeks of age and continuing for 12 weeks we have previously shown that WT female and male C57BL/6J mice almost double their body weight relative to chow fed mice [69], as observed by many other groups [283]. Strikingly, when this analysis was repeated using the same feeding paradigm in 129SvEv mice we observed almost no difference in body weight between 16 week old chow fed female (Table 5.1) and 20 week old high fat fed female (Table 5.3) or between 16 week old chow fed male (Table 5.2) and 20 week old high fat fed male mice (Table 5.4). Weekly measurements of body weight in non-fasted high fat fed mice during the 12 weeks of high fat feeding showed no evidence for a biphasic change in weight, that would have been suggestive of a toxic effect of prolonged high fat feeding, in either female (Fig. 5.2A) or male (Fig. 5.2B) WT and KO mice. This is consistent with previous studies that have observed that 129SvEv mice are resistant to DIO [281].

Despite the lack of weight gain, high fat fed female and male 129SvEv mice showed a marked increase in body fat (%) relative to chow fed mice (Fig. 5.2C). This was associated with a reduction in body muscle (%) in both high fat fed female (Table 5.3) and male 129SvEv mice (Table 5.4) relative to chow fed female (Table 5.1) and male (Table 5.2) mice (p<0.05). We observed the same pattern of increased body fat (%) and decreased body muscle (%) in high fat fed C57BL/6J mice relative to chow fed mice [69]. However, because C57BL/6J mice gain substantially more weight following high fat feeding than 129SvEv mice, the observed differences in weight and fat mass between female chow fed C57BL/6J and 129SvEv mice are reversed.
Figure 5.1. Effect of G6pc2 Deletion on Metabolic Parameters in Chow Fed 129SvEv Mice. 

Panels A-D: At 17 weeks of age chow fed mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated. Blood glucose (Panels A & B) and plasma insulin (Panels C & D) were determined as described in Methods. Results are the mean ± S.E. M. of data with the genotype, gender and number of animals indicated. WT=wild type; KO=knockout; F=female; M= male. *p < 0.014 WT versus KO (Panel A); *p < 0.01 WT versus KO (Panel D). Panels E & F: Schematics to explain the effect of G6pc2 deletion on fasting blood glucose levels. The model in Panel E proposes that in 6 hr fasted WT and G6pc2 KO mice, where insulin levels are the same, FBG is reduced in G6pc2 KO mice due to a leftward shift in the dose response curve for GSIS relative to WT mice. The model in Panel F proposes that in 6 hr fasted WT and G6pc2 KO mice, where insulin levels differ, FBG is still reduced in G6pc2 KO mice due to a leftward shift in the dose response curve for GSIS relative to WT mice.
Table 5.3. NMR Analysis of Female High Fat Fed 129SvEv \textit{G6pc2} KO Mouse Body Composition.

Body composition of 6 hour fasted, 20 week old animals following 12 weeks of high fat feeding was assessed using a mq10 NMR analyzer. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; KO=knockout.

<table>
<thead>
<tr>
<th>Gender &amp; Genotype</th>
<th>Body Weight (g)</th>
<th>Fat (g)</th>
<th>Muscle (g)</th>
<th>Free Fluid (g)</th>
<th>Fat (%)</th>
<th>Muscle (%)</th>
<th>Free Fluid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female WT</td>
<td>23.06 ± 0.69 (11)</td>
<td>4.46 ± 0.34 (11)</td>
<td>14.80 ± 0.32 (11)</td>
<td>0.58 ± 0.06 (11)</td>
<td>19.26 ± 1.23 (11)</td>
<td>64.46 ± 144 (11)</td>
<td>2.48 ± 0.23 (11)</td>
</tr>
<tr>
<td>Female KO</td>
<td>22.65 ± 0.58 (11)</td>
<td>4.30 ± 0.32 (11)</td>
<td>14.37 ± 0.37 (11)</td>
<td>0.50 ± 0.04 (11)</td>
<td>18.88 ± 1.11 (11)</td>
<td>63.55 ± 1.02 (11)</td>
<td>2.17 ± 0.15 (11)</td>
</tr>
</tbody>
</table>

Table 5.4. NMR Analysis of Male High Fat Fed 129SvEv \textit{G6pc2} KO Mouse Body Composition.

Body composition of 6 hour fasted, 20 week old animals following 12 weeks of high fat feeding was assessed using a mq10 NMR analyzer. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. WT=wild type; KO=knockout.

<table>
<thead>
<tr>
<th>Gender &amp; Genotype</th>
<th>Body Weight (g)</th>
<th>Fat (g)</th>
<th>Muscle (g)</th>
<th>Free Fluid (g)</th>
<th>Fat (%)</th>
<th>Muscle (%)</th>
<th>Free Fluid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male WT</td>
<td>26.80 ± 0.93 (10)</td>
<td>5 ± 0.78 (10)</td>
<td>16.72 ± 0.50 (10)</td>
<td>0.55 ± 0.07 (10)</td>
<td>18.18 ± 2.34 (10)</td>
<td>62.68 ± 1.63 (10)</td>
<td>2.02 ± 0.19 (10)</td>
</tr>
<tr>
<td>Male KO</td>
<td>30.20 ± 0.82 (13), *</td>
<td>6.98 ± 0.65 (13)</td>
<td>17.51 ± 0.35 (13)</td>
<td>0.60 ± 0.03 (13)</td>
<td>22.67 ± 1.75 (13)</td>
<td>58.35 ± 1.58 (13)</td>
<td>2.00 ± 0.09 (13)</td>
</tr>
</tbody>
</table>

1. Weight: *p < 0.0124
Thus, as noted above, female chow fed 129SvEv mice were ~2g heavier than C57BL/6J mice and had approximately double the fat mass [69] (Table 5.1) whereas female high fat fed C57BL/6J mice were ~12g heavier than 129SvEv mice and had approximately double the fat mass [69] (Table 5.3).

Following 12 weeks of high fat feeding we have previously shown that female, but not male, C57BL/6J G6pc2 KO mice are lighter than WT littermates and have reduced body fat [69]. In contrast, in high fat fed female 129SvEv mice we observed little difference in either body weight or body composition between WT and KO mice (Table 5.3). In addition, in high fat fed male 129SvEv mice we observed a slight increase in body weight in G6pc2 KO relative to WT mice, though body composition was unchanged (Table 5.4). This again suggests that the effect of G6pc2 deletion on body weight varies with both gender and genetic background.

**Analysis of the Effect of G6pc2 Deletion on FBG and FPI in High Fat Fed 129SvEv mice**

We next analyzed the effect of high fat feeding on FBG and FPI in 129SvEv mice. Despite 13 weeks of high fat feeding a comparison between female (Fig. 5.1A) and male (Fig. 5.1B) chow fed with female (Fig. 5.2D) and male (Fig. 5.2E) high fat fed 129SvEV WT mice revealed surprisingly no increase in FBG. Similarly, a comparison between female (Fig. 5.1C) and male (Fig. 5.1D) chow fed with female (Fig. 5.2F) and male (Fig. 5.2G) high fat fed 129SvEV WT mice revealed surprisingly no increase in FPI. In striking contrast to these results with 129SvEv mice, we previously noted marked elevations in both FBG and FPI in both female and male high fat fed C57BL/6J mice compared to chow fed mice [69].

After 13 weeks of high fat feeding a reduction in FBG was observed in both female (Fig. 5.2D) and male G6pc2 (Fig. 5.2E) KO relative to WT mice with no differences in FPI in either female (Fig. 5.2F), or male (Fig. 5.2G) mice relative to WT, similar to the observations in high fat fed C57BL/6J mice [69].
Figure 5.2. Effect of G6pc2 Deletion on Body Weight, Composition and Metabolic Parameters in High Fat Fed 129SvEv Mice.

Panels A & B: Starting at 8 weeks of age, female (Panel A) and male (Panel B) mice were fed a high-fat diet with non-fasting body weights measured weekly. Results are the mean ± S.E.M. of data from the following number of animals: female WT n=11; male WT n=11.

Panel C: Body composition was assessed in chow fed 129SvEv mice at 16 weeks of age and in high fat fed 129SvEv mice at 20 weeks of age following 12 weeks of high fat feeding. Mice were fasted for 5 hours and then weighed. One hour later body fat was determined by NMR. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; F=female; M= male. *p < 4.61E-10 high fat fed vs chow fed females; *p < 3.94E-05 high fat fed vs chow fed males.

Panels D - G: Metabolic parameters were assessed in high fat fed 129SvEv mice at 21 weeks of age following 13 weeks of high fat feeding. Mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated. Blood glucose (Panels D & E) and plasma insulin (Panels F & G) were determined as described in Methods. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; KO=knockout; F=female; M= male. *p < 0.001 female WT versus KO (Panel D); *p < 0.037 male WT versus KO (Panel E).

Panel H: Glucose tolerance was assessed in chow fed 129SvEv at ~22 weeks of age and in high fat fed 129SvEv mice at 22 weeks of age following 13 weeks of high fat feeding. IPGTTs using 2.0 g/kg glucose were performed on 6 hr fasted, conscious, chow or high fat fed WT and G6pc2 KO male mice as described in Methods. The results show the mean glucose concentrations ± S.E.M.
Analysis of the Effect of High Fat Feeding on Glucose Tolerance in 129SvEv WT and G6pc2 KO Mice

We have previously shown that deletion of G6pc2 does not affect glucose tolerance in either chow fed C57BL/6J [69] or 129SvEv mice (Chapter IV) [265], consistent with human GWAS data [108-111]. Although high fat feeding did not result in weight gain (Table 5.4) or elevated FPI (Fig. 5.2G) in male 129SvEv mice relative to chow fed mice (Table 5.2; Fig. 5.1D), IPGTTs revealed a clear impairment of glucose tolerance in both WT (p<0.0002) and G6pc2 KO (p<0.0001) high fat fed 129SvEv mice relative to chow fed mice (Fig. 5.2H), suggesting the presence of either insulin resistance and/or impaired GSIS in high fat fed 129SvEv mice. However, even in high fat fed mice, deletion of G6pc2 did not affect glucose tolerance (Fig. 5.2H).

Comparison of Pancreatic Expression of Key Genes in G6pc2 129SvEv and C57BL/6J Mice

The data derived from studies on C57BL/6J and 129SvEv mice reveal that the effect of G6pc2 deletion on body weight varies with gender and genetic background. In addition, FBG is higher in both female and male C57BL/6J mice than 129SvEv mice on both a chow and high fat diet (Figs. 5.1A & 5.2D)[69, 284, 285]. While there are likely multiple factors that account for these differences, one potential contributing factor could be variations in G6pc2 or Slc37a4 gene expression between C57BL/6J and 129SvEv mice. To address this possibility we compared pancreatic G6pc2 and Ins2 gene expression in both mouse strains. There was little difference in the ratio of G6pc2 to Ins2 gene expression between female and male chow fed 129SvEv mice (Fig. 5.3A) or between female and male chow fed C57BL/6J mice (Fig. 5.3B). There was little difference in the ratio of G6pc2 to Ins2 gene expression between chow fed female 129SvEv and C57BL/6J mice (Fig. 5.3C) or between chow fed male 129SvEv and C57BL/6J mice (Fig. 5.3D). In contrast, while there was little difference in the ratio of G6pc2 to Ins2 gene expression between female and male high fat fed 129SvEv mice (Fig. 5.3E) there was a marked difference between female and male high fat fed C57BL/6J mice (Fig. 5.3F).
Figure 5.3. Comparison of Pancreatic G6pc2 Expression in 129SvEv and C57BL/6J Mice. Pancreatic RNA was isolated following a 6 hr fast from chow fed 129SvEv (129) or C57BL/6J (C57) mice or mice fed a high fat diet for 2 weeks. G6pc2 and Ins2 expression were quantitated by Real Time PCR. Results show the ratio of G6pc2 to Ins2 expression ± S.E.M. in 3-5 pancreata. A-D are chow fed mice, E-H are high fat fed mice. *p < 0.05 versus control.
Similarly, while there was little difference in the ratio of G6pc2 to Ins2 gene expression between high fat fed female 129SvEv and C57BL/6J mice (Fig. 5.3G) there was a marked difference between high fat fed male 129SvEv and C57BL/6J mice (Fig. 5.3H). These data suggest that G6pc2 expression is induced by high fat feeding relative to Ins2 expression, which may contribute to the observed gender- and strain-specific differences between high fat fed 129SvEv and C57BL/6J mice.

**Analysis of the Effect of G6pc2 Deletion on Body Weight, FBG and FBI in High Fat Fed Mixed Genetic Background Mice**

Because the comparison of data derived from studies on C57BL/6J and 129SvEv mice reveal that the effect of G6pc2 deletion on body weight varies with gender and genetic background we repeated these high fat feeding analyses in mice with a mixed C57BL/6J X 129SvEv genetic background. After starting high fat feeding at 8 weeks of age and continuing for 8 weeks we observed no differences in body weight between female mixed genetic background WT and KO mice (Fig. 5.4A). In contrast, male mixed genetic background G6pc2 KO mice exhibited a striking protection against DIO (Fig. 5.4B). A comparison of 16 week old chow fed [96] and 16 week old high fat fed mixed genetic background mice revealed that body weight was increased by high fat feeding in both female and male WT mice (p<0.05). These data derived from mixed genetic background mice again strongly suggest that the effect of G6pc2 on body weight varies with gender and genetic background.

We next analyzed the effect of high fat feeding on FBG and FPI in mixed genetic background mice. A comparison of 16 week old chow fed [96] and 16 week old high fat fed mixed genetic background mice revealed that FBG and FPI were increased by high fat feeding in male but not female WT mice (p<0.05). A trend towards reduced FBG was observed in high fat fed female KO mice relative to WT mice (Fig. 5.4C) whereas FBG was markedly reduced in high fat fed male KO mice relative to WT mice (Fig. 5.4D). Similarly, while no difference in FPI was observed between high fat fed female KO mice relative to WT mice (Fig. 5.4E), FPI was markedly reduced in high fat fed male KO mice relative to WT mice (Fig. 5.4F).
**Analysis of the Effect of G6pc2 Deletion on the Time Course of Changes in Plasma Insulin During an IPGTT**

A key question that arises from these studies is how G6PC2, which is thought to be expressed exclusively in pancreatic islet β-cells [42, 79], could be affecting body weight. One possible explanation for the link between G6PC2 and body weight is that G6PC2 affects satiety. Thus the leftward shift in the dose response curve for GSIS observed in G6pc2 KO mice [69] might result in a faster rise in plasma insulin levels after eating, as shown diagrammatically in Fig. 5.5A. Since insulin is a satiety factor [286], this faster rise in insulin could promote a quicker cessation of feeding and ultimately reduced food intake.

In IPGTTs G6pc2 deletion has little effect on glucose tolerance in chow fed C57BL/6J [69] and 129SvEv mice (Fig. 5.2F). Similarly, human GWAS data have linked common SNPs in G6PC2 to variations in FBG [82, 266] but not altered glucose tolerance [111]. Both observations are consistent with the fact that IPGTTs are not optimal for detecting subtle shifts in the sensitivity of GSIS to glucose because they are dynamic assays, in which islets are exposed to variable glucose concentrations over the time course of the assay, rather than a sustained stimulation with a sub-maximal glucose concentration. We have recently found that G6pc2 expression in C57BL/6J mice is induced following 5 days of repeated injections with the synthetic glucocorticoid dexamethasone (Dex) (K.A.B & R.O’B., unpublished data). We hypothesized that, because a greater difference in the sensitivity of GSIS to glucose should now exist between WT and KO mice (Fig. 5.5A), the time course of GSIS might sufficiently differ between WT and KO mice following glucose injection that a difference in the time course of changes in plasma insulin between WT and KO mice might be detectable in IPGTT assays. We therefore measured plasma glucose and insulin 0.5 mins (Fig. 5.5B-E), 2 mins (Fig. 5.5F-I) and 15 mins (Fig. 5.5J-M) following injection of 2 g/kg glucose. Though the data were not statistically significant, the analysis of insulin AUC measurements show trends that suggest a change in the kinetics of insulin release in G6pc2 KO mice, with insulin AUC higher at the early 0.5 min time point (Fig. 5.5E) and lower at the later 15 min time point (Fig. 5.5M).
Figure 5.4. Effect of \(G6pc2\) Deletion on Body Weight and Metabolic Parameters in High Fat fed Mixed Background Mice. Metabolic parameters were assessed in high fat fed mixed genetic background mice at 16 weeks of age following 8 weeks of high fat feeding. Mice were fasted for 5 hours and then weighed (Panels A & B). One hour later mice were anesthetized and blood isolated. Blood glucose (Panels C & D) and plasma insulin (Panels E & F) were determined as described in Methods. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; KO=knockout; F=female; M= male. *\(p < 8.32\times10^{-5}\) WT versus KO (Panel B); *\(p < 3.45\times10^{-5}\) WT versus KO (Panel D); *\(p < 6.84\times10^{-5}\) WT versus KO (Panel F).
Figure 5.5. Effect of $G6pc2$ Deletion on the Time Course of Changes in Plasma Insulin During an IPGTT.

Panel A: Diagram proposing that deletion of $G6pc2$ will affect the time course of GSIS during an IPGTT. WT=wild type; KO=knockout.

Panels B-E: IPGTTs using 2.0 g/kg glucose performed on 6 hr fasted Dex-treated C57BL/6J $G6pc2$ WT (n=17) or KO (n=21) male mice. Results show the mean glucose or insulin concentrations at t=0 and t=0.5 mins or area under the curve (AUC) ± S.E.M. *p < 0.05 versus control.

Panels F-I: IPGTTs using 2.0 g/kg glucose performed on 6 hr fasted Dex-treated C57BL/6J $G6pc2$ WT (n=13) or KO (n=8) male mice. Results show the mean glucose or insulin concentrations at t=0 and t=2 mins or area under the curve (AUC) ± S.E.M. *p < 0.05 versus control.

Panels J-M: IPGTTs using 2.0 g/kg glucose performed on 6 hr fasted Dex-treated C57BL/6J $G6pc2$ WT (n=7) or KO (n=12) male mice. Results show the mean glucose or insulin concentrations at t=0 and t=15 mins or area under the curve (AUC) ± S.E.M. *p < 0.05 versus control.
**Analysis of the Effect of *G6pc2* Deletion on Plasma Triglyceride and Cholesterol in 129SvEv, C57BL/6J and Mixed Genetic Background Mice**

We previously observed a slight reduction in plasma triglyceride levels in female, but not male, mixed genetic background *G6pc2* KO mice, with no change in cholesterol levels [96]. We repeated these analyses in chow fed 129SvEv and C57BL/6J mice along with high fat fed 129SvEv, C57BL/6J and mixed genetic background mice. Figure 5.6 shows that a slight reduction in plasma triglyceride levels was observed in female chow fed 129SvEv *G6pc2* KO mice (Fig. 5.6A), but not in male mice (Fig. 5.6B), and not between WT and KO mice in any of the other groups examined. In contrast, Figure 5.7 shows that plasma cholesterol levels were reduced in chow fed male C57BL/6J KO mice (Fig. 5.7D), high fat fed female (Fig. 5.7G) and male (Fig. 5.7H) C57BL/6J KO mice, and high fat fed mixed genetic background male KO mice (Fig. 5.7J).

**Analysis of the relationship between *G6PC2* SNPs and metabolic parameters in humans using BioVU**

Our results in mice demonstrate that the effect of *G6pc2* deletion on triglyceride and cholesterol levels varies with gender and genetic background. We next used Vanderbilt’s BioVU DNA databank to determine whether *G6PC2* affects these parameters in humans. BioVU individuals with extant genotyping at the intronic *G6PC2* SNP rs560887 were screened to identify associations with cholesterol and triglyceride measurements. The rs560887-G allele, which enhances *G6PC2* pre-mRNA splicing [79], was associated with increased cholesterol (total cholesterol: $\beta = 1.0$, $p = 0.039$; LDL-C: $\beta = 1.1$, $p = 0.006$), but not triglyceride levels ($\beta = 0.90$, $p = 0.46$) or HDL-C ($\beta = -0.07$, $p = 0.75$) (Table 5.5). We further analyzed the population by sex and found that rs560887-G significantly associated with increased LDL-C in males ($p = 0.009$) but not in females ($p=0.15$), although SNP and sex interaction is not significant ($p=0.30$) (Table 5.5). Rs560887 did not associate with diabetes status ($p=0.37$). Thus, as in mice, the impact in humans of modulating *G6PC2* expression on plasma lipids is dependent on gender.
Figure 5.6. Effect of G6pc2 Deletion on Plasma Triglyceride in 129SvEv, C57BL/6J and Mixed Genetic Background Mice. 

Panels A-D: At 17 weeks of age chow fed 129SvEv or C57BL/6J mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Panels E-H: At 21 weeks of age, following 13 weeks of high fat feeding, 129SvEv and C57BL/6J mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Panels I & J: At 16 weeks of age, following 8 weeks of high fat feeding, mixed genetic background mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Plasma triglyceride was determined as described in Methods. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT=wild type; KO=knockout; F=female; M=male.

*p < 0.01 WT versus KO (Panel A).

A) 129SvEv Chow  B) 129SvEv Chow  C) C57BL/6J Chow  D) C57BL/6J

E) 129SvEv HFF  F) 129SvEv HFF  G) C57BL/6J HFF  H) C57BL/6J

I) Mixed HFF  J) Mixed HFF
Figure 5.7. Effect of G6pc2 Deletion on Plasma Cholesterol in 129SvEv, C57BL6J and Mixed Genetic Background Mice.

Panels A - D: At 17 weeks of age chow fed 129SvEv or C57BL/6J mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Panels E - H: At 21 weeks of age, following 13 weeks of high fat feeding, 129SvEv and C57BL/6J mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Panels I & J: At 16 weeks of age, following 8 weeks of high fat feeding, mixed genetic background mice were fasted for 5 hours and then weighed. One hour later mice were anesthetized and blood isolated.

Plasma cholesterol was determined as described in Methods. Results are the mean ± S.E.M. of data with the genotype, gender and number of animals indicated. WT= wild type; KO= knockout; F= female; M= male. *p < 0.0069 WT versus KO (Panel D); *p < 0.02 female WT versus KO (Panel G); *p < 7.01E-06 male WT versus KO (Panel H); *p < 0.001 WT versus KO (Panel J).
Table 5.5 Association Between *G6PC2* SNP rs560887 and Plasma Lipid Measurements Using Electronic Health Record (EHR)-Derived Phenotype Analyses.

Plasma lipid measurements were obtained from routine lipid panels in Vanderbilt University Medical Center's EHR repository. For each laboratory of each individual, the associations are tested against the median of all lab results for that test. All associations were adjusted for age, sex, and body mass index using linear regression. Cholesterol: total cholesterol; LDL-C: calculated low-density lipoprotein; HDL-C: calculated high-density lipoprotein. The unit for all measurements is mg/dL.

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<th>P-value</th>
<th>GG</th>
<th>GA</th>
<th>AA</th>
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Discussion

Our results demonstrate that the effect of \textit{G6pc2} deletion in mice on FBG, body weight and body composition closely parallel human GWAS data in that the effect of \textit{G6pc2} deletion on FBG is largely independent of gender and genetic background whereas the effect of \textit{G6pc2} deletion on body weight and fat mass is highly dependent on gender, genetic background, as well, at least in mice, on diet.

With respect to FBG, we previously showed that FBG is reduced in both female and male chow fed and high fat fed \textit{G6pc2} KO mice on a pure C57BL/6J genetic background [69]. We have also shown that FBG is reduced in female and male chow fed \textit{G6pc2} KO mice on a mixed genetic background [96]. We show here that FBG is also reduced in both female (Fig. 5.2D) and male (Fig. 5.2E) high fat fed \textit{G6pc2} KO mice on a pure 129SvEv genetic background. FBG is also reduced in female chow fed 129SvEv \textit{G6pc2} KO mice (Fig. 5.1A) with a similar trend seen in 17 week old males (Fig. 5.1B) and a statistically significant decrease seen in an older cohort of males [265]. Similarly, FBG is reduced in male high fat fed mixed genetic background \textit{G6pc2} KO mice (Fig. 5.4D). FBG was not reduced in female high fat fed mixed genetic background \textit{G6pc2} KO mice (Fig. 5.4C), though the n value in this study was relatively low. These observations are largely consistent with human GWAS data showing an association between \textit{G6PC2} and FBG in multiple different populations [76, 81, 112-115, 278, 279].

With respect to FPI, we previously showed that FPI is unchanged in both female and male chow fed and high fat fed \textit{G6pc2} KO mice on a pure C57BL/6J genetic background [69]. We have also shown that FPI is unchanged in female and male chow fed \textit{G6pc2} KO mice on a mixed genetic background [96]. We show here that FPI is also unchanged in both female (Fig. 5.2F) and male (Fig. 5.2G) high fat fed \textit{G6pc2} KO mice on a pure 129SvEv genetic background. Similarly, FPI is unchanged in female high fat fed mixed genetic background \textit{G6pc2} KO mice (Fig. 5.4E). A reduction in FPI was observed in male high fat fed mixed genetic background \textit{G6pc2} KO mice (Fig. 5.4F) but this is presumably secondary to the marked effect of \textit{G6pc2} deletion on body weight in males (Fig. 5.4B). These observations are consistent with human GWAS data showing no association between \textit{G6PC2} and FPI in multiple different populations [82, 266, 287, 288].
one apparent exception to these matching mouse and human data are male chow fed 129SvEv G6pc2 KO mice where a trend towards reduced FBG (Fig. 5.1B) was associated with a reduction in FPI (Fig. 5.1D). However, in a slightly older cohort of chow fed 129SvEv mice we recently reported that FBG was reduced in male G6pc2 KO mice relative to WT with no change in FPI [265]. We speculate that a difference in FPI between WT and KO mice could arise depending on the kinetics of the fall in blood glucose and plasma insulin during fasting (Figs. 5.1E & F).

In contrast to these data showing largely consistent effects of G6pc2 deletion on FBG and FPI regardless of gender, diet and genetic background, the effect of G6pc2 deletion on body weight and body composition is highly dependent on these variables. We previously showed that female, but not male, G6pc2 KO mice on a pure C57BL/6J genetic background had reduced body weight and body fat on both a chow and high fat diet relative to WT mice [69]. In contrast, we show here that deletion of G6pc2 in female mice on the 129SvEv genetic background has no effect on body weight or body fat on either a chow (Table 5.1) or high fat (Table 5.3) diet relative to WT mice. Similarly, deletion of G6pc2 in female mice on a mixed 129SvEv X C57BL/6J genetic background has no effect on body weight on either a chow [96] or high fat (Fig. 5.4A) diet relative to WT mice. In males deletion of G6pc2 on the 129SvEv genetic background was associated with reduced body weight on a chow diet (Table 5.2) but increased body weight on a high fat diet (Table 5.4). In contrast, deletion of G6pc2 in male mice on a mixed 129SvEv X C57BL/6J genetic background had no effect on body weight on a chow diet [96] whereas this conferred a marked protection against DIO on a high fat diet (Fig. 5.4B). Overall our results suggest that FBG is a much more tightly regulated variable than body weight. Thus while FBG levels are relatively similar in chow fed C57BL/6J [69], 129SvEv (Fig. 5.1) and mixed [96] genetic background mice, the increase in body weight and body fat in response to high fat feeding is markedly different in C57BL/6J [69] and 129SvEv mice (Fig. 5.2). Interestingly, the response to DIO varies remarkably even within inbred mice through poorly understood epigenetic mechanisms [289-291].
As with the effects of \textit{G6pc2} deletion on body weight and body composition, the effects on triglyceride and cholesterol levels also vary with gender, genetic background and diet. A reduction in triglyceride levels was observed in female chow fed mice on a mixed [96] and 129SvEv genetic background (Fig. 5.6A) whereas a reduction in cholesterol levels was observed in male chow fed mice on a C57BL/6J genetic background (Fig. 5.7D), female (Fig. 5.7G) and male (Fig. 5.7H) high fat fed mice on a C57BL/6J genetic background and male high fat fed mice on a mixed genetic background (Fig. 5.7J). BioVU studies show that the rs560887-A allele, which confers reduced \textit{G6PC2} expression [79], is associated with a reduction in cholesterol but not triglycerides in humans, with a sex-specific effect for males (Table 5.5).

Although FBG levels are relatively similar in chow fed C57BL/6J [69] and 129SvEv mice (Fig. 5.1), we observed that, in 6 hr fasted mice, the FBG concentration in male 129SvEv mice (Fig. 5.1B) is lower than that seen in male C57BL/6J mice [69] (p<0.05) whereas the FPI concentration is similar in male 129SvEv mice (Fig. 5.1D) and male C57BL/6J mice [69]. This difference could potentially be explained by the enhanced insulin sensitivity observed in chow fed 129SvEv versus C57BL/6J mice [275]. Whether insulin sensitivity also differs between female chow fed 129SvEv and C57BL/6J mice has not been determined [275] but clear gender differences exist. Thus body weight and fat mass are higher in 16 week old female 129SvEv mice (Table 5.1) than female C57BL/6J mice [69] (p<0.05) whereas these parameters do not differ between 16 week old male 129SvEv mice (Table 5.2) and male C57BL/6J mice [69]. In addition, FBG concentrations in 6 hr fasted female 129SvEv mice (Fig. 5.1A) are lower than those seen in female C57BL/6J mice [69] (p<0.05) whereas FPI concentrations are higher in female 129SvEv mice (Fig. 5.1C) than female C57BL/6J mice [69] (p<0.05). While multiple factors may explain the lower FBG in chow fed female (Fig. 5.1A) and male (Fig. 5.1B) 129SvEv mice relative to C57BL/6J mice [69], including the relative rates of non-insulin dependent glucose disposal, a comparison of \textit{G6pc2} expression between chow fed 129SvEv and C57BL/6J mice suggests that differences in \textit{G6pc2} expression do not contribute (Fig. 5.3A-D). On the other hand, we observed a selective induction of \textit{G6pc2} expression in male C57BL/6J mice by high fat feeding
that might contribute to the observed gender- and strain-specific differences between high fat fed 129SvEv and C57BL/6J mice (Fig. 5.3E-H).

A key question that remains to be addressed is how G6PC2, which is thought to be expressed exclusively in pancreatic islet β-cells [42, 78], could be affecting body weight. One possibility, as proposed by Li et al. [111], is that the differences in body weight they observed in humans are secondary effects due to altered insulin signaling efficacy that arise due to an effect of G6PC2 on the pulsatility of insulin secretion. Another possibility is that G6PC2 expression in other tissues that affect body weight has been overlooked. Indeed while RNA blotting showed no evidence for G6PC2 expression in brain [66] and transgenic mouse studies gave inconsistent results [292, 293], one group has reported G6pc2 expression in the mouse hypothalamus [294], a region critical for the control of body weight [295]. However, this expression was only detected using very high template concentrations and PCR cycles [294]. Moreover, while low levels of expression were detected, it is unlikely to be biologically consequential since expression of the enzymatically more active G6pc3 isoform was detected at much higher levels [42, 294]. One other potential explanation for the link between G6PC2 and body weight is that G6PC2 affects satiety. Thus the leftward shift in the dose response curve for GSIS observed in G6pc2 KO mice [69] might result in a faster rise in plasma insulin levels after eating (Fig. 5.5A). Since insulin is a satiety factor [286], this faster rise in insulin could promote a quicker cessation of feeding and ultimately reduced food intake. Though the data were not statistically significant, the experiments shown in Figure 5.5 showed trends that supported his hypothesis. Interestingly, our hypothesis that G6PC2 affects the timing of GSIS during glucose tolerance tests could also explain the counterintuitive observation that the rs560887-G allele, which confers elevated G6PC2 expression [79], is associated with elevated FBG but also higher insulin levels at the 30 minute time point in a glucose tolerance test [111]. Future studies to determine whether deletion of G6pc2 affects food intake or energy expenditure [296] and especially studies on β-cell-specific G6pc2 KO mice will provide insight into these possibilities.
In summary, our data suggest the influence of G6pc2 on FBG is largely independent of diet, gender and genetic background whereas its effect on body weight and fat mass is highly dependent on these variables. This suggests that modifier genes influence some aspects of G6pc2 function, a conclusion that is consistent with other studies showing that the influence of G6PC2 SNPs on the risk for type 2 diabetes also varies between population [74, 112, 113, 114, 278].
VI. ANALYSIS OF THE EFFECT OF 11-DEHYDROCORTICOSTERONE TREATMENT ON C57BL/6J AND 129SVEV WT AND G6PC2 KO MICE

Introduction

As T2D prevalence continues to rise, it is becoming increasingly important to better understand the drivers of β-cell exhaustion in the context of insulin resistance. Because there is a strong link between the phenotype of Cushing’s disease patients and metabolic syndrome, there have been many studies focusing on the role that glucocorticoids and glucocorticoid metabolism play in regulating glucose metabolism. Previous studies have indicated that transgenic mice overexpressing 11β-HSD1 in either WAT or liver, resulting in increased local tissue glucocorticoid levels (Fig. 6.1), develop idiopathic metabolic syndrome [297]. Specifically, this study showed that these mice are insulin resistant and glucose intolerant. Conversely, 11β-HSD1 null mice are protected from metabolic disease and exhibit fasting hypoglycemia, increased whole body insulin sensitivity and increased basal and insulin stimulated glucose uptake [298]. Moreover, selective inhibition of 11β-HSD1, thereby increasing 11-DHC levels, in cell culture [299] or in vivo by a selective inhibitor [300], results in enhanced insulin sensitivity in adipose, liver and muscle tissue. These studies further demonstrated that 11β-HSD1 inhibition resulted in decreased fasting blood glucose, decreased hepatic glucose production and increased insulin signaling as measured by serine 307 phosphorylation of the Insulin Receptor Substrate 1 protein [299, 300]. As such, 11β-HSD1 has been intensively investigated as a target for therapy in T2D.

While it has been established that targeting 11β-HSD1 in the muscle, liver or adipose tissue improves insulin sensitivity and represses FBG, the data regarding the role of 11β-HSD1 specifically in the β-cell has been less well explored. Two studies indicate that 11β-HSD1 plays a role in regulating insulin secretion directly. The first study showed that a mild increase in 11β-HSD1 protein expression in the islet, resulting in increased cortisone levels, was associated with enhanced insulin secretion, which contradicts studies done in vivo [160]. The second showed that deletion of 11β-HSD1 in the islet, i.e. increased 11-DHC levels, results in diminished capacity for GSIS both in vitro and in vivo [153, 159], also contradicting in vivo
studies. Similarly, while not directly studying 11β-HSD1, treatment of islets with 11-DHC results in decreased insulin secretion, supporting the 11β-HSD1 inhibition studies [158,160,301]. It remains unclear how overexpression of 11β-HSD1 results in insulin resistance and metabolic syndrome yet increased β-cell 11β-HSD1 expression enhances GSIS. Despite the confounding data, it is clear that modulation of 11β-HSD1 alters the dynamics of GSIS, and as such it is important to continue exploring the role 11β-HSD1 plays specifically in the β-cell prior to determining if it is an optimal target for treatment or prevention of metabolic syndrome symptoms. One aspect of 11β-HSD1 activity and glucocorticoid metabolism in the β-cell that remains unclear is whether there are additional upstream modulators of the pathway that regulate the conversion of inactive to active glucocorticoids (Fig. 6.1).

As indicated in Figure 6.1, the inter-conversion of 11-DHC to corticosterone in rodents is driven by the presence of G6P. Previous studies in the liver have shown that G6P transport by SLC37A4 (G6PT) into the ER lumen requires the presence of G6PC1, indicating that G6P transport is linked to G6PC1 activity [302]. Whether G6P transport into the β-cell ER lumen through G6PT requires the presence of G6PC2 is unknown. If it is coupled, we hypothesized that in the absence of G6pc2, there would be impaired corticosterone generation in β-cells due to diminished G6P levels in the ER lumen. On the other hand, if G6PC2 is not coupled to G6PT and therefore G6P transport does not require G6PC2, we hypothesized that the absence of G6PC2 would promote conversion of G6P to 6-phosphogluconolactone and hence promote corticosterone generation in β-cells (Fig. 6.1). Whether increased corticosterone generation would results in impaired or enhanced insulin secretion in mice remains to be determined as the available literature regarding glucocorticoid treatments effects on insulin secretion is contradictory as mentioned in the introductory section. To investigate these hypotheses, WT and G6pc2 KO 129SvEv and C57BL/6J mice were exposed to 11-DHC supplemented water for 3-6 weeks [241] prior to the analysis of FBG and glucose tolerance.
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Figure 6.1. 11β-HSD1 Modulates Glucocorticoid Metabolism in the Islet β-cell. The conversion of inactive 11-DHC to active cortisol in rodents is dependent on the coupled reactions seen in this diagram. As the conversion is dependent on G6P levels in the ER lumen, hydrolysis of G6P to glucose by G6PC2 could affect the local GC concentrations in β-cells. Alternatively, if G6PC2 is coupled to G6P transport, as has been demonstrated with G6PC1 in the liver, this could also impact local GC concentrations.
Results

Analysis of FBG and Gene Expression from 11-DHC Treated C57BL/6J WT and G6pc2 KO Mice

5 weeks of 11-DHC supplementation resulted in significant weight gain in both C57BL/6J WT and KO mice, with no significant difference in weight gain between genotypes (Table 6.1). As the literature supports the role of glucocorticoid treatment driving an increase in central adiposity, this increased weight gain may be attributed to increased fat deposition although control, age matched mice were not measured at this time point (Table 6.1) [129, 303-305]. After 3 or 5 weeks of 11-DHC supplementation, both WT and G6pc2 KO C57BL/6J mice had improved glucose tolerance relative to non-treated controls (Fig. 6.2). After 3 weeks of treatment, G6pc2 KO mice had significantly improved glucose tolerance relative to the WT littermates (Fig. 6.2). While at 5 weeks of treatment G6pc2 KO mice trended towards improved glucose tolerance relative to WT treated mice, this improvement was not statistically significant (p=0.06, Fig. 6.2). Importantly, Turban et al. showed that enhanced corticosterone generation by β-cells selectively overexpressing 11β-HSD1 results in insulin hypersecretion rather inhibition [159]. Our data supports this finding if we assume that enhanced insulin secretion explains the improvement in glucose tolerance, rather than an improvement in insulin sensitivity [159]. Future studies will measure FPI and corticosterone levels in order to further support these conclusions. Both G6pc2 and G6pt gene expression were induced following 6 weeks of 11-DHC treatment in WT mice (Fig. 6.3). Surprisingly, while 11-DHC suppressed FBG in both WT and KO mice, there was no observed difference in FBG levels between 11-DHC treated WT and KO C57BL/6J mice (Fig. 6.4).
Table 6.1. Characterization of 14 week old male C57BL/6J WT and G6pc2 KO mice after 6 weeks of 11-DHC supplementation.

<table>
<thead>
<tr>
<th></th>
<th>Initial Weight (g)</th>
<th>Week 6 Weight (g)</th>
<th>% Change</th>
<th>Fat (g)</th>
<th>Muscle (g)</th>
<th>Free Fluid (g)</th>
<th>Fat %</th>
<th>Muscle %</th>
<th>Free Fluid %</th>
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<tr>
<td>WT</td>
<td>23.68 ± 0.76 (12)</td>
<td>29.26 ± 1.43 (12)</td>
<td>23.30 ± 2.68 * (12)</td>
<td>5.46 ± 0.77 (8)</td>
<td>16.57 ± 0.85 (8)</td>
<td>0.30 ± 0.03 (8)</td>
<td>22 ± 1</td>
<td>59 ± 1</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>KO</td>
<td>24.91 ± 0.48 (14)</td>
<td>29.63 ± 0.50 (14)</td>
<td>20.42 ± 3.37 ** (14)</td>
<td>6.81 ± 0.43 (10)</td>
<td>17.36 ± 0.18 (10)</td>
<td>0.28 ± 0.04 (10)</td>
<td>19 ± 3</td>
<td>57 ± 4</td>
<td>1 ± 0.1</td>
</tr>
</tbody>
</table>

1. % Change: p<0.05 for WT weight week 0 vs WT week 6
2. % Change: p<0.05 for KO weight week 0 vs KO week 6

Figure 6.2. 11-DHC Treatment Improves the Glucose Tolerance of C57BL/6J WT and G6pc2 KO Mice. 8 week old male mice were given water supplemented with 11-DHC for 6 weeks. Glucose tolerance was assessed at week 3 and week 5.
Figure 6.3. Analysis of G6pc2 and G6pt Gene Expression in 11-DHC treated C57BL/6J WT Mice. 8 week old male mice were placed on 11-DHC water for 6 weeks. After 6 weeks, WT mice were fasted for 6 hours before being isolating pancreatic RNA. Control samples were obtained from age matched mice.

Figure 6.4. Analysis of FBG in 11-DHC treated C57BL/6J WT and G6pc2 KO Mice. WT and KO mice have significantly reduced FBG relative to non-treated controls following 6 weeks of 11-DHC treatment (p<0.001).
Analysis of FBG and Gene Expression from 11-DHC Treated 129SvEv WT and G6pc2 KO Mice

In contrast to the C57BL/6J mice, after 6 weeks of 11-DHC supplementation, there were no significant changes in body composition or weight in 11-DHC treated 129SvEv WT or G6pc2 KO mice (Table 6.2). 11-DHC treatment in G6pc2 KO mice resulted in a mild, yet significant, improvement in glucose tolerance relative to WT 11-DHC treated mice (Fig. 6.5). There were no significant changes in glucose tolerance between 11-DHC treated and non-treated WT or KO mice (Fig 6.5). Consistent with the minimal change in glucose tolerance, a significant induction of G6pc2 or G6PT gene expression in WT mice was not observed following 11-DHC treatment (Fig. 6.6). Moreover, the difference in FBG between WT and KO mice was not enhanced (Fig. 6.7). Overall these data suggest limited generation of corticosterone in 129SvEv mice. Future studies will repeat these experiments and measure both corticosterone levels and FPI.

Discussion

While the studies described in this chapter are preliminary, they do begin to elucidate the role that G6pc2 could be playing in glucocorticoid metabolism in the β-cell (Fig. 6.1). In C57BL/6J mice, the dramatic improvement of glucose tolerance in both WT and G6pc2 KO mice indicates that 11-DHC treatment may result in enhanced GSIS or insulin sensitivity (Fig. 6.2). The improvement in glucose tolerance was observed both at a short 3-week and a longer 5-week 11-DHC treatment time and correlated with significant induction of G6pc2 gene expression in C57BL/6J mice (Fig. 6.3). The more likely explanation is that there is enhanced GSIS, as the literature showing that glucocorticoids cause insulin resistance is extensive [129]. Moreover, based on my studies outlined in chapter IV, we observed enhanced GSIS following Dex injection. These data strongly support the conclusion that the improved glucose tolerance in 11-DHC treated mice is due to enhanced GSIS.
Table 6.2. Characterization of 14 week old male 129/SvEv WT and G6pc2 KO mice after 6 weeks of 11-DHC supplementation.

<table>
<thead>
<tr>
<th></th>
<th>Initial Weight (g)</th>
<th>Week 5 Weight (g)</th>
<th>% Change</th>
<th>Fat (g)</th>
<th>Muscle (g)</th>
<th>Free Fluid (g)</th>
<th>Fat %</th>
<th>Muscle %</th>
<th>Free Fluid %</th>
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<tr>
<td>WT</td>
<td>22.54 ± 0.57 (9)</td>
<td>22.79 ± 0.41 (9)</td>
<td>4 ± 0.01 (9)</td>
<td>2.47 ± 0.21 (4)</td>
<td>15.28 ± 0.33 (4)</td>
<td>0.51 ± 0.01 (4)</td>
<td>11 ± 1</td>
<td>67 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>KO</td>
<td>23.10 ± 0.84</td>
<td>24.04 ± 0.98 (11)</td>
<td>4 ± 0.02 (11)</td>
<td>2.47 ± 0.27 (6)</td>
<td>14.40 ± 0.49 (6)</td>
<td>0.68 ± 0.11 (6)</td>
<td>12 ± 1</td>
<td>73 ± 1</td>
<td>2 ± 1</td>
</tr>
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</table>

Figure 6.5. 11-DHC Treatment in 129/SvEv Mice Improves Glucose Tolerance in G6pc2 KO Mice. 8 week old male mice were given water supplemented with 11-DHC for 6 weeks. Glucose tolerance was assessed in week 5 and showed that KO, but not WT, mice had improved glucose tolerance relative to non-treated controls (p<0.05).
**Figure 6.6. Analysis of G6pc2 and G6pt Gene Expression in 11-DHC treated 129SvEv WT Mice.** 8 week old male mice were placed on 11-DHC water for 6 weeks. After 6 weeks, WT mice were fasted for 6 hours before isolating pancreatic RNA. Control samples were obtained from age matched mice.

**Figure 6.7. Analysis of FBG in 11-DHC treated 129SvEv WT and G6pc2 KO Mice.** WT, but not KO, mice have significantly reduced FBG relative to non-treated controls following 6 weeks of 11-DHC treatment (p<0.05).
In contrast to the C57BL/6J mice, there was not a significant improvement of glucose tolerance in 129SvEv WT mice, which correlated with the lack of G6pc2 gene induction (Fig. 6.5-6.6). There was, however, a significant enhancement of glucose tolerance in 11-DHC treated KO mice. Interestingly, throughout these studies I observed that the 129SvEv mice appeared to drink less water relative to C57BL/6J, which may explain the difference in magnitude of improvement of glucose tolerance (Fig. 6.2 and 6.5). Future studies need to measure the amount of water consumed from each cage in order to better determine the amount of 11-DHC that each mouse strain is being exposed to.

If G6pc2 did not affect corticosterone generation in β-cells, we expected that FBG would be repressed in 11-DHC treated WT and G6pc2 KO mice due to corticosterone generation in peripheral tissues and that the difference in FBG between WT and KO mice would be changed in 11-DHC treated animals due to the induction of G6pc2 expression. These data are consistent with the model shown in Fig 6.1 that proposes coupling of G6pc2 and G6pt in β-cells. As noted above, we predict that glucocorticoids will be generated by 11-DHC metabolism in other tissues. Future studies will measure circulating corticosterone levels as well as insulin secretion. We predict that elevated circulating corticosterone explains the FBG results in WT and KO mice. We hypothesize that improvement in glucose tolerance in 11-DHC KO mice relative to WT mice is due to the decrease in peak glucose levels and hence an increase in the influence of G6pc2 deletion on glucose tolerance as explained by figure 4.11.
Introduction

Elevated FBG has been associated with increased risk for the development of T2D and CAM [70-72]. Previous studies have shown that an increase in FBG of 9-18 mg/dl is associated with a ~30% increased risk of CAM [71]. Conversely, a reduction in FBG of ~9 mg/dl is associated with a 25% reduction in risk of CAM [72]. Multiple groups have performed GWAS in an effort to identify genes associated with variations in FBG and, as mentioned previously, the rs560887 SNP located in the third intron of the G6PC2 locus has been identified as the strongest common genetic determinant of FBG levels in terms of significance and effect size, accounting for ~1% of total variance in FBG [73, 76, 79, 82, 114, 266, 278].

The role of G6PC2 in islet β-cells has been extensively covered in the introduction of this dissertation. Figure 1.1 highlights the main function of G6PC2 to hydrolyze G6P to glucose and a free phosphate, thereby creating a futile cycle that opposes the actions of glucokinase (Fig. 1.4). Previous data supports the role of G6PC2 as a negative regulator of GSIS and as contributing to glucose cycling in β-cells [69] [276, 277]. Deletion of G6pc2 results in leftward shift in the dose response curve for GSIS [69]. At sub-maximal glucose levels this shift results in enhanced GSIS from G6pc2 KO relative to WT mouse islets [69]. Under fasting conditions, where insulin levels are the same in WT and G6pc2 KO mice, this shift results in reduced FBG in KO mice [69, 96].

Common variants associated with variations in FBG were thought to account for a low percentage (~10%; Ref. [306]) of total heritable variation, leading to speculation that rare (minor allele frequency <5%), high impact variants undetected by GWAS might account for the remaining 90% of heritability [120]. However, more recent studies have suggested that the combined effects of multiple common variants have the potential to largely account for missing heritability [126-128]. Nevertheless, the identification of high impact rare variants has provided tremendous insight into β-cell biology [123, 307]. For example, while common SNPs in the glucokinase (GCK) GCK locus have modest effects on FBG [82, 266], rare inactivating
variants in the GCK locus have been shown to cause neonatal diabetes mellitus or maturity-onset diabetes in youth while rare activating variants cause hyperinsulinemia [123, 307]. Evolutionarily this is logical, as rare variants with significant detrimental effects on health would be selected against and therefore not be propagated in the human population. These data also highlight an important caveat in the interpretation of GWAS data: the effect size of common genetic variants does not necessarily reflect the importance of the gene in relation to the disease or phenotype being studied. As observed with GCK and G6PC2, despite the greater effect size of common G6PC2 variants on FBG, deletion of the Gck gene in mice is lethal [122] whereas deletion of G6pc2 results in a mild reduction in FBG [69, 96].

Because the identification of high impact rare variants in GCK has provided tremendous insight into β-cell biology [123, 307], we were interested in identifying variants in G6PC2 that have a significant effect on enzyme activity and/or protein expression. This current study describes a systematic functional analysis of 22 non-synonymous G6PC2 SNPs using a novel in situ functional assay for glucose-6-phosphatase activity.

Results

Analysis of the Effect of Human G6PC2 Codon Variation on Protein Expression

Before beginning the functional analysis of non-synonymous human G6PC2 SNPs we sought to maximize human G6PC2 protein expression in transient transfection assays. Plasmids encoding V5 His-tagged variants of human G6PC2 and mouse G6pc2 [55, 56, 66] were transiently transfected into COS cells. Figure 7.1A shows that human G6PC2 and mouse G6pc2 RNA were expressed at similar levels but mouse G6pc2 protein expression was much higher than human G6PC2 (Fig. 7.1B).
Figure 7.1. Analysis of Human \textit{G6PC2} and Mouse \textit{G6pc2} mRNA and Protein Expression. COS 7 cells were transiently transfected, as described in Materials and Methods, with expression vectors encoding either wild type (WT) mouse (m) G6pc2, human (h) G6PC2 or the empty pcDNA3 vector. Following transfection, cells were incubated for 18-20 hr in serum-containing medium. Cells were then harvested and either RNA (Panel A) or protein (Panel B) expression were assayed as described in Materials and Methods. A representative agarose gel (Panel A) or Western blot (Panel B) are shown. The faint band of the same size in the empty vector transfected cells represents background plasmid contamination of our PCR reagents/tubes.
Chimeras of human G6PC2 and mouse G6pc2 were generated to investigate whether this difference in protein expression was associated with a particular region of the human G6PC2 or mouse G6pc2 proteins (Fig. 7.2A). The results show that chimeric protein expression decreased as the proportion of human G6PC2 coding sequence increased (Fig. 7.2B). The region of G6PC2 between AAs 72 and 192 appeared to have the greatest impact on the difference in expression between mouse G6pc2 and human G6PC2 (Fig. 7.2B). In this region 110/121 AAs are conserved between mouse G6pc2 and human G6PC2. This suggested that multiple differences between human and mouse codons potentially explained the difference in protein expression. We therefore next investigated the effect of switching individual human G6PC2 codons that are rarely present in human mRNAs, with codons that code for the same amino acid (AA) but are more commonly found in human mRNAs. In some instances this resulted in a switch to the same codon used to encode the equivalent AA in mouse G6pc2 (Fig. 7.3A; 7.1). In other cases this resulted in a switch to a codon that was distinct from the codon used to encode the equivalent AA in mouse G6pc2 (Fig. 7.3B; Table 7.2). Switching the codons encoding three AAs, 58, 67 and 333, resulted in a slight improvement in human G6PC2 expression but the effect of combining these codon changes was not additive (Fig. 7.3A). Changing two other codons, encoding AAs 179 and 263, further reduced human G6PC2 expression (Fig. 7.3B). These data suggest that the molecular basis for the increased expression of mouse G6pc2 versus human G6PC2 is complex and involves differences in translation efficiency and/or stability that are conferred by multiple codons and/or AAs, respectively.

**Characterization of a Novel Assay for the Measurement of Glucose-6-Phosphatase Activity In Situ**

Because the activity of G6pc1 appears to be regulated by unknown factors [308], it is unclear whether glucose-6-phosphatase activity assayed *in vitro* truly reflects activity in intact cells. Therefore, before beginning the functional analysis of non-synonymous human G6PC2 SNPs, we first developed a novel assay for the measurement of glucose-6-phosphatase activity *in situ.*
Figure 7.2. Analysis of Human G6PC2:Mouse G6pc2 Chimeric Protein Expression. 832/13 cells were transiently transfected, as described in Materials and Methods, with expression vectors encoding either wild type mouse (m) G6pc2, human (h) G6PC2 or the indicated chimeric proteins (Panel A). Following transfection, cells were incubated for 18-20 hr in serum containing medium. Cells were then harvested and protein expression assayed as described in Materials and Methods (Panel B). A representative blot is shown.
**Figure 7.3. Analysis of the Effect of Human G6PC2 Codon Variation on Protein Expression.** 832/13 cells were transiently transfected, as described in Materials and Methods, with expression vectors encoding either WT mouse (m) G6pc2, human (h) G6PC2 or G6PC2 variants in which the codon used to encode the indicated AAs had been optimized as shown in Tables 7.1 and 7.2. Following transfection, cells were incubated for 18-20 hr in serum containing medium. Cells were then harvested and protein expression assayed as described in Materials and Methods. In some instances codon optimization resulted in a switch to the same codon used to encode the equivalent AA in mouse G6pc2 (Panel A; Table 7.1). In other cases this resulted in a switch to a codon that was distinct from the codon used to encode the equivalent AA in mouse G6pc2 (Panel B; Table 7.2). Representative blots are shown.

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<th>B)</th>
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Table 7.1. Comparison of Codon Usage in Human G6PC2 mRNA with Common Codon Usage in Human mRNAs. The Table shows that the codons used to encode the indicated AAs in human G6PC2 are not the most commonly used codons to encode these AAs in human proteins. The Table also shows that the codons that are commonly used to encode these AAs in human proteins are the same as the codons used to encode these AAs in mouse G6pc2. The effect on human G6PC2 protein expression of changing some of these codons to the most frequently used codon was assessed as described in Figure 7.3A.

Codon usage in human mRNAs is described at the following website:
http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=9606&aa=1&style=N

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</table>
**Table 7.2. Comparison of Codon Usage in Human G6PC2 mRNA with Common Codon Usage in Human mRNAs.** The Table shows that the codons used to encode the indicated AAs in human G6PC2 are not the most commonly used codons to encode these AAs in human proteins. The Table also shows that the codons that are commonly used to encode these AAs in human proteins are also different to the codons used to encode these AAs in mouse G6pc2. The effect on human G6PC2 protein expression of changing these codons to the most frequently used codon was assessed as described in Figure 7.3B.

In this analysis we just focused on codons for AAs that are conserved between mouse G6pc2 and human G6PC2. In other words, we did not optimize codons that encode AAs that are unique to human G6PC2.

<table>
<thead>
<tr>
<th>AA#</th>
<th>Human G6PC2 Codon</th>
<th>Frequency per 1000 Human cDNAs</th>
<th>Most Frequently Used Codon</th>
<th>Mouse G6pc2 Codon</th>
<th>Frequency per 1000 Human cDNAs</th>
<th>Frequency Difference</th>
<th>Effect on hG6PC2 Expression</th>
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</thead>
<tbody>
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<td>219</td>
<td>CTT</td>
<td>13.19</td>
<td>CTG</td>
<td>CTC</td>
<td>39.64</td>
<td>26.45</td>
<td>N.C.</td>
</tr>
<tr>
<td>263</td>
<td>CTT</td>
<td>13.19</td>
<td>CTG</td>
<td>CTC</td>
<td>39.64</td>
<td>26.45</td>
<td>Decreased</td>
</tr>
<tr>
<td>225</td>
<td>CTT</td>
<td>13.19</td>
<td>CTG</td>
<td>CTC</td>
<td>39.64</td>
<td>26.45</td>
<td>N.C.</td>
</tr>
<tr>
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<td>CTT</td>
<td>13.19</td>
<td>CTG</td>
<td>CTC</td>
<td>39.64</td>
<td>26.45</td>
<td>N.C.</td>
</tr>
<tr>
<td>336</td>
<td>GTT</td>
<td>11.03</td>
<td>GTG</td>
<td>GTG</td>
<td>28.12</td>
<td>17.09</td>
<td>N.C.</td>
</tr>
<tr>
<td>179</td>
<td>GTT</td>
<td>11.03</td>
<td>GTG</td>
<td>GTC</td>
<td>28.12</td>
<td>17.09</td>
<td>Decreased</td>
</tr>
<tr>
<td>11</td>
<td>ATA</td>
<td>7.49</td>
<td>ATC</td>
<td>ATT</td>
<td>20.82</td>
<td>13.33</td>
<td>N.C.</td>
</tr>
<tr>
<td>303</td>
<td>ATA</td>
<td>7.49</td>
<td>ATC</td>
<td>ACA</td>
<td>20.82</td>
<td>13.33</td>
<td>N.C.</td>
</tr>
<tr>
<td>108</td>
<td>GGT</td>
<td>10.75</td>
<td>GGC</td>
<td>GGC</td>
<td>22.22</td>
<td>11.47</td>
<td>N.C.</td>
</tr>
<tr>
<td>77</td>
<td>GGT</td>
<td>10.75</td>
<td>GGC</td>
<td>GGC</td>
<td>22.22</td>
<td>11.47</td>
<td>N.C.</td>
</tr>
</tbody>
</table>
Newgard and colleagues [309, 310] have previously described a highly glucose responsive INS-1 cell line variant, designated 832/13. Rat G6pc1 [311] and liver pyruvate kinase (Pklr) [312] fusion gene expression are robustly induced by glucose in 832/13 cells. Figure 7.4A shows that, following transient transfection of 832/13 cells with luciferase fusion genes containing Pklr promoter sequence between -206 and +1 or G6pc1 promoter sequence between -7248 and +62, glucose markedly stimulated reporter gene expression, confirming published reports [311, 312]. Mannitol, a control for the osmotic effect of glucose, had no effect (Fig. 7.4A). Deletion of the Pklr promoter region between -206 and -101 markedly reduced the effect of glucose on Pklr-luciferase fusion gene expression whereas deletion of the G6pc1 promoter region between -7248 and -1641 had little effect on glucose-stimulated G6pc1-luciferase fusion gene expression (Fig. 7.4B). A comparison of the EC_{50} for glucose-stimulated Pklr-luciferase and G6pc1-luciferase expression showed that Pklr-luciferase fusion gene expression was more sensitive to glucose (Fig. 7.4C).

Since the 832/13 cell line is derived from rat islets [309, 310] these cells do not express endogenous G6pc2 because, in contrast to all other species examined to date, G6pc2 is a pseudogene in rats [66]. It therefore occurred to us that these cells could be used to assay glucose-6-phosphatase enzyme activity in situ by measuring the ability of G6pc1 expression to blunt glucose-stimulated Pklr-luciferase or G6pc1-luciferase fusion gene expression (Fig. 7.5A). We hypothesized that G6pc1 would repress glucose-stimulated fusion gene expression by stimulating G6P hydrolysis [313], therefore opposing the action of the endogenous glucokinase in these cells [314] and thereby reducing glycolytic flux. To test this hypothesis plasmids encoding wild type (WT) G6pc1 or a catalytically dead (D) variant were co-transfected with the Pklr-luciferase and G6pc1-luciferase fusion genes. WT and catalytically dead G6pc1 were expressed at similar levels (Fig. 7.5B).
Figure 7.4. Glucose-Regulated Fusion Gene Expression in 832/13 Cells. 832/13 cells were transiently co-transfected, as described in Materials and Methods, with an expression vector encoding Renilla luciferase (0.5 μg) and Pklr- luciferase or G6pc1- luciferase fusion genes (2 μg) containing the promoter regions from -206 to +1 and -7253 to +66, respectively, (Panels A and C) or the indicated promoter regions (Panel B). Following transfection, cells were incubated for 18-20 hr in serum-free medium in the presence of the indicated concentrations of glucose (Glc) or mannitol (Mann). Cells were then harvested and luciferase activity assayed as described in Materials and Methods. Results are presented as the ratio of firefly:Renilla luciferase activity (Panels A and B) or a percentage of the induction achieved with 30 mM glucose (Panel C). Results represent the mean ± S.E.M. of 3 experiments using independent preparations of all fusion gene constructs in which each experimental condition was assayed in triplicate.
Figure 7.5. Overexpression of G6pc1 Suppresses Glucose-Stimulated Fusion Gene Expression in 832/13 Cells.

Panel A: Schematic illustrating how the extent of glucose cycling catalyzed by glucokinase and G6pc1 determines intracellular G6P levels and hence activation of fusion gene expression through ChREBP and CREB. Panel B: Western blot showing that wild type (W) and catalytically dead (D) G6pc1 are expressed at similar levels following expression in 832/13 cells as described in Figure 7.3. A representative blot is shown. Panel C and D: 832/13 cells were transiently co-transfected, as described in Materials and Methods, with the -206/+1 Pkl-luciferase or -7248/+62 G6pc1-luciferase fusion genes (2 μg), an expression vectors encoding Renilla luciferase (0.5 μg) and either 2 μg (Panel D) or the indicated amounts (Panel C) of expression vectors encoding either WT or catalytically dead (D) G6pc1. The total DNA added was kept constant using the empty pcDNA3 vector. Following transfection, cells were incubated for 18-20 hr in serum-free medium in the presence of the indicated glucose concentrations (Panel D) or 30 mM glucose (Panel C). Cells were then harvested and luciferase activity assayed as described in Materials and Methods. Results were calculated as the ratio of firefly:Renilla luciferase activity and are presented as a percentage relative to that in 30 mM glucose-treated cells transfected with the empty pcDNA3 vector (2 μg) (Panel C) or as a percentage relative to that in 30 mM glucose-treated cells in the presence of catalytically dead G6pc1 (Panel D). Results represent the mean ± S.E.M. of 3 experiments using independent preparations of both fusion gene constructs in which each experimental condition was assayed in triplicate.
In the catalytically dead variant AA 83 was changed from arginine to alanine, which abolishes G6P hydrolysis [315]. Figures 7.5C and 7.5D show that WT G6pc1 repressed glucose-stimulated Pklr-luciferase and G6pc1-luciferase fusion gene expression relative to the expression obtained in the presence of catalytically dead G6pc1.

Most importantly, Figures 7.5C and 7.5D demonstrate that the effect of WT G6pc1 on glucose-stimulated Pklr-luciferase and G6pc1-luciferase fusion gene expression was not equivalent with G6pc1 mediating a greater repression of the latter. For the purpose of studying the impact of SNPs on glucose-6-phosphatase enzyme activity, subsequent experiments therefore examined the repression of glucose-stimulated G6pc1-luciferase fusion gene expression by glucose-6-phosphatase.

**Analysis of the Effect of Human G6PC2 SNPs on Glucose-6-Phosphatase Activity**

We have previously shown that glucose-6-phosphate activity is abolished in G6pc2 KO mouse islets strongly suggesting that G6pc2 has phosphohydrolase activity [69]. However, while several groups have attempted to detect G6P hydrolysis following overexpression of human G6PC2 or mouse G6pc2 [65, 66, 316], only one group has been successful [67]. Petrolonis et al. demonstrated that the rate of G6P hydrolysis by G6PC2 overexpressed in COS7 cells was 20-40 fold lower than that of G6PC1 [67]. This suggests that there are inherent technical difficulties in demonstrating G6P hydrolysis following overexpression of G6PC2.

Because of the low enzyme activity of G6PC2 and difficulty with achieving high human G6PC2 expression (Figs. 7.1-7.3), we decided to focus on non-synonymous human G6PC2 SNPs that alter AAs that are conserved in the highly related [66] and much more enzymatically active human G6PC1 and mouse G6pc1 isoforms of the glucose-6-phosphatase catalytic subunit (Fig. 7.6; Table 7.3). Specifically, we decided to analyze the effect of these G6PC2 SNPs indirectly by examining their effect on mouse G6pc1 enzyme activity. We hypothesize that G6PC2 SNPs that affect the function of mouse G6pc2 are highly likely to affect the function of human G6PC2 because of the strong conservation of catalytically important amino acids
between these proteins [66]. Supporting this hypothesis is the observation that of the 56 AAs in human G6PC1 mutation of which gives rise to glycogen storage disease (GSD) type 1a [43], 51 are conserved or represent conserved changes in human G6PC2 (Table 7.4). Based on this logic, we searched available databases for non-synonymous human G6PC2 SNPs that alter AAs that are conserved in mouse G6pc2 and the highly related and much more enzymatically active human G6PC1 and mouse G6pc1 isoforms of the glucose-6-phosphatase catalytic subunit. We identified 22 such non-synonymous human G6PC2 SNPs (Fig. 7.6; Table 7.3). These SNPs change AAs in a number of different regions of G6PC2 (Table 7.3), based on the predicted membrane topology of human G6PC1 [317]. We analyzed the effect of these G6PC2 SNPs indirectly by examining their effect on mouse G6pc1 enzyme activity using our novel in situ assay.

For these experiments we transfected 0.05 μg of plasmids encoding various G6pc1 variants, which confers a sub-maximal repression of glucose-stimulated G6pc1-luciferase fusion gene expression (Fig. 7.5C). This approach allowed for the identification of both inhibitory and activating variants. Using this assay we determined that the AA changes associated with the rs144254880 (Arg79Gln), rs149663725 (Gly114Arg) and rs2232326 (Ser324Pro) SNPs markedly reduce G6pc1 enzyme activity (Table 7.3) without affecting protein expression (Figs. 7.7A & B). For simplicity and comparison with the effect of these variants on human G6PC2 protein expression, these conserved AAs are numbered based on the position of the AA in human G6PC2 rather than their actual location in mouse G6pc1 (Fig. 7.6). The AA changes associated with the rs142189264 (Ser30Phe), rs199682245 (Asn68Ile) and rs150538801 (Phe256Leu) SNPs also reduced G6pc1 enzyme activity (Table 7.3), though to a lesser degree, but again without affecting protein expression (Fig. 7.7C). The AA changes associated with several other SNPs had statistically significant though minor effects on G6pc1 enzyme activity (Table 7.3), without affecting protein expression (Figure 7.8).
Figure 7.6. Conservation of Amino Acids Between Human G6PC2, Mouse G6pc2, Human G6PC1 and Mouse G6pc1. Sequence alignment showing the conservation of AAs between human (h) G6PC2, mouse (m) G6pc2, human G6PC1 and mouse G6pc1. Residues highlighted in green represent AAs mutation of which in G6PC1 causes GSD type 1a [43]. Residues highlighted in pink represent AAs that are changed by human G6PC2 SNPs that were identified using the UCSC Genome Browser (https://genome.ucsc.edu/) and HumSAVR (http://omictools.com/humsavar-tool) databases. Residues highlighted in yellow represent conserved AAs in human G6PC2, mouse G6pc2, human G6PC1 and mouse G6pc1 that are changed by a human G6PC2 SNP and where mutation in G6PC1 can cause GSD type 1a. Identities are indicated by filled circles and similarities by vertical bars.
**Figure 7.7. Analysis of the Effect of Amino Acid Changes on Mouse G6pc1 Protein Expression.** 832/13 cells were transiently transfected, as described in Materials and Methods, with expression vectors encoding either WT mouse (m) G6pc1 or G6pc1 variants in which the indicated amino acid (AA) had been changed as shown in Table 1. Following transfection, cells were incubated for 18-20 hr in serum-containing medium. Cells were then harvested and protein expression assayed as described in Materials and Methods. In some instances these AA changes did not affect G6pc1 protein expression (Panels A and B). In other cases they resulted in reduced expression (Panel C). Representative blots are shown. The individual panels shown in Panel B were all derived from the same blot. For simplicity and comparison with Figure 7.9, these AAs are numbered based on the position of the equivalent conserved AA in human G6PC2 (Figure 7.4).
Figure 7.8. Analysis of the Effect of Amino Acid Changes on Mouse G6pc1 Protein Expression. 832/13 cells were transiently transfected, as described in Materials and Methods, with expression vectors encoding either wild type (WT) mouse (m) G6pc1 or G6pc1 variants in which the indicated amino acid (AA) had been changed as shown in Table 1. Following transfection, cells were incubated for 18-20 hr in serum-containing medium. Cells were then harvested and protein expression assayed as described in Materials and Methods. With the exception of AA 8, these AA changes did not markedly affect G6pc1 protein expression. Representative blots are shown. For simplicity and comparison with Figure 7.9, these AAs are numbered based on the position of the equivalent AA in human G6PC2 (Fig. 7.6).
Table 7.3. Analysis of the Effect of Amino Acids Changed by Human G6PC2 SNPs on Human G6PC2 and Mouse G6pc1 Protein Expression and Activity. Amino acids (AAs) changed by human G6PC2 SNPs that are conserved in human G6PC2, mouse G6pc2, human G6PC1 and mouse G6pc1 were identified using the UCSC Genome Browser (https://genome.ucsc.edu/) and HumSAVR (http://omictools.com/humsavar-tool) databases. The G6PC2 domain affected by each AA change was predicted by comparison with the proposed structure of G6PC1 [317]. The Table shows the effect of these SNPs on G6pc1 enzyme activity based on comparison with WT G6pc1 as assessed 646 using a novel in situ enzyme assay (Figs. 7.5 & 7.6). This assay measures the ability of G6pc1 to suppress glucose-stimulated fusion gene expression (Figs. 7.5 & 7.6). Results for each variant represent the mean ± S.E.M. of 3 experiments using two independent preparations of each expression vector construct in which each experimental condition was assayed in triplicate. Some of these G6PC2 SNPs change AAs that are not only conserved in G6PC1 but where mutation of these AAs in G6PC1 causes GSD type 1a [318]. In each case the AA associated with GSD type 1a is shown in parentheses. In each case the G6PC2 SNP changes the AA to one distinct from that associated with GSD type 1a. For simplicity and comparisons between human G6PC2 and mouse G6pc1 the AAs in mouse G6pc1 are numbered based on the position of the equivalent conserved AA in human G6PC2 (Figure 7.4). N.D., not determined; N.C., no change.

<table>
<thead>
<tr>
<th>hG6PC2 SNP</th>
<th>Base #</th>
<th>AA#</th>
<th>GSD Type1a Mutation</th>
<th>Domain Location</th>
<th>hG6PC2 Expression</th>
<th>mG6pc Expression</th>
<th>% WT mG6pc Activity</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs388882511</td>
<td>GGA23GAA</td>
<td>Gly6Glu</td>
<td>No</td>
<td>N terminus</td>
<td>Decreased</td>
<td>Decreased</td>
<td>90.13 ± 2.97</td>
<td>0.03</td>
</tr>
<tr>
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<td>Gin16His</td>
<td>Yes (Arg)</td>
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<td>N.D.</td>
<td>N.C.</td>
<td>110.04 ± 1.71</td>
<td>0.001</td>
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<td>Ser50Phe</td>
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<td>N.D.</td>
<td>N.C.</td>
<td>67.22 ± 1.39</td>
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<td>Val53Ile</td>
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<td>N.D.</td>
<td>N.C.</td>
<td>109.98 ± 2.07</td>
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<td>Asn68Ile</td>
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<td>N.C.</td>
<td>52 ± 1.07</td>
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<td>Thr107Arg</td>
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<tr>
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<td>GGC340GCC</td>
<td>Gly114Arg</td>
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<td>In loop</td>
<td>N.D.</td>
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<td>53.32 ± 0.88</td>
<td>0.000001</td>
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<tr>
<td>rs187707693</td>
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<td>Tyr124Cys</td>
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<td>N.D.</td>
<td>N.C.</td>
<td>104 ± 0.92</td>
<td>0.01</td>
</tr>
<tr>
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<td>Met126Val</td>
<td>No</td>
<td>In membrane 3</td>
<td>N.D.</td>
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<td>103.96 ± 5.75</td>
<td>0.53</td>
</tr>
<tr>
<td>rs138726309</td>
<td>CAT529TAT</td>
<td>His177Tyr</td>
<td>Yes (Pro)</td>
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<td>92 ± 2.69</td>
<td>0.03</td>
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<tr>
<td>rs201094274</td>
<td>AGT609AGA</td>
<td>Ser203Arg</td>
<td>No</td>
<td>In loop</td>
<td>N.D.</td>
<td>N.C.</td>
<td>115.33 ± 3.65</td>
<td>0.003</td>
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<tr>
<td>rs2232323</td>
<td>TAC620TTC</td>
<td>Tyr207Ser</td>
<td>Yes (Cys)</td>
<td>In loop</td>
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<td>88.19 ± 6.53</td>
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</tr>
<tr>
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<td>TTC644TTC</td>
<td>Phe215Ser</td>
<td>No</td>
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<td>N.D.</td>
<td>N.C.</td>
<td>105 ± 5.09</td>
<td>0.42</td>
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<tr>
<td>rs375572810</td>
<td>TAC664CAC</td>
<td>Tyr222His</td>
<td>No</td>
<td>In membrane 6</td>
<td>N.D.</td>
<td>N.C.</td>
<td>94.59 ± 2.37</td>
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<tr>
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<td>His250Tyr</td>
<td>No</td>
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<td>N.D.</td>
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</tr>
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<td>83.55 ± 2.58</td>
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<td>Leu301Ser</td>
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<td>86.94 ± 5.85</td>
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<td>Pro313Leu</td>
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<td>83 ± 5.48</td>
<td>0.04</td>
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<td>Pro340Leu</td>
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<td>55.31 ± 4.93</td>
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Table 7.4 Amino Acids in Human G6PC1 Whose Mutation Causes Glycogen Storage Disease Type 1a are Highly Conserved in Mouse G6pc1, Mouse G6pc2 and Human G6PC2. The Table shows AAs in human G6PC1 whose mutation causes glycogen storage disease (GSD) type 1a [43] and whether these AAs are conserved or similar in mouse G6pc1, mouse G6pc2 and human G6PC2.

<table>
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</tr>
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Interestingly, the AA changes associated with the rs368382511 (Gly8Glu), rs374055555 (Arg293Trp) and rs2232327 (Pro340Leu) SNPs markedly reduced G6pc1 protein expression (Figs. 7.7D & E) but only the latter had a marked effect on enzyme activity (Table 7.3). This result suggests that these AA changes may actually be increasing the specific activity of G6pc1.

Table 7.1 and Figure 7.6 show that there are six SNPs in G6PC2 that alter AAs that are conserved in G6PC1 and where mutation of these AAs in G6PC1 causes GSD type 1a [43]. However, these SNPs change the residue associated with GSD type 1a to an AA distinct from that that causes GSD type 1a. For example, rs372008743 changes a glutamine at residue 16 to a histidine whereas the mutation associated with GSD type 1a involves a change from a glutamine at residue 16 to an arginine (Table 7.3; Ref. [43]). For four of these 6 SNPs the AA change associated with the G6PC2 SNP had little effect on G6pc1 enzyme activity or protein expression (Table 7.3) suggesting that the change is silent. However, for two of these 6 SNPs the AA change associated with the G6PC2 SNP markedly affected G6pc1 enzyme activity (rs144254880; Arg79Gln) (Table 7.1) or expression (rs374055555; Arg293Trp) (Figs. 7.7D & E).

**Analysis of the Effect of Human G6PC2 SNPs on Protein Expression**

We next analyzed the effect of several human non-synonymous G6PC2 SNPs on human G6PC2 protein expression (Table 7.3). We began by analyzing the 3 SNPs that were associated with reduced mouse G6pc1 protein expression (Figs. 7.7D & E), namely rs368382511 (Gly8Glu), rs374055555 (Arg293Trp) and rs2232327 (Pro340Leu). Figures 7.9A & B show that rs374055555 (Arg293Trp) and rs2232327 (Pro340Leu) also confer reduced expression of human G6PC2 in COS cells, with a trend towards reduced expression observed with rs368382511 (Gly8Glu). We next analyzed three SNPs, namely rs138726309 (His177Tyr), rs2232323 (Tyr207Ser) and rs492594 (Val219Leu) that Mahajan et al. [287] recently showed reduced human G6PC2 protein expression in 832/13 cells.
Figure 7.9. Analysis of the Effect of Human G6PC2 SNPs on Human G6PC2 Protein Expression. COS 7 cells were transiently transfected, as described in Materials and Methods, with expression vectors encoding either wild type (WT) human (h) G6PC2 or G6PC2 variants in which the indicated amino acid (AA) had been changed as shown in Table 1. Following transfection, cells were incubated for 18-20 hr in serum-containing medium. Cells were then harvested and protein expression assayed as described in Materials and Methods. The variants shown either reduced (Panels A-D) or had little effect (Panel E) on human G6PC2 protein expression. Data were quantitated by scanning with the results in Panels B and D showing the mean ± S.E.M. of 4 experiments. Representative blots are shown.
Figures 7.9C & D show that rs138726309 (His177Tyr) and rs2232323 (Tyr207Ser) also confer reduced expression of human G6PC2 in COS cells. In contrast, the rs492594 (Val219Leu) variant had little effect on human G6PC2 expression in COS cells (Fig. 7.9E). The AA changes associated with rs138726309 (His177Tyr) and rs2232323 (Tyr207Ser) did not affect mouse G6pc1 protein expression (Fig. 7.7A). The rs492594 (Val219Leu) variant alters an AA that is not conserved in mouse G6pc2, human G6PC1 or mouse G6pc1 (Table 7.5).

Finally, we analyzed two additional SNPs at the C terminus of human G6PC2, namely rs137857125 (Pro313Leu) and rs2232326 (Ser324Pro). Figures 7.9C & D show that these SNPs also confer reduced expression of human G6PC2 in COS cells. In contrast, the AA changes associated with these SNPs did not affect mouse G6pc1 protein expression (Fig. 7.7B). Strikingly, these results suggest that despite the conservation of key AAs involved in enzyme activity between mouse G6pc1, mouse G6pc2, human G6PC1 and human G6PC2 [43, 315, 319] the mutation of conserved AAs has variable effects on human G6PC2 and mouse G6pc1 protein expression.

Discussion

This study focused on 22 non-synonymous SNPs in human G6PC2 that change AAs that are conserved between human G6PC2, mouse G6pc2, human G6PC1 and mouse G6pc1 (Table 7.3) (Fig. 7.6), though database analyses identified multiple additional non-synonymous G6PC2 SNPs that affect AAs in G6PC2 that are not conserved across all four isoforms (Table 7.5). We show that the AA changes associated with the rs144254880 (Arg79Gln), rs149663725 (Gly114Arg), rs2232326 (Ser324Pro), rs142189264 (Ser30Phe), rs199682245 (Asn68Ile) and rs150538801 (Phe256Leu) SNPs reduced G6pc1 enzyme activity in situ (Table 7.3) without affecting protein expression (Figs. 7.7A-C). We also show that the AA changes associated with the rs368382511 (Gly8Glu), rs374055555 (Arg293Trp) and rs2232327 (Pro340Leu) SNPs markedly reduced G6pc1 protein expression (Figs. 7.7D & E).
Table 7.5. Human G6PC2 SNPs that Alter Amino Acids that are not Conserved in Human G6PC2, Mouse G6pc2, Human G6PC1 and Mouse G6pc1.

Human G6PC2 SNPs that change AAs that are not uniformly conserved in human G6PC2, mouse G6pc2, human G6PC1 and mouse G6pc1 were identified using the UCSC Genome Browser (https://genome.ucsc.edu/) and HumSAVR (http://omictools.com/humsavar-tool) databases. The G6PC2 domain affected by each AA change was predicted by comparison with the proposed structure of G6PC1 [317]. **, this residue has been associated with variations in FBG in healthy individuals who do not have diabetes [287].

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Finally, we show that the rs368382511 (Gly8Glu), rs374055555 (Arg293Trp), rs2232327 (Pro340Leu), rs138726309 (His177Tyr), rs2232323 (Tyr207Ser), rs137857125 (Pro313Leu) and rs2232326 (Ser324Pro) SNPs confer reduced expression of human G6PC2 (Figs. 7.8A-D).

Once the challenge of achieving high human G6PC2 expression is overcome (Figs. 7.1-7.3), future studies will aim to determine whether the SNPs that affect mouse G6pc1 enzyme activity also affect human G6PC2 enzyme activity. This seems highly likely since site directed mutagenesis studies [315, 319] and the analysis of mutations causing GSD type 1a [43] have shown AAs that are essential for high G6PC1 enzyme activity are conserved between mouse G6pc1, mouse G6pc2, human G6PC1 and human G6PC2. Indeed, of the 56 AAs in human G6PC1 mutation of which gives rise to GSD type 1a [43], 51 are conserved or represent conserved changes in human G6PC2 (Table 7.6). It is striking that the G6PC2 SNPs rs138726309 (His177Tyr) and rs2232323 (Tyr207Ser), that have been linked to variations in FBG [287, 288, 320], affect AAs mutation of which in G6PC1 causes GSD type 1a (Table 7.3) [43]. Site directed mutagenesis studies [321] and the analysis of mutations causing Dursun syndrome [322] have similarly shown a conservation of catalytically important AAs between G6PC1 and the G6PC3 isoform of glucose-6-phosphatase, initially referred to as UGRP, even though they share only a 36% overall AA conservation [55]. Furthermore, there is a statistically significant association between residues that are associated with GSD type 1a and Dursun syndrome [323], supporting the notion that SNPs that alter conserved residues in all three G6PC1 isoforms will likely have similar effects on enzyme activity because catalytically important residues are conserved in all three isoforms.

Future studies will also use Vanderbilt University's BioVU biobank to examine whether SNPs that affect human G6PC2 protein expression or activity are associated with altered phenotypic characteristics in humans, such as FBG and T2D risk. BioVU is a DNA biobank linked to a de-identified version of the Vanderbilt electronic health records, called the Synthetic Derivative (SD) [249, 250]. The SD can be screened, using a procedure referred to as a PheWAS, to identify associations between specific SNPs and human diseases as well as associations with altered plasma hormone/metabolite levels [324-328]. Of
particular interest will be the medical records of individuals with \textit{G6PC2} SNPs that result in frameshift mutations or premature termination (Table 7.6).

Mahajan et al. [287] recently showed that the rs138726309 (His177Tyr) and rs2232323 (Tyr207Ser) SNPs result in reduced human G6PC2 protein expression in HEK293 and INS-1E cells, which we confirmed in COS cells (Figs. 7.8C & D). They also showed that another SNP rs492594 (Val219Leu), that changes an AA that is not conserved in mouse G6pc2, human G6PC1 or mouse G6pc1 (Table 7.5), also results in reduced human G6PC2 protein expression in HEK293 cells though not in INS-1E cells [287]. We observed that this SNP also does not appear to affect human G6PC2 protein expression in COS cells (Fig. 7.8E). This suggests that for this particular SNP, unknown cell line-dependent factors influence its action on G6PC2 expression. As with the initially described GWAS SNP, rs560887 [82, 266], Mahajan et al. [287] showed that all three of these SNPs are associated with variations in fasting plasma glucose (FPG). Horikoshi et al. [320] have also shown that the rs138726309 (His177Tyr) is associated with variations in FPG. In addition, Wessel et al. [329] have shown that rs138726309 (His177Tyr) and rs2232323 (Tyr207Ser), as well as two additional non-synonymous SNPs, rs2232326 (Ser324Pro) and rs146779637 (Arg283STOP) are associated with variations in FPG. We showed that the rs2232326 (Ser324Pro) SNP results in altered G6PC2 protein expression in COS cells (Figs. 7.8C & D). Interestingly, for reasons that are unclear, Mahajan et al. [287], in contrast to Wessel et al. [329], did not observe an association between rs146779637 (Arg283STOP) and FPG. Mahajan et al. [287] speculated that the lack of association with FPG was because this variant might retain activity despite the removal of the terminal 72 AAs of G6PC2. This variant clearly merits further study, especially since the data suggest a potential difference with human G6PC1, whose activity is susceptible to C terminal truncation [315].

Previous studies have suggested a complex relationship between G6PC2 and T2D risk with apparently conflicting results in different populations [76, 112, 278, 330]. Interestingly, Mahajan et al. [287] showed that the rs492594 (Val219Leu) SNP is associated with altered risk for T2D. In contrast, in their study of non-synonymous \textit{G6PC2} SNPs, Wessel et al. [329] reported no association between \textit{G6PC2}
and T2D risk. The rs492594 (Val219Leu) G6PC2 variant was not included in the studies of Wessel et al. [329] so potential reasons for this apparent discrepancy between G6PC2 variation and T2D risk remain unclear. These results may indicate that the rs492594 (Val219Leu) G6PC2 variant has a unique effect on β-cell function, unrelated to the control of glycolytic flux, especially since our results (Fig. 7.8) and the results of Mahajan et al. [287] suggest that the rs492594 (Val219Leu) variant reduces G6PC2 protein expression less than the rs138726309 (His177Tyr) and rs2232323 (Tyr207Ser) variants that were included in the studies of Wessel et al. [329]. Indeed, we have previously speculated that G6PC2 may affect β-cell endoplasmic reticulum calcium retention, in addition to its action on glycolytic flux [78]. Indirect support for such a function for G6PC2 was recently suggested by the observation that deletion of the sorcin gene, which regulates endoplasmic reticulum calcium retention, resulted in elevated G6pc2 expression [331].

Our study also describes a novel assay for the measurement of glucose-6-phosphatase activity in situ (Figs. 7.4 & 7.5). G6pc1 is unstable [332] and much remains unknown about the factors regulating G6pc1 activity [308] so this assay has the advantage that G6pc1 activity can be studied in an endogenous environment rather than in isolated and/or permeabilized microsomes. However, there are several caveats associated with this assay. Firstly, even though the amount of G6pc1 expressed was sufficient to achieve a sub-maximal repression of glucose-stimulated G6pc1-luciferase gene expression (Fig. 7.5), this assay will not have the same linearity relative to an in vitro assay given the influence of other intracellular factors on G6pc1 activity. Secondly, in this assay apparent changes in G6pc1 activity could arise indirectly due to a change in sub-cellular distribution. Finally, because G6pc1 is located in the endoplasmic reticulum with its active site directed towards the lumen, the glucose-6-phosphatase activity of G6pc1 in situ is dependent on transport of its substrate G6P into the lumen by a G6P/Pi transporter, encoded by the SLC37A4 gene [42, 78]. Pan et al. [302] have shown that G6PC1 and SLC37A4 are functionally coupled. Therefore, AA changes that affect this coupling will also appear to affect the inherent glucose-6-phosphatase activity of G6pc1 in the in situ assay. Future studies comparing the activity of specific G6pc1 variants in this in situ assay and
the standard *in vitro* assay may lead to the identification of variants that affect aspects of \( G6pc1 \) function other than G6P hydrolysis.

In the course of developing our novel assay we made several interesting observations about glucose-regulated gene expression. Collier et al. [312] demonstrated that the effect of glucose on rat \( Pklr \) gene expression in 832/13 cells is mediated by the carbohydrate response element binding protein (ChREBP), which binds a carbohydrate response element (ChoRE) located between -188 and -172 in the rat \( Pklr \) promoter [333]. Consistent with this observation, deletion of the promoter region between -206 and -101 markedly reduced the effect of glucose on \( Pklr \)-luciferase fusion gene expression (Fig. 7.4B). In contrast, Pederson et al. [311] demonstrated that the effect of glucose on rat \( G6pc1 \) expression in 832/13 cells is mediated by two promoter elements, a ChoRE located between -3616 and -3600 that binds ChREBP [311], and a cAMP response element (CRE) located between -163 and -156 that binds CRE binding protein (CREB) [272]. Pederson et al. [311] found that deletion of the G6PC1 ChoRE reduced the glucose response by \( \sim 80\% \). However, Figure 7.4B shows that deletion of the promoter region between -7248 and -1641 had little effect on glucose-stimulated \( G6pc1 \)-luciferase fusion gene expression, suggesting that differences possibly related to cell passage number or growth conditions have altered the relative importance of ChREBP and CREB in glucose signaling to the \( G6pc1 \) promoter in our 832/13 cells. Interestingly, \( Pklr \)-luciferase fusion gene expression was more sensitive to glucose suggesting that the signaling pathways used by glucose to regulate ChREBP and CREB are distinct (Fig. 7.4C). Consistent with this idea, the effect of WT \( G6pc1 \) on glucose-stimulated \( Pklr \)-luciferase and \( G6pc1 \)-luciferase fusion gene expression was not equivalent with \( G6pc1 \) mediating a greater repression of the latter (Fig. 7.5C & D). This result not only suggests that the glucose-signaling pathways to ChREBP and CREB are distinct and that \( G6pc1 \) preferentially influences the latter. Finally, we also observed that catalytically dead \( G6pc1 \) partially represses glucose-stimulated fusion gene expression (Figs. 7.5C & D). This may reflect activation of the endoplasmic reticulum stress response [334] and could also explain why catalytically dead \( G6pc1 \) has a greater effect on \( G6pc1 \) versus \( Pklr \) fusion gene expression since the former promoter contains a stress
response element [335]. Interestingly, over expression of G6PC2 in mice causes diabetes due to activation of the ER stress response [336].
VIII. SUMMARY AND FUTURE DIRECTIONS

Thesis Summary

The work described in this dissertation aims to provide insight into the role that G6pc2 plays in modulating FBG during glucocorticoid induced stress, while also identifying the role G6pc2 is playing in modulating BMI and how G6PC2 SNPs affect protein expression and activity. Briefly, as was described in the introduction, G6pc2 functions to create a substrate cycle with glucokinase and, through hydrolyzing G6P, it modulates FBG under certain physiological conditions by modulating glycolytic flux and the amount of G6P metabolized by the \( \beta \)-cell. The work described here aimed to identify physiological conditions under which G6pc2 activity or expression was regulated, as well as the mechanisms by which such regulation occurs. Moreover, I further identified how, by using a variety of stress inducing experimental paradigms, induction of G6pc2 gene expression affects glucose metabolism, specifically FBG. Additionally, due to the novel association of human G6PC2 SNPs with BMI and adiposity in Mexican Americans, I aimed to determine whether G6pc2 KO mice were protected from DIO. Finally, by using a systematic approach, I worked to analyze whether rare human G6PC2 SNPs affect either protein expression or activity, as assayed using a novel *in vitro* assay (Fig. 7.6A). Because my analysis of G6PC2 SNPs on protein expression and enzyme activity was indirect due to technical issues in expressing the protein in tissue culture, these analyses need to be repeated once this hurdle is overcome.

Because the synthetic glucocorticoid Dex was found to stimulate human G6PC2 promoter activity, I extended these studies by characterizing the effect of three different models of stress (Dex injections, physical restraint and 11-DHC) on FBG and glucose tolerance in WT and G6pc2 KO 129SvEv and C57BL/6J mice. Briefly, I showed that stress induces G6pc2 gene expression and this results in improved glucose tolerance in all three models (Fig. 4.7H&I, 4.10A&G, 6.2). The improvement in glucose tolerance was greater in KO mice relative to WT in the 129SvEv physical restraint, C57BL/6J 11-DHC and C57BL/6J Dex models, consistent with enhanced sensitivity of GSIS to glucose. Moreover, there was a significant reduction
in FBG of KO treated animals relative to WT in all three paradigms following a 6hr fast (Fig. 3.3J, 4.7F, 4.10C&E, 6.4)). This is consistent with GWAS data that associate \textit{G6PC2} with FBG variation in humans. These data indicate that G6PC2 plays an important role in regulating the set point for FBG (Fig. 1.5).

As noted previously, the improvement in glucose tolerance that I observed in my stress paradigms is not characteristic of other models and data in the glucocorticoid metabolism field. The literature more frequently supports the role of glucocorticoid treatment and stress resulting in increased FBG, insulin resistance and impaired glucose intolerance [129, 169, 173, 337]. The difference between my findings in relation to the field can be explained in a number of ways. The first is that many of these papers used rats to study the effects of glucocorticoid treatment, and as previously mentioned, \textit{G6pc2} is a pseudogene in rats [147, 210, 211, 227, 338]. Secondly, there are differences in the amount of time animals were fasted prior to FBG measurements. In my studies, mice were fasted for 6hrs prior to experiments, however a majority of the published studies were performed on overnight fasted rats. However, while the 6hr data opposes the generally accepted trends regarding the effect of glucocorticoids on glucose metabolism, when I repeated my studies on 24hr fasted mice, I was able to replicate those studies and detect impaired glucose tolerance and enhanced FBG in physically restrained WT and \textit{G6pc2} KO mice relative to controls (Fig. 4.10E). The final explanation for the inconsistencies between my data and other published studies can be attributed to different experimental methods and paradigms, i.e. there is not a consistent dose, delivery method or type of glucocorticoid used [206].

Because the rs560887 SNP in \textit{G6PC2} is associated with variations in BMI and adiposity, I wanted to determine if \textit{G6pc2} KO mice were protected from DIO. The findings outlined in chapter V highlight the fact that there are gender, genotype and background specific effects of high fat feeding in mice. Moreover, due to the observed background differences, it seems that the effect of high fat feeding induced \textit{G6pc2} gene expression is dependent on strain specific modifier genes. Interestingly, cholesterol levels were significantly decreased in high fat fed C57BL/6J and mixed genetic background \textit{G6pc2} KO mice relative to WT (Fig. 5.6 G,H&J). Future studies should focus on how the absence of \textit{G6pc2} could affect cholesterol
metabolism in these mice. I speculate that it is most likely through an indirect mechanism driven be the observed decrease in FBG relative to WT high fat fed mice.

Finally, in an effort to identify rare G6PC2 variants that would be predicted to have significant effects on FBG, I systematically characterized the effect that human G6PC2 SNPs have on protein expression and enzyme activity. These studies identified numerous SNPs that were conserved at catalytically important residues that had significant effects on activity and/or protein expression (Table 7.3). While the activity of human G6PC2 was not successfully studied, this body of data will provide a database of SNPs that can be used to characterize human SNPs when an improved system for studying human G6PC2 expression and activity is available. Future studies should aim to improve human G6PC2 expression in tissue culture systems so as to better understand how these SNPs affect activity in vitro. Once the effect of these human SNPs have been characterized, we can use BioVU to address whether these SNPs correlate with marked changes in FBG. Finally, future work needs to determine if the differences in protein expression in human G6PC2 and mouse G6pc1 were caused by differences in translation efficiency and/or stability. Because our RNA analysis of mouse G6pc2 and human G6pc2 in COS7 cells indicates that there is more endogenous mouse G6pc2 RNA present relative to human (Fig. 7.1), this potentially could explain part of the difference in protein expression that is observed (Fig. 7.2). However, I do not think that the different levels of RNA expression fully accounts for the difference in protein expression because the magnitude of the difference in mouse G6pc2 protein expressed relative to human is much larger than that at the RNA level. In order to determine if there is a difference in protein stability between mouse and human G6PC2, a pulse-proteolysis experiment could be performed in transiently transfected COS7 cells [339].

Further Studies to Elucidate the Role of G6pc2 in Pancreatic β-cells

The studies described here further aid in understanding the role that G6pc2 plays in vivo, specifically in conditions of elevated glucocorticoid levels. While I examined multiple experimental
paradigms looking at the effect of stress on \textit{G6pc2} expression and its role in glucose metabolism, more work needs to be done to examine the mechanisms by which glucocorticoids affect glucose cycling in islets and subsequently affects FBG. With the advancements made by Dr. Jamey Young in studying glucose cycling \cite{68}, we can now design experiments to directly examine how glucose cycling is modulated by elevated glucocorticoid levels. By using Dr. Young’s stable isotope method, altered G6Pase activity and glucose cycling in islets under conditions of enhanced glucocorticoid levels can be directly linked to the presence of \textit{G6pc2} by using WT islets, with the expectation that glucocorticoids will increase glucose cycling rates and decrease glycolytic flux following glucocorticoid treatment. Interestingly, work published prior to the identification of \textit{G6PC2} and \textit{G6PT} showed that G6Pase activity and glucose cycling are enhanced following Dex treatment \cite{90, 230}. The findings presented in chapter III can explain these previous findings in that Dex stimulation of \textit{G6pc2} and \textit{G6pt} gene expression would be expected to increase glucose cycling, increase G6Pase activity and blunt insulin secretion, as these older studies observed \cite{90, 230}. It would be valuable to repeat these studies in order to confirm that these data can be explained by an action of \textit{G6pc2} on glucose cycling and glycolytic flux; using WT islets we should be able to replicate their data, however, using \textit{G6pc2} glucocorticoid treated KO islets, we expect glucose cycling to be abolished.

These isolated islet studies could be performed in two ways, the first is to isolate islets from glucocorticoid treated or stressed animals and the second is to directly treat isolated islets with glucocorticoids. There are caveats to both methods. One concern with isolating islets from glucocorticoid treated animals is that the isolation process will effect glucose cycling rates or that glucocorticoid stimulated changes in the expression of key \textbeta-cell genes could be lost in the process of islet isolation, as has previously been reported. Alternatively, the concern with directly treating isolated islets with glucocorticoids is that multiple genes have been shown to be regulated differently \textit{in vitro} relative to \textit{in vivo} function; so this method may not accurately reflect the effect of glucocorticoids on glucose cycling and glycolytic flux \textit{in vivo} \cite{68, 90, 230, 340}. A final concern with isolated islet studies is that, while they highlight important aspects of \textbeta-cell biology, they are not representative of the effect that glucocorticoids
have in vivo. As it has been well established that glucocorticoid treatment causes whole body insulin resistance [129, 147, 169], performing studies in isolated islets is not ideal in that these studies are executed in the absence of insulin resistance.

In addition to assessing the effect that stress has on glucose cycling in WT and G6pc2 KO islets, a paradigm which detects a difference in insulin secretion between WT and G6pc2 KO glucocorticoid treated mice, would provide further support for the models highlighted in this thesis. In my studies, I focused on FBG and did not attempt to highlight a difference in GSIS, but our model predicts that, at submaximal glucose concentrations, G6pc2 KO mice would have enhanced insulin secretion relative to WT (Fig. 1.6 & 4.11). Future studies directly examining GSIS in stressed mice using either perfused pancreata or clamp studies are necessary because they will further support the findings in this thesis and provide increased evidence for the role of G6pc2 as a negative regulator of GSIS. 11-DHC supplementation (chapter VI) and long-term corticosterone treatment, as discussed in the next section, may prove to be more successful in detecting a difference in insulin secretion. Performing these studies will further elucidate the function of G6pc2 in modulating FBG and GSIS via increased rates of glucose cycling during stress.

While data presented in Chapter III-VI show that, under basal conditions, there are no differences in glucose tolerance between WT and G6pc2 KO mice, consistent with GWAS data [69], we do see improved glucose tolerance in KO mice relative to WT in the Dex injection, 11-DHC and physical restraint paradigms, consistent with enhanced sensitivity of GSIS to glucose. Additionally, while not directly related to the function of G6pc2, future studies should examine the mechanisms driving the improvement in glucose tolerance that I observed in all three stress paradigms. Traditionally, it has been accepted that glucocorticoid treatment results in an elevation of FBG caused by whole body insulin resistance, hepatic glycogenolysis and inhibition of insulin secretion [129]. However, the data presented here does not support this model following a 6hr fast. While it is evident the G6pc2 functions to protect against hypoglycemia in WT mice following glucocorticoid treatment, it is unclear what the benefit of improved glucose tolerance in stressed mice would be (Fig. 4.7H&I, 4.10A, 6.2 & 6.5). While this may be explained by
differences in fasting times, as previously discussed, another explanation is that I measured FBG and glucose tolerance at a time point when β-cells were still functioning and able to hyper-secrete insulin, thereby resulting in repressed FBG and enhanced glucose tolerance. Data presented in chapters III & IV show that, following glucocorticoid treatment or restraint, FPI levels are significantly elevated (Fig. 4.7G, 4.10B&F), consistent with enhanced GSIS. While we did not directly show enhanced insulin secretion, previous literature reports hyperinsulinemia following Dex injections, which supports our model [129, 141, 185, 211, 226]. However, following a more chronic paradigm such as 11-DHC or corticosterone treatments, we may observe that the β-cells cannot adequately secrete insulin at a level necessary to overcome the whole body, glucocorticoid induced, insulin resistance. As such, we would expect in these paradigms to see impaired glucose tolerance and enhanced FBG relative to controls. These chronic paradigms could also demonstrate impaired β-cell function, as seen in other paradigms in the literature, and allow us to further detect differences in WT and G6pc2 KO glucose metabolism in vivo. These models would also better mimic the human conditions of Cushing’s disease or diseases that require long-term glucocorticoid treatments such as rheumatoid arthritis. Further support for this idea comes from the observation that a subset of 129SvEv mice treated with Dex become diabetic, mimicking glucocorticoid induced diabetes in humans (Fig. 4.7 C&D).

**Analysis of the Effect of Corticosterone Pellets in C57BL/6J and 129SvEv WT and G6pc2 KO Mice**

An alternate model to study the effect of enhanced glucocorticoid levels is to use slow releasing corticosterone pellets in adrenalectomized mice [341-344]. There are multiple differences in this model relative to the previously studied effects of physical restraint (chapter III), Dex injections (chapter IV) and 11-DHC (chapter VI) treatment. The first is that it is a chronic model using the endogenous active glucocorticoid in adrenalectomized mice. By removing the adrenal gland, we will be able to study the effect of chronic stress with less interference from the naturally produced corticosterone. The next difference is
that by chronically elevating glucocorticoids, we will enhance whole body glucocorticoid levels in a sustained fashion. This will occur because, once the animals are adrenalectomized, their natural circadian rhythm of glucocorticoid secretion will be abolished and, due to the slow-release nature of the pellet, there will be a constant release of corticosterone into the system, thereby resulting in a constant level of glucocorticoid exposure to all tissues [344]. While the 11-DHC studies were also chronic, the pellet studies differ in that the 11-DHC mice still exhibit circadian control over endogenous glucocorticoid secretion. Moreover, they differ because we will be administering the endogenously active corticosterone versus the inactive 11-DHC. Finally, by using slow release pellets in adrenalectomized mice, it would allow us to more closely mimic Cushing’s disease, which, in humans, is typically caused by an adenoma on the adrenal gland that results in chronic and uninhibited secretion of cortisol. These patients do not exhibit negative feedback of the HPA axis (Fig. 1.13) and demonstrate increased central adiposity, weight gain, high blood pressure, bone loss and in some cases T2D [148, 202, 203, 345]. Overall, using slow release pellets will allow us to examine the role that G6pc2 plays in chronically stressed mice versus acutely stressed.

Because of the chronic nature of the paradigm, I hypothesize that there will be widespread insulin resistance, resulting in impaired glucose tolerance and increased FBG, with KO mice still having relatively reduced FBG compared to WT. In comparison to the other models studied, I expect that FBG will be significantly increased in treated animals compared to control animals, as observed in 24hr fasted Dex treated mice, as opposed to the decrease in FBG we observed in the 6hr fasted models. As mentioned in previous sections, we hypothesize that the models studied in chapters III, IV and VI demonstrated a relative reduction in FBG because of the experimental paradigms that were studied, not necessarily because of the biological function of glucocorticoids. However, in this chronic model, I expect that the β-cells will not be able to compensate for the whole body insulin resistance by hyper-secreting insulin to adequate levels to balance this resistance over the long-term of the study. Because glucocorticoid levels are expected to remain significantly elevated over several weeks, I expect this to ultimately result in hyperglycemia, insulin resistance, increased adiposity and weight gain. By abolishing endogenous glucocorticoid regulation and
increasing glucocorticoid levels to a supra-physiological level, this would allow us to examine the role G6pc2 plays in modulating glucose metabolism and GSIS in periods of extreme stress.

**Characterization of a β-cell Specific G6pc2 KO Mouse**

Although G6pc2 is thought to be exclusively expressed in islet β-cells [346], G6pc2 transcripts have been identified in liver [347] and hypothalamus [294], although at trace levels relative to G6pc1 and G6pc3. Moreover, while the data presented in this thesis and previous studies [69, 96] support the role of G6pc2 in regulating glucose metabolism via its function in the islet β-cell, it is possible that there could be developmental compensation that occurred following germline deletion of G6pc2. Justification for this hypothesis comes from the observation that compensatory gene expression changes have been documented to occur during development following a germline mutation of many other genes [348]. If this hypothesis were correct, than adult mice lacking G6pc2 could show a more severe phenotype than mice lacking G6pc2 since conception. Once these mice have been designed, FBG, glycolytic flux, glucose cycling and GSIS should all be analyzed. Performing these KO studies in adult mice will allow us to determine whether G6pc2 regulates glucose homeostasis solely through its function in islet β-cells as well as determine if inactivation of G6pc2, in the absence of developmental compensation, results in a more severe phenotype. By performing these studies we will be able to determine if targeted G6PC2 inhibition in adults will prevent and/or treat T2D. If there is developmental compensation from conception in G6pc2 KO mice, it is possible that a more severe phenotype in adults may be detrimental such that G6PC2 inhibition by pharmacological agents would be beneficial in decreasing the chances for T2D or a cardiovascular event.

Additionally, as the mechanism that connects G6pc2 to cholesterol metabolism remains unclear, G6pc2 expression in other cell types might explain why C57BL/6J and mixed genetic background KO mice have reduced cholesterol levels. Therefore examining the role of G6pc2 in a β-cell specific KO mouse is pertinent to improved interpretation of these findings.
Analysis of a Secondary Role of G6PC2 in Modulating Calcium Flux: Preliminary Data and Future Directions

Finally, we hypothesize that G6pc2 may have an additional function in pancreatic islet β-cells to modulate calcium flux at submaximal glucose levels. In addition to regulating glucose cycling and modulating the set point for FBG, we hypothesize that G6pc2 could affect calcium retention in the ER mediated through the generation of P_i from the hydrolysis of G6P. Support for this hypothesis comes from studies performed in liver microsomes that showed that G6P and glucose-6-phosphatase activity drives the accumulation of calcium in the ER. Subsequently, there have been several studies looking at the potential role of the liver G6PC1 isoform in modulating ER calcium retention in the liver (Fig. 1.4) [349-353]. Researchers have proposed that ATP dependent ER calcium accumulation is stimulated by G6Pase activity and potentially can regulate calcium-activated exocytosis of insulin granules [352, 353]. Moreover, with increasing concentrations of P_i, as would be predicted with increased hydrolysis of G6P in WT islets, there is a biphasic and P_i-dependent calcium uptake into the ER, consistent with this hypothesis [351]. As it is known that increasing glucose concentrations drives an increase in the ER calcium pool in β-cells [354], it is possible, given this data, that there is a relationship between β-cell G6PC2 activity and calcium flux [355]. Therefore is has been hypothesized that if G6PC2 activity can regulate calcium signaling in β-cells, as G6pc1 has been shown to in the liver, this function could alter the kinetics of GSIS [349, 355].

Preliminary data shown in Fig. 8.1 indicates that there is a selective improvement of glucose tolerance in C57BL/6J G6pc2 Heterozygous (Het) mice using a low 0.75g/kg glucose dose. Similarly I saw that a 2.0g/kg glucose dose in 129SvEv Het mice also improved glucose tolerance (Fig. 8.2). As 129SvEv mice are more insulin sensitive, a 0.75g/kg glucose dose cannot be used in an IPGTT because the blood glucose does not increase to an adequate level to allow us to identify differences between genotypes. The improved glucose tolerance in both C57BL/6J and 129SvEv Het mice suggest that there could be an additional positive function of G6pc2, which, we think, is to modulate ER calcium retention.
We propose that there is not a difference in glucose tolerance between WT and G6pc2 KO mice because, while the WT mice benefit from increased calcium retention, this benefit is offset by reduced glycolytic flux. In contrast, in KO mice the benefit of increased glycolytic flux is offset by the loss of G6pc2 modulating ER calcium levels, leading to similar glucose tolerance relative to WT mice (Fig.8.1&2). However, I further hypothesize in Het mice that a single copy of G6pc2 is sufficient to retain control of ER calcium levels, which is combined with the benefit of a partial decrease in glycolytic flux, leading to improved glucose tolerance. This improvement in glucose tolerance in C57BL/6J Het mice is lost when a high glucose dose (2.0 g/kg) is used (Fig. 8.3), consistent with the role of G6pc2 in modulating ER calcium retention being only important at submaximal glucose concentrations. Also, at a high glucose dose, G6pc2’s ability to modulate glycolytic flux is minimal due to the low Km for G6P. Further experiments need to be performed to determine if these changes in glucose tolerance of Het mice depending on glucose dosage are in fact due to alterations in calcium homeostasis. These studies can be done by analyzing GSIS from isolated islets incubated with varied doses of glucose concentrations. I expect that Het islets would exhibit enhanced GSIS relative to WT and KO at submaximal glucose concentrations (Het>KO>WT), with no observed differences at maximal glucose concentrations.
Figure 8.1. C57BL/6J G6pc2 HET Mice Have Improved Glucose Tolerance Using a 0.75 g/kg Glucose Dose. Het mice have significantly improved glucose tolerance relative to WT and KO mice (p<0.05).

Figure 8.2. 129SvEv G6pc2 HET Mice Have Improved Glucose Tolerance Using a 2.0 g/kg Glucose Dose. Het mice have significantly improved glucose tolerance relative to WT and KO mice (p<0.05).
Figure 8.3. Glucose Tolerance is the Same Between WT, HET and KO C57BL/6J Mice Using a 2.0 g/kg Glucose Dose. Following IP injection of 2.0 g/kg there was no significant difference in glucose tolerance between genotypes.
These studies can then be extended to look at ER calcium oscillations in collaboration with Dr. David Jacobson using an ER-targeted calcium indicator. I expect decreased ER calcium and altered ER calcium oscillations in KO islets, with no difference between Het and WT at submaximal glucose concentrations. By performing these studies, we will better be able to understand the contribution of G6pc2 to both GSIS and ER calcium regulation and whether this explains our observations regarding glucose tolerance in these mice.

**Analysis of a Secondary Role of G6PC2 in Preventing Hypoglycemia: Preliminary Data and Future Directions**

Preliminary BioVU studies done in collaboration with the Denny laboratory have revealed that both non-diabetic and T2D patients with the rs560887-A allele significantly associate with the presence of hypoglycemic events (data in submission). As mentioned earlier, this locus is associated with variation in FBG and, the A allele specifically, is associated with reduced FBG, consistent with these BioVU findings. To extend these findings in mice, we performed preliminary fasting experiments on 18hr fasted mice. We have previously shown that, following a 6-hour fast, FBG levels are reduced in G6pc2 KO mice on a mixed, C57BL/6J and 129SvEv genetic background [69, 96, 265]. In contrast, following an 18-hour fast, FBG levels were not reduced in C57BL/6J G6pc2 KO mice relative to WT mice (Figure 8.4) or in 129SvEv G6pc2 KO mice relative to WT mice (Figure 8.5). This result initially suggested that 18-hour fasting, which represents an extreme physiological challenge to mice that is associated with a ~10% reduction in body weight and near complete depletion of liver glycogen, is not analogous to transient hypoglycemic events in humans since G6PC2 appeared to only influence blood glucose in the latter. However, we noted a slight trend towards increased FPI in both C57BL/6J G6pc2 KO mice relative to WT mice (Figure 8.6) and 129SvEv G6pc2 KO mice relative to WT mice (Figure 8.7). This raised the possibility that an enhanced counter-regulatory response was obscuring the influence of G6pc2 at low glucose levels in mice. Figure 8.8 shows that this is indeed the case in C57BL/6J mice where corticosterone levels are significantly elevated in C57BL/6J G6pc2 KO mice relative to WT.
**Figure 8.4. FBG is Not Reduced in 18hr Fasted C57BL/6J G6pc2 KO Mice.** Mice were fasted for 18hrs when we performed a retro-orbital bleed for glucose measurements n=10-20.

**Figure 8.5. FBG is Not Reduced in 18hr Fasted 129SvEv G6pc2 KO Mice.** Mice were fasted for 18hrs when we performed a retro-orbital bleed for glucose measurements n=10-20.
Figure 8.6. FPI Levels Are Trending Higher in 18hr Fasted C57BL/6J G6pc2 KO Mice. Mice were fasted for 18hrs when we performed a retro-orbital bleed. Samples were submitted to the Vanderbilt Hormone Assay Core for insulin measurements n=10-20.

Figure 8.7. FPI Levels Are Trending Higher in 18hr Fasted 129SvEv G6pc2 KO Mice. Mice were fasted for 18hrs when we performed a retro-orbital bleed. Samples were submitted to the Vanderbilt Hormone Assay Core for insulin measurements n=10-20.
Figure 8.8. 18hr Fasted C57BL/6J G6pc2 KO Mice Have Significantly Elevated Plasma Corticosterone Levels. Mice were fasted for 18hrs when we performed a retro-orbital bleed. Samples were submitted to the Vanderbilt Hormone Assay Core for measurements n=5; p<0.05.

Figure 8.9. 18hr Fasted 129SvEv G6pc2 KO Mice Have Similar Plasma Corticosterone Levels. Mice were fasted for 18hrs when we performed a retro-orbital bleed. Samples were submitted to the Vanderbilt Hormone Assay Core for measurements n=5.
In 129SvEv mice corticosterone levels were much higher than in C57BL/6J mice and no difference was apparent between WT and G6pc2 KO mice (Fig. 8.9). This suggests that G6pc2 influences FBG at low glucose levels in C57BL/6J mice as in humans but in 129SvEv mice this effect is obscured by a strong counter-regulatory response to prolonged fasting. Moreover, this finding demonstrates that G6PC2 variants affect blood glucose set-point in GSIS even in diabetic populations. Future studies should further examine how the absence of G6pc2 drives this counter-regulatory response in KO mice.
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