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Pancreatic islet beta cell-specific deletion of *G6pc2* reduces fasting blood glucose

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Abstract

The *G6PC1*, *G6PC2* and *G6PC3* genes encode distinct glucose-6-phosphatase catalytic subunit (G6PC) isoforms. In mice, germline deletion of *G6pc2* lowers fasting blood glucose (FBG) without affecting fasting plasma insulin (FPI) while, in isolated islets, glucose-6-phosphatase activity and glucose cycling are abolished and glucose-stimulated insulin secretion (GSIS) is enhanced at submaximal but not high glucose. These observations are all consistent with a model in which G6PC2 regulates the sensitivity of GSIS to glucose by opposing the action of glucokinase. *G6PC2* is highly expressed in human and mouse islet beta cells however, various studies have shown trace *G6PC2* expression in multiple tissues raising the possibility that G6PC2 also affects FBG through non-islet cell actions. Using real-time PCR we show here that expression of *G6pc1* and/or *G6pc3* are much greater than *G6pc2* in peripheral tissues, whereas *G6pc2* expression is much higher than *G6pc3* in both pancreas and islets with *G6pc1* expression not detected. In adult mice, beta cell-specific deletion of *G6pc2* was sufficient to reduce FBG without changing FPI. In addition, electronic health record-derived phenotype analyses showed no association between *G6PC2* expression and phenotypes clearly unrelated to islet function in humans. Finally, we show that germline *G6pc2* deletion enhances glycolysis in mouse islets and that glucose cycling can also be detected in human islets. These observations are all consistent with a mechanism by which G6PC2 action in islets is sufficient to regulate the sensitivity of GSIS to glucose and hence influence FBG without affecting FPI.

Key Words

- ▶ diabetes II
- ▶ islet cells
- ▶ metabolism
- ▶ pancreatic beta cell

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Introduction

Glucose-6-phosphatase catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and inorganic phosphate and is located in the endoplasmic reticulum (ER) membrane (Hutton & O'Brien 2009). It is thought to exist as a multi-component enzyme system in which a

G6P transporter, encoded by the *SLC37A4* gene, delivers substrate from the cytosol to the active site of a glucose-6-phosphatase catalytic subunit (G6PC) in the lumen of the ER with transporters for inorganic phosphate and glucose returning the reaction products back to the

cytosol (Hutton & O'Brien 2009). The *G6PC* gene family is comprised of three members, *G6PC1*, *G6PC2* and *G6PC3* (Hutton & O'Brien 2009). *G6PC1*, also known as *G6PC* or *G6Pase*, is predominantly expressed in liver and kidney where it catalyzes the terminal step in the gluconeogenic and glycogenolytic pathways (Hutton & O'Brien 2009). *G6PC3* is widely expressed, with especially high expression in kidney, testis, skeletal muscle and brain (Hutton & O'Brien 2009). While *G6PC3* catalyzes G6P hydrolysis *in vitro* (Shieh *et al.* 2003), its key substrate *in vivo* is thought to be the G6P analog 1,5-anhydroglucitol-6-phosphate (Veiga-da-Cunha *et al.* 2019). Based on Northern blotting and immunohistochemistry, *G6PC2*, also known as *IGRP*, was thought to be only expressed in pancreatic islets in mice (Arden *et al.* 1999) and humans (Martin *et al.* 2001). However, human RNA-Seq data, available through the GTEx database, suggest very low expression of *G6PC2* in other tissues, including liver, skeletal muscle, and hypothalamus, raising the possibility that *G6PC2* is functionally important in non-islet tissues.

The beta cell glucose sensor, glucokinase, catalyzes the formation of G6P from glucose and was thought to be the key factor that determines the rate of beta cell glycolytic flux (Iynedjian 2009, Matschinsky & Wilson 2019). This glycolytic rate determines the sensitivity of glucose-stimulated insulin secretion (GSIS) to glucose and hence the influence of beta cells on fasting blood glucose (FBG) (Iynedjian 2009, Matschinsky & Wilson 2019). However, in isolated germline *G6pc2*-knockout (KO) islets glucose-6-phosphatase activity (Pound *et al.* 2013) and glucose cycling (Wall *et al.* 2015), calculated as the percentage of the G6P generated from glucose that is converted back to glucose, are abolished. This results in a leftward shift in the dose response curve for GSIS (Pound *et al.* 2013) such that under fasting conditions, insulin levels are the same in wild type (WT) and germline *G6pc2* KO mice but FBG is reduced in KO mice (Wang *et al.* 2007, Pound *et al.* 2013). These data challenge the existing dogma and suggest a new paradigm in which a glucokinase/*G6PC2* futile cycle, rather than glucokinase alone, determines the rate of beta cell glycolytic flux and hence the sensitivity of GSIS to glucose. Molecular studies have shown that the 'A' allele of the rs560887 SNP in the *G6PC2* gene reduces *G6PC2* RNA splicing (Baerenwald *et al.* 2013) and hence expression of full-length *G6PC2*. Consistent with the new paradigm, genome wide association studies (GWAS) have linked the rs560887 'A' allele to reduced FBG (Bouatia-Naji *et al.* 2008, Chen *et al.* 2008).

The experiments described here provide further support for this new model by showing that glucose

cycling also exists in human islets and that germline deletion of *G6pc2* enhances glycolysis in mouse islets. In addition, gene expression analyses, electronic health record-derived phenotype analyses, and studies in a novel mouse model in which *G6pc2* is specifically deleted in beta cells all suggest that *G6PC2* is not active in non-islet tissues, and instead imply that an islet-specific action of *G6PC2* is sufficient to mediate the effect of *G6PC2* on FBG.

Materials and methods

Animal care

The Vanderbilt University Medical Center Animal Care and Use Committee approved all protocols used. Mice were maintained on a standard rodent chow diet (calorie contributions: 28% protein, 12% fat, 60% carbohydrate (14% disaccharides); LabDiet 5001; PMI Nutrition International). Food and water were provided *ad libitum*.

Generation of germline *G6pc2* KO mice

The generation of germline *G6pc2* KO mice on a pure C57BL/6J genetic background has been previously described (Wang *et al.* 2007, Pound *et al.* 2013). The generation of beta cell-specific (BCS) *G6pc2* KO mice is described in the Supplementary material (see section on [supplementary materials](#) given at the end of this article).

Mouse and human islet isolation

Mouse islets were isolated by the Vanderbilt Islet Procurement and Analysis Core as previously described (Syring *et al.* 2016). Human islets were obtained by A.C.P. through the NIDDK-funded Integrated Islet Distribution Program (<https://iidp.coh.org/>) and handled and assessed as previously described (Kayton *et al.* 2015). Human islets designated as 'Group 1' (Kayton *et al.* 2015) based on islet perfusion analyses (Supplementary Fig. 4) were used for these studies.

Analysis of gene expression

Tissue RNA was isolated using the ToTALLY RNA kit, whereas islet RNA was isolated using the RNAqueous kit (Ambion, Carlsbad, CA, USA). The Turbo DNA-free DNase Treatment Kit (Ambion) was then used to remove trace genomic DNA followed by cDNA generation using the iScript DNA Synthesis Kit (Bio-Rad). Gene expression was then quantitated by PCR using the dUTP-containing

FastStart SYBR Green Master Mix in conjunction with Uracil-DNA-Glycosylase (Roche). Fold induction of gene expression was calculated using the $2(-\Delta\Delta C(T))$ method (Livak & Schmittgen 2001). PCR primer sequences are provided in the Supplementary material.

Phenotypic analysis of fasted germline and BCS *G6pc2* KO mice

Mice were fasted for 5 h 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). EDTA (5 μ L; 0.5 M) was then added to blood samples prior to isolation of plasma by centrifugation. Insulin samples were assayed using RIA (Morgan & Lazarow 1963) by the Vanderbilt Hormone Assay and Analytical Services Core.

Hematoxylin, eosin and histochemical analysis of tissue morphology

Mice were killed at 8 months of age and the pancreata were formalin fixed. Pancreata were then paraffin embedded and sections cut at a depth of 5 μ m, dewaxed and stained in hematoxylin and eosin by the Vanderbilt Translational Pathology Shared Resource. Details on the analysis of pancreatic cryo-sections by immunofluorescence are provided in the Supplementary material.

Electronic health record (EHR)-based analyses of human research subjects

EHR-based analyses were 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 (Roden *et al.* 2008, Pulley *et al.* 2010). The methods used to perform genome-wide association studies (PheWAS) have been previously published (Denny *et al.* 2010, 2013). The methods used to perform laboratory value-wide association studies (LabWAS) are described in the GitHub repository [<https://bitbucket.org/juliasealock/labwas/src/master/>].

The BioVU sample was restricted to a European American (EA) population ($n=50,115$) and an African

American (AA) population ($n=9640$), based on global genetic ancestry estimated from principal components. We used the PheWAS package in R to perform 1212 logistic regressions to determine whether any Phecodes (hierarchical clustering of International Classification of Disease (ICD) codes) were significantly associated with our SNPs of interest after adjusting for sex, age (defined for each individual as median age across their medical record) and the top four principal components of ancestry (Denny *et al.* 2010, 2013). For each phenotype, we required a minimum number of 100 cases for inclusion in the PheWAS. We used the LabWAS package to perform 453 linear regressions to determine whether any laboratory values were associated with SNP genotype. The SNPs tested were common in both EA and AA populations.

Glucose cycling

Glucose cycling was analyzed as previously described (Wall *et al.* 2015). Briefly, aliquots of ~100 islets were incubated for 24 or 72 h at 28°C in RPMI-1640 medium containing 5 mM or 11 mM glucose in a volume of 175 μ L. Islets were incubated in either naturally labeled glucose or [1,2,3,4,5,6- 2 H₇]glucose (D7-glucose) (98% isotopic purity per site; Cambridge Isotope Laboratories, Inc., Andover, MA, USA). Following the 24- or 72-h incubation, islets were resuspended by pipetting and pelleted by centrifugation. The supernatant was retained for GC-MS analysis of glucose concentration and mass isotopomer distribution, with glucose concentration determined through comparison to a standard curve. Glucose cycling and glucose uptake rates were calculated as described previously (Wall *et al.* 2015).

Glycolysis

Glycolysis was analyzed as described (Ashcroft *et al.* 1972, Wang & Iynedjian 1997, Tamarit-Rodriguez *et al.* 1998). Briefly, aliquots of ~100 WT and germline *G6pc2* KO islets were incubated for 2 h at 37°C in Krebs bicarbonate buffer containing 5.6 mM glucose spiked with 1 μ L D-[5- 3 H]glucose (Perkin Elmer; 10Ci/mmol; 1 mCi/mL) in a volume of 100 μ L. Following the incubation, islets were pelleted by centrifugation. The supernatant was retained for analysis of 3 H₂O generation, whereas DNA content of the cell pellet was measured using the Hoechst reagent (Sigma) as described (Ashcroft *et al.* 1972).

Statistical analyses

Mouse data were analyzed either using a Student's *t*-test, two sample assuming equal variance or one-way ANOVA,

assuming normal distribution and equal variance, as indicated. *Post hoc* analyses were performed using the Bonferroni correction for multiple comparisons. *P* values indicating the level of significance are shown in the figure legends. EHR associations were analyzed with logistic and linear regressions using the PheWAS package in R and the LabWAS package in R. PheWAS and LabWAS results were deemed significant if the *P* value of the association passed a Bonferroni multiple testing correction to account for 1212 logistic and 453 linear regressions that were performed, respectively.

Results

G6pc isoforms exhibit tissue specific expression in male mice

While published data strongly suggest that G6PC2 is active in islets, a major unanswered question is whether it is also active in other tissues. The observation from GTEx data that G6PC2 is expressed at low levels in multiple tissues raises this possibility, though it is important to note that there is currently no evidence for detectable G6PC2 protein expression outside of islets. Whether G6PC2 is functionally active in these tissues is likely to depend in part on its expression relative to other G6PC isoforms. We therefore used real-time PCR to compare the tissue specific expression of mouse *G6pc1* (Fig. 1A), *G6pc2* (Fig. 1B), and *G6pc3* (Fig. 1C), as well as *Slc37a4* (Fig. 1D). Of the tissues tested, *G6pc2* expression was only detected in pancreas (Fig. 1B). In contrast, *G6pc1* was expressed predominantly in liver and kidney (Fig. 1A), whereas *G6pc3* expression was detected in all tissues examined (Fig. 1C). Similarly, expression of *Slc37a4*, which is known to couple with G6PC1 and G6PC3 (Pan *et al.* 2011), was detected in all tissues examined (Fig. 1D). These real-time PCR data are similar to the results of expression analyses for *G6pc1* (Shelly *et al.* 1993), *G6pc2* (Arden *et al.* 1999), *G6pc3* (Boustead *et al.* 2004) and *Slc37a4* (Gerin *et al.* 1997) performed using Northern blotting. These results may indicate that the pattern of *G6pc2* expression differs between mouse and human or, more likely, that this apparent difference simply reflects the increased sensitivity of RNA-Seq versus real-time PCR with the former method being able to detect extremely low expression. For example, RNA-Seq data demonstrate that G6PC2 is expressed in liver at ~0.018% of the level in islets (Ku *et al.* 2012), a difference too small to easily detect using real-time PCR. Even if *G6pc2* is expressed at trace levels in these non-pancreatic tissues, because both

Figure 1

Comparison of tissue-specific *G6pc* isoform expression in male mice. Comparison of *G6pc1* (Panel A), *G6pc2* (Panel B), *G6pc3* (Panel C) and *Slc37a4* (Panel D) expression in brain, heart, kidney, liver, pancreas and testis from 2-month-old non-fasted male mice. *G6pc* and *Slc37a4* expression were quantitated relative to *Ppia* (cyclophilin A) expression in the indicated tissue and then expressed relative to that in either kidney (Panel A) or pancreas (Panels B, C and D). Comparison of *G6pc1*, *G6pc2* and *G6pc3* expression in pancreas (Panel E), islets (Panel F), liver (Panel G) and kidney (Panel H) from 2-month-old non-fasted male mice. *G6pc* expression was quantitated relative to *G6pc2* expression in pancreas (Panel E) or islet (Panel F) or relative to *G6pc1* expression in liver (Panel G) or kidney (Panel H). Results represent the mean data $\pm$ S.E.M. derived from tissues isolated from three male mice.

G6PC1 (Lei *et al.* 1993) and G6PC3 (Shieh *et al.* 2003) have much higher catalytic activity than G6PC2 (Petrotonis *et al.* 2004), these results suggest that G6PC2 is highly unlikely to directly affect metabolism in these tissues.

The relative expression of G6pc isoforms varies within specific tissues in male mice

We next examined the relative expression of *G6pc* isoforms within individual tissues. Within pancreas (Fig. 1E) and islets (Fig. 1F) *G6pc2* expression is higher than that of *G6pc3*, and *G6pc1* expression is not detected. These results suggest that G6PC3 is unlikely to compensate for the absence of G6PC2 in pancreatic islets. Indeed, glucose-6-phosphatase activity (Pound *et al.* 2013) and glucose cycling (Wall *et al.* 2015) are abolished rather than reduced

in germline *G6pc2* KO islets. Similarly, *G6pc1* expression is higher than that of *G6pc3* in both liver (Fig. 1G) and kidney (Fig. 1H) suggesting that G6PC3 is unlikely to be able to compensate for the absence of G6PC1 in either tissue, explaining why inactivating mutations in G6PC1 result in glycogen storage disease type 1a despite the presence of G6PC3 (Chou & Mansfield 2008).

G6pc isoform expression, FBG and FPI change with aging in male mice

To potentially further establish a critical role for pancreatic G6PC2 in the regulation of FBG, we examined the correlation between the induction of *G6pc2* expression during development with the ability of G6PC2 to regulate FBG. *G6pc2* expression is markedly induced during the weaning period between days 12 and 21 after birth relative to *Slc2a2*, which encodes the GLUT2 glucose transporter (Fig. 2A). *Ins2* expression shows a modest induction over

this same time period (Fig. 2B). *G6pc2* expression changes little between day 21 and day 56 (2 months) (Fig. 2A) and remains unchanged even in old mice (17 months) (Fig. 2C). In contrast, expression of *G6pc1* (Fig. 2D) and *G6pc3* (Fig. 2E) decline markedly in liver and kidney in old mice. *G6pc3* expression remains unchanged in other tissues in old mice (Supplementary Fig. 1) while *Slc37a4* expression is selectively reduced in kidney (Fig. 2F). The induction of *G6pc2* expression at P21 did not correlate with a reduction in FBG in germline *G6pc2* KO relative to WT mice; a reduction was only apparent at P56 (Fig. 2G). Since FBG (Fig. 2G) and especially FPI (Fig. 2H) increase markedly between P21 and P56, we hypothesize that the absence of a difference in FBG between WT and germline *G6pc2* KO mice at P21 is simply because the dose response curve for GSIS is at a low point where it is not affected by G6PC2 (Supplementary Fig. 2). A trend toward reduced blood glucose levels is also observed in non-fasted adult *G6pc2* KO mice (Fig. 2I), with no change in plasma

Figure 2

Analysis of changes in *G6pc* isoform expression, FBG and FPI with aging in male mice. Comparison of pancreatic *G6pc2* (Panel A) and *Ins2* (Panel B) expression in P12 (non-fasted) and 6 h fasted P21 and P56 male mice. *G6pc2* and *Ins2* expression were quantitated relative to *Slc2a2* expression and then expressed relative to that at P12. Results represent the mean data $\pm$ s.e.m. derived from pancreata isolated from 3–5 mice. $*P < 0.05$ vs P12, one-way ANOVA. Comparison of *G6pc2* (Panel C), *G6pc1* (Panel D), *G6pc3* (Panel E) and *Slc37a4* (Panel F) expression in pancreas, kidney or liver in 2- versus 17-month-old non-fasted male mice. *G6pc* and *Slc37a4* expression were quantitated relative to *Ppia* (cyclophilin A) expression in the indicated tissue and then expressed relative to expression at 2 months. Results represent the mean data $\pm$ s.e.m. derived from tissues isolated from three male mice. $*P < 0.05$ vs 2 months. Comparison of FBG (Panel G) and FPI (Panel H) in P12 (non-fasted) and 6-h fasted P21 and P56 male WT and germline *G6pc2* KO mice. FBG data represent the mean $\pm$ s.e.m. derived from WT, $n = 10$; KO, $n = 7$ (P12), WT, $n = 9$; KO, $n = 9$ (P21) and WT, $n = 21$; KO, $n = 24$ (P56) mice. FPI data represent the mean $\pm$ s.e.m. derived from WT, $n = 10$; KO, $n = 7$ (P12), WT, $n = 9$; KO, $n = 9$ (P21) and WT, $n = 15$; KO, $n = 23$ (P56) mice. $*P < 0.05$ vs WT. Comparison of glucose (Panel I), insulin (Panel J) and glucagon (Panel K) in 14–16-week-old non-fasted WT ($n = 7$) and germline *G6pc2* KO ($n = 7$) mice. Data represent the mean $\pm$ s.e.m.

insulin (Fig. 2J) or glucagon (Fig. 2K). Since the weaning period is associated with the switch to a relatively high carbohydrate diet, we hypothesize that this induction of *G6pc2* represents part of the beta cell maturation process (Stolovich-Rain *et al.* 2015) and is important for enabling the independent offspring to tightly regulate blood glucose levels.

FBG is reduced in male beta cell-specific (BCS) *G6pc2* KO mice

We reasoned that a second approach for addressing whether G6PC2 is also active in other tissues would be through the generation and analysis of beta cell-specific (BCS) *G6pc2* KO mice. If the effect of *G6pc2* deletion on FBG were retained it would argue that G6PC2 primarily regulates FBG through an islet-dependent mechanism. BCS *G6pc2* KO mice were generated by breeding mice with a floxed *G6pc2* allele in which LoxP sites surround *G6pc2* exon 3, which encodes the third transmembrane domain (Arden *et al.* 1999), with MIP1-*CreERT2* knock in mice in which expression of the *CreERT2* recombinase gene is driven by the endogenous mouse *insulin1* promoter (Thorens *et al.* 2015). Treatment of these mice with tamoxifen allows for the removal of exon 3 from the floxed *G6pc2* allele in beta cells in adult mice thereby inactivating the gene. Sequence analysis indicates that, were splicing of *G6pc2* exon 2 to exon 4 to occur, an in-frame G6PC2 variant lacking only exon 3 encoded amino acids would not be generated.

Neither the presence of LoxP sites in the *G6pc2* gene nor the presence of the MIP1-*CreERT2* gene prior to tamoxifen treatment had major effects on pancreatic *G6pc2*, *Slc37a4* or *Ins2* RNA expression in homozygous floxed relative to WT mice (Fig. 3A, B, C, D, E and F). In contrast, tamoxifen treatment (by three days of IP injections) of homozygous floxed mice expressing the MIP1-*CreERT2* gene led to a selective ~60% reduction in pancreatic *G6pc2* RNA (Fig. 3G, H and I) and protein (Fig. 3J and K) expression in floxed mice relative to WT mice, similar to the efficiency of gene deletion reported by Thorens *et al.* (2015) using these MIP1-*CreERT2* mice.

Neither the presence of LoxP sites in the *G6pc2* gene nor the presence of the MIP1-*CreERT2* gene prior to tamoxifen treatment affected FBG and FPI (Fig. 4A, B, C and D). In contrast, tamoxifen treatment led to a clear reduction in FBG in floxed mice (Fig. 4E) with no change in FPI (Fig. 4F) indicating that the loss of G6PC2 selectively in beta cells is sufficient to regulate FBG. As expected, the difference in FBG between WT and floxed mice (~15 mg/

dL; WT *n* = 14; floxed *n* = 17) (Fig. 4E) is slightly less than that observed in germline *G6pc2* KO mice (~21 mg/dL; WT *n* = 31; KO *n* = 17) (Pound *et al.* 2013), consistent with the difference between a partial reduction in *G6pc2* expression in BCS *G6pc2* KO mice compared to a complete loss of *G6pc2* expression in germline KO mice. These data also suggest that developmental compensation, a common feature in germline KO mouse studies, has not affected the phenotype of germline *G6pc2* KO mice, consistent with the absence of evidence for compensatory changes in pancreatic, islet and liver gene expression comparing WT and *G6pc2* KO mice (Supplementary Fig. 3).

Human biobank studies find no evidence for extra-pancreatic actions of G6PC2

To complement our mouse studies we searched for evidence for potential extra-pancreatic functions of G6PC2 in humans using the Vanderbilt University's Medical Center (VUMC) BioVU Biobank, a DNA biobank linked to a de-identified version of the Vanderbilt electronic health records, called the Synthetic Derivative (SD) (Roden *et al.* 2008, Pulley *et al.* 2010). Systematic and efficient approaches have been developed that involve screening the SD with specific SNPs to identify both novel phenotype-variant associations and plasma hormone/metabolite associations, referred to as PheWAS and LabWAS analyses, respectively (Denny *et al.* 2010, 2011, 2013, Ritchie *et al.* 2013, Shameer *et al.* 2014). For these analyses we used the *G6PC2* rs560887 SNP that has been shown to affect *G6PC2* RNA splicing (Baerenwald *et al.* 2013) and has been linked by GWAS to variations in FBG (Bouatia-Naji *et al.* 2008, Chen *et al.* 2008) and hemoglobin A1c (HbA1c) (Soranzo *et al.* 2010). PheWAS analyses showed that the *G6PC2* rs560887 'A' allele is associated with increased risk for acute pancreatitis in non-diabetic European Americans (EAs) (*G6PC2* OR = 1.39, CI = 1.20–1.62, *P* = 2.05E-05) after Bonferroni correction for the 1212 phenotypes tested (Table 1). Similar, though less statistically significant, trends were observed in the AA sample and in the total (multi-ancestry) population (Table 1). The association with acute pancreatitis was not observed in EAs with type 2 diabetes (T2D) (Table 1) or in African Americans with T2D (AAs; data not shown). Because *G6PC2* is not expressed in pancreatic exocrine or ductal tissue (Hutton & Eisenbarth 2003), this suggests that reduced islet *G6PC2* expression can influence non-islet pancreatic tissue function. *G6PC2* was not associated with T2D in the total population (Table 1). In contrast, BioVU analyses using the established rs13266634 T2D-associated

Figure 3

Analysis of *G6pc2* RNA and G6PC2 protein expression in floxed *G6pc2* male mice. Comparison of pancreatic *G6pc2* (Panels A, D and G), *Slc37a4* (Panels B, E and H) and *Ins2* (Panels C, F and I) expression in 16-week-old 6-h fasted male mice in the presence or absence of the MIP1-CreERT2 allele and in the presence or absence of tamoxifen (Tam) treatment. Pancreatic *G6pc2*, *Ins2* and *Slc37a4* expression were quantitated relative to *Ppia* (cyclophilin A) and then expressed relative to that in WT mice. Results represent the mean data $\pm$ s.e.m. derived from tissues isolated from the indicated number of male mice. Comparison of insulin and G6PC2 protein expression in representative sections of pancreata isolated from WT, germline and BCS *G6pc2* KO mice (Panel J). Quantitation of G6PC2 protein expression (Panel K). Data represent the mean $\pm$ s.e.m. from 100 cells for each group. FL, floxed; KD, knockdown. A full colour version of this figure is available at <https://doi.org/10.1530/JME-20-0031>.

SNP in the *SLC30A8* gene (Yaghootkar & Frayling 2013) did detect an association with T2D (Table 1).

LabWAS analyses showed that the *G6PC2* rs560887 'A' allele was associated with reduced blood glucose (Effect estimate = -0.05, s.e. = 0.01, $P = 6.56 \times 10^{-24}$) in the total population. Similar trends were observed in the total EA population and in non-diabetic EAs but not EAs with T2D (Table 2) or in AAs (data not shown). Given the differences in sample sizes we cannot rule out the possibility that the lack of association in these groups is simply related to power. The *G6PC2* rs560887 'A' allele was also associated

with reduced hemoglobin A1c (HbA1c) but only in non-diabetic EAs (Table 2). These results are consistent with GWAS data (Bouatia-Naji *et al.* 2008, Chen *et al.* 2008, Soranzo *et al.* 2010) and suggest that the influence of G6PC2 on blood glucose and HbA1c is lost under diabetic conditions. Surprisingly, the *G6PC2* rs560887 'A' allele was associated with increased taurine levels in the total population (effect estimate = 0.32, s.e. = 0.08, $P = 7.28 \times 10^{-5}$) with similar trends in the total EA and non-diabetic EA populations (Table 2). It is well established that insulin regulates amino acid metabolism (Rooyackers & Nair

Figure 4

Analysis of FBG and FPI in floxed *G6pc2* male mice. Comparison of FBG (Panels A, C and E) and FPI (Panels B, D and F) in 16-week-old 6-h fasted male mice in the presence or absence of the *MIP1-CreERT2* allele and in the presence or absence of tamoxifen (Tam) treatment. Results represent the mean data $\pm$ s.e.m. derived from the indicated number of male mice. * $P < 0.05$ vs WT.

1997), so the simplest hypothesis is that reduced islet *G6PC2* expression influences the kinetics of insulin secretion which then affects amino acid metabolism. Overall these PheWAS and LabWAS results complement the conclusions of the mouse studies by not finding evidence for extra-pancreatic functions of *G6PC2*.

To follow-up on these observations from human population studies, we examined whether germline deletion of *G6pc2* results in pancreatitis in mice. Pancreatic sections from ~8-month-old 6-h fasted mice showed no evidence of pancreatitis, with no necrosis, edema, hemorrhage, steatosis, or fibrosis within the pancreatic parenchyma and no increase in inflammatory infiltrate compared to WT controls. Furthermore, acinar cell morphology appeared normal with no evidence of degranulation, vacuolization, or luminal

Table 1 Analysis of the association between *G6PC2* SNP rs560887 with disease phenotypes using Electronic Health Record (EHR)-derived analyses. Data show an association between *G6PC2* SNP rs560887 and acute pancreatitis in European Americans (EAs) without T2D and no association with T2D, in contrast to SLC30A8 SNP rs13266634.

Gene	SNP	Population	Phenotype	Description	Beta	s.e.	OR	P	n_total	n_cases	n_controls	n_no_snp	LCI	UCI	Bonferroni	FDR
G6PC2	rs560887	Total	577.1	Acute pancreatitis	0.13	0.05	1.14	0.00709997	37343	411	36932	10	1.04	1.26	FALSE	FALSE
G6PC2	rs560887	All EA	577.1	Acute pancreatitis	0.15	0.05	1.16	0.00606234	31878	339	31539	6	1.04	1.28	FALSE	FALSE
G6PC2	rs560887	EA Non-T2D	577.1	Acute pancreatitis	0.33	0.08	1.39	2.05E-05	21357	145	21212	5	1.20	1.62	TRUE	TRUE
G6PC2	rs560887	EA T2D	577.1	Acute pancreatitis	2.5E-03	0.09	1.00	0.97696475	6155	140	6015	0	0.85	1.19	FALSE	FALSE
G6PC2	rs560887	Total	250.2	Type 2 diabetes	0.01	0.01	1.01	0.43847308	32759	7026	25733	9	0.98	1.04	FALSE	FALSE
SLC30A8	rs13266634	Total	250.2	Type 2 diabetes	-0.06	0.01	0.94	3.56E-05	32782	7035	25747	16	0.92	0.97	TRUE	TRUE

Total refers to the total EHR population. Phenotypes refer to the numerical abbreviation for individual phenotypes in the Vanderbilt EHRs. All associations were adjusted for the covariates median age of record, sex, and EHR-reported race using linear regression. FDR, false discovery rate; LCI, lower confidence interval; n_no_snp, number of records with a missing predictor; UCI, upper confidence interval.

Table 2 Analysis of the association between *G6PC2* SNP rs560887 with laboratory analytes using Electronic Health Record (EHR)-derived analyses.

Gene	SNP	Population	Analyte	Effect Estimate	s.e.	OR	P	n	LCI	UCI	Bonferroni	FDR
<i>G6PC2</i>	rs560887	Total	Blood glucose	-0.05	0.01	0.95	6.56E-24	38301	0.94	0.96	TRUE	TRUE
<i>G6PC2</i>	rs560887	Total	HbA1c glycated	-0.01	0.01	0.99	0.21060335	14328	0.97	1.01	FALSE	FALSE
<i>G6PC2</i>	rs560887	Total	Taurine	0.32	0.08	1.38	7.28E-05	158	1.22	1.53	TRUE	TRUE
<i>G6PC2</i>	rs560887	All EA	Blood glucose	-0.05	0.01	0.95	2.08E-22	32686	0.94	0.96	TRUE	TRUE
<i>G6PC2</i>	rs560887	All EA	HbA1c glycated	-0.01	0.01	0.99	0.15780832	12271	0.97	1.00	FALSE	FALSE
<i>G6PC2</i>	rs560887	All EA	Taurine	0.27	0.08	1.31	0.00171088	132	1.15	1.47	FALSE	FALSE
<i>G6PC2</i>	rs560887	Non T2D EA	Blood glucose	-0.07	0.01	0.93	3.97E-38	21728	0.92	0.94	TRUE	TRUE
<i>G6PC2</i>	rs560887	Non T2D EA	HbA1c glycated	-0.05	0.01	0.96	3.83E-05	4353	0.93	0.98	TRUE	TRUE
<i>G6PC2</i>	rs560887	Non T2D EA	Taurine	0.29	0.09	1.34	0.00243233	114	1.16	1.53	FALSE	FALSE
<i>G6PC2</i>	rs560887	T2D EA	Blood glucose	0.01	0.01	1.01	0.18306124	6444	0.99	1.04	FALSE	FALSE
<i>G6PC2</i>	rs560887	T2D EA	HbA1c glycated	0.02	0.01	1.02	0.18513367	5159	0.99	1.04	FALSE	FALSE
<i>G6PC2</i>	rs560887	T2D EA	Taurine	0.30	0.51	1.35	0.58240028	12	0.36	2.35	FALSE	FALSE

Data show an association between *G6PC2* SNP rs560887 and blood glucose, hemoglobin A1c (HbA1c) and blood taurine in either the total EHR population (Total) or specific European American (EA) sub-populations. No associations were observed in the African American (AA) population or separately in AAs with or without T2D (data not shown). For each laboratory value, we tested for associations between the median of all lab values for an individual against the number of minor alleles for that individual. All associations were adjusted for covariates median age of record, sex, and EHR-reported race using linear regression. The units for all measurements in any given lab were consistent within the lab (e.g. all blood glucose values were reported in mg/dL). FDR, false discovery rate.

dilation (Fig. 5A). However, expression of *Prss1*, which encodes trypsinogen, was elevated in 16-week-old 6-h fasted germline *G6pc2* KO mice (Fig. 5B). The expression of *Ctrb1* (chymotrypsinogen), *Amy2a2* (amylase) and *Cpa1* (carboxypeptidase A1) were unchanged (Fig. 5B) as were *Ins2*, *Slc30a8* (ZnT8) and *Slc37a4*, though *Slc2a2* expression was slightly reduced (Fig. 5C). Surprisingly the opposite result was observed in 16-week-old 6-h fasted BCS *G6pc2* KO mice in which expression of *Prss1* was reduced (Fig. 5D).

Glycolysis is enhanced in germline *G6pc2* KO relative to WT islets

Our model predicts that deletion of *G6pc2* will increase glycolytic flux in islets. While glucose cycling analyses can quantitate G6P production (Wall *et al.* 2015) such experiments do not quantitate the rate of glycolysis. It is important to address this key difference because glycolysis, rather than the rate of G6P production, is the determinant of the magnitude of GSIS (Iynedjian 2009, Matschinsky & Wilson 2019). This distinction arises because G6P has multiple alternate metabolic fates, including conversion to fructose-6-phosphate, glucose-1-phosphate, 6-phosphogluconolactone and inositol-3-phosphate. The well-established glycolytic assay that measures the generation of $^3\text{H}_2\text{O}$ from D-[5- ^3H]glucose (Ashcroft *et al.* 1972, Wang & Iynedjian 1997, Tamarit-Rodriguez *et al.* 1998) was used to compare glycolytic rates in WT and germline *G6pc2* KO islets. Consistent with our model, glycolysis was elevated in KO islets at 5.6 mM glucose (Fig. 6).

Glucose cycling exists in human islets

Using a novel stable isotope-based methodology that eliminates assumptions associated with the use of radioisotopes, we have recently shown that mouse islets exhibit significant rates of glucose cycling (Wall *et al.* 2015). The same methodology was used to address the key question as to whether glucose cycling also occurs in human islets. Kayton *et al.* (2015) have recently used islet perfusion assays to define five patterns of GSIS in human islets, designated as Groups 1 – 5. Group 1 islets exhibit optimal responses to secretagogues, whereas GSIS shows distinct abnormalities in Groups 2–5 islets (Kayton *et al.* 2015). Glucose uptake and cycling were compared at both 5 mM and 11 mM glucose in human islet preparations from four donors that were defined as Group 1 using perfusion assays (Supplementary Fig. 4). Significant rates

Figure 5

Analysis of pancreatitis and acinar gene expression in germline *G6pc2* KO and floxed *G6pc2* male mice. (Panel A) Comparison of pancreatic tissue in WT and germline *G6pc2* KO mice. Results are representative of sections analyzed from three WT and three KO mice. Size bars represent 100 μ m. (Panels B and C) Comparison of pancreatic gene expression in 6-h fasted 16-week-old male WT and germline *G6pc2* KO mice. Gene expression was quantitated relative to *Ppia* (cyclophilin A) and then expressed relative to that in WT mice. Results represent the mean data $\pm$ S.E.M. derived from pancreata isolated from five mice. * $P < 0.05$ vs WT. (Panel D) Comparison of pancreatic gene expression in 6-h fasted 16-week-old male WT and BCS *G6pc2* KO mice following tamoxifen treatment. The RNA samples were a subset of those used in (G), (H) and (I) of Fig. 5. Gene expression was quantitated relative to *Ppia* (cyclophilin A) and then expressed relative to that in WT mice. Results represent the mean data $\pm$ S.E.M. derived from pancreata isolated from five mice. * $P < 0.05$ vs WT. A full colour version of this figure is available at <https://doi.org/10.1530/JME-20-0031>.

of glucose uptake and glucose cycling were detected and were typically greater at 11 mM than 5 mM though, for reasons that are unclear, this was not uniform and the islet preparation that exhibited decreased glucose uptake at 11 mM was distinct from the preparation that exhibited decreased glucose cycling at 11 mM (Fig. 7). At 5 mM glucose the level of glucose uptake inversely correlated with the level of glucose cycling (Fig. 7). Since the rate of glucose cycling is determined by quantitating the glucose isotopomers generated by cycling that are transported out of the islets and back into the media, one possible interpretation of this observation is that in cells with high glycolytic flux, re-cycled glucose preferentially re-enters the glycolytic pathway rather than leaving the cell, leading to an underestimate of the rate of cycling. Another possible interpretation is that the rate of G6P

dephosphorylation is fairly constant despite changes in glucose uptake, so glucose cycling represents a smaller percentage of glucose uptake when the glucose uptake rate is high. The mean rate of glucose cycling is clearly lower in human (Fig. 7) than mouse (Wall *et al.* 2015) islets. This correlates with higher basal GSIS (Conrad *et al.* 2016) and lower *G6PC2* expression (Xin *et al.* 2016) in human versus mouse islets as well as lower FBG in healthy humans (American Diabetes Association 2019) than C57BL/6J mice (Pound *et al.* 2013).

Discussion

The experiments described here provide further support for the concept that G6PC2 activity in pancreatic islets creates a glucokinase/G6PC2 futile cycle that determines

Figure 6

Comparison of glycolytic rates in WT and germline *G6pc2* KO islets. Data show the rates of glycolysis in isolated islets from WT and germline *G6pc2* KO mice at 5.6 mM glucose and represent the mean $\pm$ S.E.M. of 5–7 islet samples. * $P < 0.05$ vs WT.

the rate of beta cell glycolytic flux and hence the sensitivity of GSIS to glucose rather than this rate being determined by glucokinase alone. Specifically, we show that deletion of *G6pc2* enhances glycolysis in mouse islets (Fig. 6) and glucose cycling also exists in human islets (Fig. 7). In addition, gene expression analyses show that G6PC2 is unlikely to be important in non-islet tissues where the more active G6PC isoforms, G6PC1 and G6PC3, are more abundantly expressed (Fig. 1) and that deletion of *G6pc2* specifically in beta cells is sufficient to reduce FBG (Figs 3 and 4). Future studies will be designed to confirm that GSIS is enhanced at sub-maximal glucose in perfused pancreata and isolated islets from BCS *G6pc2* KO mice as previously observed in germline *G6pc2* KO mice (Pound *et al.* 2013).

Figure 7

Comparison of glucose uptake and cycling rates in Group 1 human islets. Data show the rates of glucose uptake (Panel A) and cycling (Panel B) in individual preparations of Group 1 islets at either 5 mM or 11 mM glucose from four donors (designated A, B, C and D). Information on the individual human donors is presented in Supplementary Table 1 and information on the insulin secretory profile of each islet preparation, as assessed by islet perfusion, is presented in Supplementary Fig. 4.

Several interesting observations arose from the human biobank studies. First, we observed a strong association between *G6PC2* and blood glucose levels (Table 2) even though the BioVU population represents individuals who visit VUMC with a wide range of medical issues and who were not all under overnight fasting conditions when blood was isolated. This suggests that G6PC2 can influence blood glucose under non-fasting conditions, consistent with the concept that altered *G6PC2* expression affects the sensitivity of GSIS to glucose over a range of glucose concentrations rather than just at fasting glucose levels (Supplementary Fig. 2). Second, the results show that altered *G6PC2* expression in humans can affect the risk of acute pancreatitis (Table 1) and that deletion of *G6pc2* in mice alters *Prss1* expression (Fig. 5B and D). Since altered *Prss1* expression may affect the risk of pancreatitis (Le Marechal *et al.* 2006, Boulling *et al.* 2016) this provides a potential mechanistic link between these observations. Since *G6PC2* is expressed selectively in pancreatic islet cells (Hutton & Eisenbarth 2003) the mechanism by which it affects *Prss1* expression and especially why the direction of the effect differs with germline and BCS deletion is unclear but will be the subject of future studies. Finally, we observed that *G6PC2* was not associated with altered risk for T2D (Table 1), matching GWAS data that have failed to establish a consistent link with T2D risk (Shi *et al.* 2017). It will be of considerable interest to identify individuals with mutations that have a major effect on G6PC2 function to determine whether an association with T2D risk then emerges. Elevations in FBG in the prediabetic state correlate with (Brunzell *et al.* 1976) and appear sufficient to drive (Brereton *et al.* 2014, Wang *et al.* 2014) the decline in beta cell function, likely mediated by reactive oxygen species (ROS), that underlies the progression from prediabetes to T2D (Weir & Bonner-Weir 2004, Halban *et al.* 2014). However, unless G6PC2 has unknown additional effects on beta cell metabolic flux, a therapy designed to inhibit G6PC2 and thereby lower FBG may fail to prevent this progression because the reduced FBG would be associated with unchanged glycolytic flux and therefore unchanged generation of damaging ROS. Future studies will compare the generation of ROS in WT and KO islets. While a therapy directed at G6PC2 may not affect T2D risk it would likely still be beneficial in preventing cardiovascular-associated mortality (CAM) in individuals with elevated FBG. Thus, in Europeans, an increase in FBG levels from less than 90 mg/dL to between 99 and 108 mg/dL is associated with 30% increased risk of CAM (Coutinho *et al.* 1999), and in Asians a reduction in FBG from 99 to 90 mg/dL is associated with a 25%

reduction in the risk of CAM (Lawes *et al.* 2004). The risk of CAM increases still further in individuals with the high FBG levels characteristic of diabetes (Coutinho *et al.* 1999, Khaw *et al.* 2001, DECODE 2003, Lawes *et al.* 2004).

In mice, *G6pc2* expression is ~20 fold higher in beta than alpha cells (Xin *et al.* 2016), G6PC2 protein is undetectable in alpha cells (Hutton & Eisenbarth 2003), and glucagon levels are unchanged in germline *G6pc2* KO mice (Wang *et al.* 2007). The reduction in FBG in germline *G6pc2* KO mice with unchanged FPI is therefore solely due to the shift in the sensitivity of GSIS to glucose, consistent with our glycolysis and BCS *G6pc2* KO mouse data. In contrast, in humans *G6PC2* expression is only ~5-fold higher in beta than alpha cells (Xin *et al.* 2016) such that altered alpha cell glycolysis could contribute to the reduction in FBG in humans with reduced *G6PC2* expression.

In summary, these studies show that islet-specific G6PC2 activity is sufficient to regulate FBG, although the possibility that G6PC2 plays some role in tissue-specific metabolism outside the pancreas cannot be excluded.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-20-0031>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K J B performed most of the mouse phenotyping studies, gene expression analyses and some of the manuscript writing. M R and M L W performed the human islet glucose cycling studies. K S and S B G performed association studies with EHR data and manuscript editing. J X performed the immunohistochemical studies. K E S performed some of the mouse

studies. J K O performed some of the gene expression analyses. G P performed human islet perfusion analyses. O P M contributed to the design of experiments and manuscript editing. L K D contributed to design of EHR studies and manuscript editing. A C P contributed to design of the human islet glucose cycling studies and edited the manuscript. W H L contributed to design of the immunohistochemical studies. J D Y contributed to design of the human islet glucose cycling studies. R M O performed some of the mouse studies and manuscript writing.

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