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Diabetes genes identified by genome-wide association studies are regulated in mice by nutritional factors in metabolically relevant tissues and by glucose concentrations in islets

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Abstract

Background: Genome-wide association studies (GWAS) have recently identified many new genetic variants associated with the development of type 2 diabetes. Many of these variants are in introns of known genes or between known genes, suggesting they affect the expression of these genes. The regulation of gene expression is often tissue and context dependent, for example occurring in response to dietary changes, hormone levels, or many other factors. Thus, to understand how these new genetic variants associated with diabetes risk may act, it is necessary to understand the regulation of their cognate genes.

Results: We identified fourteen type 2 diabetes-associated genes discovered by the first waves of GWAS for which there was little prior evidence of their potential role in diabetes (*Adam30*, *Adamts9*, *Camk1d*, *Cdc123*, *Cdkal1*, *Cdkn2a*, *Cdkn2b*, *Ext2*, *Hhex*, *Ide*, *Jazf1*, *Lgr5*, *Thada* and *Tspan8*). We examined their expression in metabolically relevant tissues including liver, adipose tissue, brain, and hypothalamus obtained from mice under fasted, non-fasted and high fat diet-fed conditions. In addition, we examined their expression in pancreatic islets from these mice cultured in low and high glucose. We found that the expression of *Jazf1* was reduced by high fat feeding in liver, with similar tendencies in adipose tissue and the hypothalamus. *Adamts9* expression was decreased in the hypothalamus of high fat fed mice. In contrast, the expression of *Camk1d*, *Ext2*, *Jazf1* and *Lgr5* were increased in the brain of non-fasted animals compared to fasted mice. Most notably, the expression levels of most of the genes were decreased in islets cultured in high glucose.

Conclusions: These data provide insight into the metabolic regulation of these new type 2 diabetes genes that will be important for determining how the GWAS variants affect gene expression and ultimately the development of type 2 diabetes.

Keywords: Type 2 diabetes, Gene expression, Genome-wide association, High fat diet, Feeding and fasting, Mice, Pancreatic islets, Glucotoxicity

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Background

Nearly 350 million people world-wide are currently affected by diabetes, and the number of people with type 2 diabetes mellitus is increasing at an alarming rate [1]. Type 2 diabetes results when the β -cells of the pancreas are no longer capable of producing sufficient insulin to meet the body's demands. Thus β -cell dysfunction is a key component of type 2 diabetes pathology. Although the increased prevalence of obesity and resulting insulin resistance is contributing to the increased prevalence of type 2 diabetes, many obese individuals are insulin resistant but do not develop diabetes [2]. Genetic factors, many of which have been proposed to affect β -cell function, play an important role in determining an individual's risk within this context [3-6]. In a small number of individuals, type 2 diabetes is caused by rare single gene mutations, but for most individuals type 2 diabetes results from the combined effects of many common single-nucleotide polymorphisms (SNPs), each of which have a small effect on risk and likely interact with each other and with environmental and lifestyle factors [7].

Genome-wide association studies (GWAS) have recently revealed many novel SNPs associated with type 2 diabetes. These include SNPs located in the regions near *TCF7L2*, *HHEX-IDE*, *EXT2*, *FTO*, *SLC30A8*, *IGF2BP2*, *CDKAL1*, and *CDKN2A-CDKN2B* [8-13]. A second phase of studies identified many additional variants, including those near *JAZF1*, *TSPAN8-LGR5*, *THADA*, *ADAMTS9*, *NOTCH2-ADAM30*, *CDC123-CAMK1D*, and *KCNQ1* [14,15]. The two genes in which common variants were previously convincingly associated with type 2 diabetes, *PPARG* and *KCNJ11*, were also identified in these GWAS [12,16,17]. More recently, numerous other SNPs have been identified in additional GWAS and meta-analyses [18].

The association between variants in *TCF7L2* and type 2 diabetes, which was identified initially in pre-GWAS studies, has been almost universally replicated and has the largest effect on diabetes risk [19,20]. This transcription factor is known to play a role in *WNT* signalling and pancreatic development [20]. Variation in *FTO* has been shown to influence risk of type 2 diabetes through its effects on promoting obesity [8]. Variation within *IDE*, the insulin degrading enzyme, has previously been associated with risk of type 2 diabetes in both humans and rats, although these findings were not consistently replicated [21-26]. *SLC30A8* is a zinc transporter expressed in β -cells and is known to be involved in insulin granule formation and insulin secretion [27-29]. *IGF2BP2* encodes insulin like growth factor 2 mRNA binding protein 2, which plays a role in RNA stability and localization and has been suggested to affect pancreatic development [30]. *NOTCH2* is a transcription factor

important for pancreatic development [31] and the Notch pathway plays a role in adult beta-cell survival [32]. *KCNQ1* is a voltage-gated potassium channel that has been shown to affect insulin secretion [33]. However, the mechanisms by which the remainder of these genes affect diabetes risk are largely unknown. Thus, we sought to obtain evidence of their potential role in metabolic disease.

Most of the diabetes-associated SNPs were found in non-coding regions of the genome and are thus likely to affect gene regulation. In order to understand how these genes affect type 2 diabetes and how the SNPs associated with diabetes affect gene expression, we need to first understand the physiological processes that regulate the expression of these genes. We examined the expression patterns of these potential new diabetes-susceptibility genes to determine which are expressed in tissues important for the development of type 2 diabetes. This may also suggest the potential mechanism(s) by which alterations in these genes affect diabetes risk (e.g. insulin secretion versus insulin sensitivity). We also sought to determine whether any of these genes are regulated by conditions known to alter the expression of metabolically relevant genes. We examined the expression of these genes under fasting and non-fasting conditions (e.g. in response to insulin), which might be altered if they affect peripheral insulin sensitivity. Consumption of diets high in fats and sugars is associated with risk of developing type 2 diabetes [34] and many genes that are critical for β -cell function are regulated by glucose [35]. Thus, we also compared their expression in fasted mice consuming a normal chow diet or a diet high in fat and sugar, and examined the expression of these genes in mouse pancreatic islets cultured under low and high glucose concentrations. Here we show that most of the diabetes-associated genes are expressed in many metabolically relevant tissues and the expression levels of several of these genes were decreased by high fat feeding or were increased in the fed state in the brain. In addition, we found most of these genes are down-regulated by increased glucose concentrations in mouse islets.

Results

Tissue distribution of gene expression

High throughput gene expression profiling has previously been performed to identify gene expression patterns across a wide variety of tissues [36], but such microarray-based data must be complemented by accurate and quantitative analysis. Thus, we performed qPCR analysis of the recently identified diabetes susceptibility genes across a panel of tissues (Table 1) to determine their relative expression levels in each tissue. Most of these genes were expressed in many metabolically relevant tissues including pancreatic islets, liver, white and

Table 1 Relative expression patterns of GWAS diabetes genes in chow fed mice

	Isl	Brain	Hypo	P-AT	BAT	Liver	SkM	Hrt	St	SI	LI	Lung	Kid	Spl	Ov	Ut	Thy
<i>Adam30</i>	NE	Low	NE	Avg	NE	NE	Avg	Avg	Avg	NE	NE	NE	NE	Avg	NE	NE	Avg
<i>Adamts9</i>	Low	Low	Avg	Avg	Avg	Avg	NE	Avg	Avg	NE	Avg	Avg	Avg	Avg	High	High	Avg
<i>Camk1d</i>	Avg	Avg	Avg	Avg	Avg	Avg	Low	Avg	Avg	NE	High	NE	Avg	Avg	Avg	Avg	High
<i>Cdc123</i>	Avg	Avg	Avg	Avg	Avg	Avg	Low	Avg	Avg	NE	Avg	Low	Avg	Avg	High	Avg	Avg
<i>Cdkal1</i>	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Low	Avg	Avg	Avg	Avg	Avg
<i>Cdkn2a (Arf)</i>	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg	Avg
<i>Cdkn2a</i>	NE	NE	NE	Avg	Avg	NE	NE	NE	NE	NE	Avg	NE	Avg	Avg	NE	NE	Low
<i>Cdkn2b</i>	High	Avg	Avg	Avg	Avg	Avg	NT	Avg	NT	High	NT	NT	Avg	NT	High	Low	Low
<i>Ext2</i>	Low	Avg	Avg	Avg	Avg	Avg	Low	Avg	Avg	Avg	Avg	Low	Avg	Avg	High	Avg	Avg
<i>Hhex</i>	Low	Avg	Avg	Avg	Avg	High	Low	Avg	Low	Low	Avg	Low	Avg	High	Avg	High	Avg
<i>Ide</i>	Avg	Avg	Avg	Avg	High	Avg	NT	Avg	NT	Avg	NT	NT	NT	NT	High	Low	Low
<i>Jazf1</i>	Avg	Avg	Avg	Avg	High	NE	NE	Avg	Low	Low	Avg	Low	Low	Low	Avg	High	Avg
<i>Lgr5</i>	NE	High	Avg	Avg	Low	Avg	Low	Avg	Avg	NE	Avg	Low	NE	Avg	High	High	Avg
<i>Thada</i>	Avg	Avg	Avg	Avg	Avg	Avg	NE	Avg	Avg	NE	Avg	Low	Avg	Avg	High	Avg	Avg
<i>Tspan8</i>	Low	Low	Low	Avg	Avg	Low	Low	Avg	High	NE	High	NE	Avg	High	Avg	High	Low

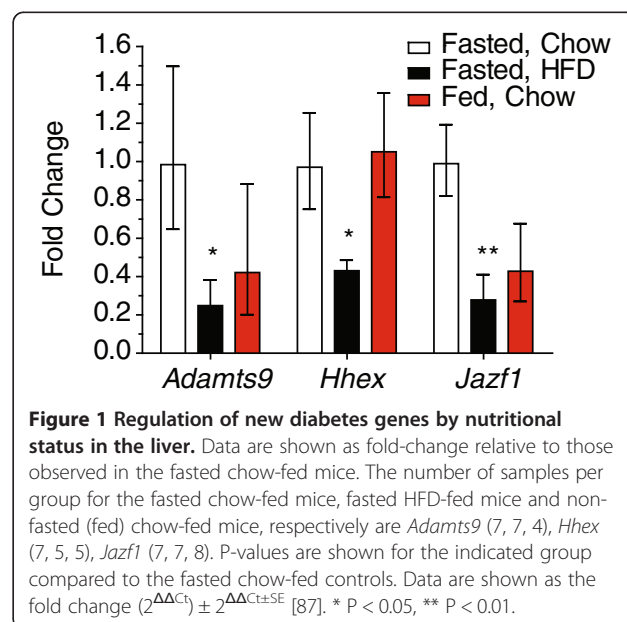
Expression levels are relative within a gene, but comparisons between genes cannot be made. Isl = islets, Hypo = hypothalamus, P-AT = parametrial white adipose tissue, BAT = brown adipose tissue, SkM = skeletal muscle (soleus), Hrt = heart, St = stomach, SI = small intestine (duodenum), LI = large intestine (distal), Kid = kidney, Spl = spleen, Ov = ovary, Ut = uterus, Thy = thymus. NE = not expressed, Ct values within 2 cycles of negative control; avg. = average expression (within 5-fold (2.3 cycles) of average expression across all tissues); high = >5 fold higher (Ct values >2.3 cycles lower) than average expression; low = >5 fold lower (Ct value >2.3 cycles higher) than average expression. NT = not tested.

brown adipose tissue, skeletal muscle, and heart. In addition, most were expressed in the hypothalamus and in regions of the brain outside the hypothalamus. Few of these genes were robustly expressed in skeletal muscle or small intestine. We detected expression of all the genes in pancreatic islets except *Adam30*, *Cdkn2a*, and *Lgr5*. Together, these studies point to several metabolically relevant tissues as potential key sites of action of the diabetes susceptibility genes identified by the GWAS.

Regulation of GWAS diabetes genes by dietary status in the liver

To determine whether these new type 2 diabetes susceptibility genes are regulated by nutritional manipulations, we examined whether there was a change in their expression between tissues from fasted and non-fasted chow-fed mice, or between tissues from fasted chow and fasted high fat diet-fed mice. We used a common control group (fasted, chow-fed mice) to which the non-fasted, chow-fed mice and the fasted, high fat diet-fed mice were compared. As the liver is a key metabolic organ and a major target of insulin action, we first examined the expression of these genes in the liver (Figure 1). The expression of *Jazf1* was decreased by approximately 70% in mice fed a high fat diet compared to chow-fed controls. Similar differences were observed for *Adamts9* (75% decrease) and *Hhex* (60% decrease). Whereas we could detect expression of the *Arf* isoform of *Cdkn2a* in chow-fed mice, its expression was detected in only 2 samples from the high fat diet-fed mice. In

contrast to many classical transcriptional targets of insulin signalling, none of the genes examined in this study had significantly altered expression in the livers of fasted versus non-fasted mice. Again, while it was found in most of the liver samples from the chow-fed fasted control mice, the expression of *Cdkn2a (Arf)* was detected only sporadically in the livers of non-fasted mice. While *Cdkn2b* was expressed in all the samples, its expression did not differ between groups. No differences in



expression were observed for *Camk1d*, *Cdc123*, *Cdkal1*, *Ext2*, *Ide*, *Lgr5*, *Thada*, or *Tspan8* (not shown), although expression of *Cdc123* was decreased ~35% in high fat diet-fed mice ($P = 0.07$). Thus, while the hepatic expression of some of these new diabetes genes is regulated by chronic consumption of diets high in fat and sugar, we do not find evidence that they are acutely regulated in response to feeding and fasting as might be expected for key insulin-responsive genes.

Regulation of GWAS diabetes genes by dietary status in adipose tissue

Adipose tissue is a critical insulin-responsive tissue. Fat also produces many factors, adipokines, that may contribute to whole body insulin sensitivity. We examined whether any of these diabetes susceptibility genes are regulated by nutritional status in adipose tissue (Figure 2). *Cdkn2b* and *Thada* expression levels were decreased ~50 and 30%, respectively, in fasted chow versus high fat diet-fed mice. *Jazf1* expression may also be reduced (~45%, $P = 0.06$, not shown). In contrast *Ide* expression levels were ~1.6 fold higher in high fat-fed mice. The expression of *Adam30* was decreased over 80% in fasted versus non-fasted mice, although this did not reach statistical significance ($P = 0.06$). None of the remaining genes expressed in adipose tissue showed significant changes in expression for either condition (Figure 2), suggesting little involvement of these new diabetes genes in adipose tissue function. This is in contrast to many of the newly identified obesity genes [37].

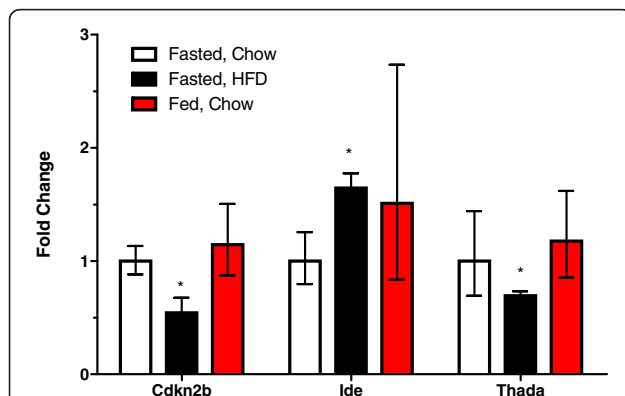


Figure 2 Regulation of new diabetes genes by nutritional status in adipose tissue. Data are shown as fold-change relative to those observed in the fasted chow-fed mice ($n = 7$ for the fasted chow-fed group, 6 for the fasted HFD-fed mice and 8 for the fed chow-fed mice). P-values are for the comparison between the indicated group and the fasted, chow-fed control mice. Data are shown as the fold change ($2^{\Delta\Delta Ct} \pm 2^{-\Delta\Delta Ct \pm SE}$) [87]. * $P < 0.05$.

Regulation of GWAS diabetes genes by dietary status in the brain

Recent studies have shown important neuronal contributions to the regulation of metabolism, insulin sensitivity and islet function, particularly within regions of the hypothalamus [38-41]. Thus, we examined the expression of the recently identified type 2 diabetes susceptibility genes in the hypothalamus (Figure 3) and the remainder of the brain (Figure 4). In the hypothalamus, the expression of *Adamts9* and *Camk1d* were decreased by ~80%, in fasted chow versus fasted high fat diet-fed mice (Figure 3). *Cdkn2b* expression was similarly decreased, although this did not reach statistical significance (not shown). The mRNA levels of *Jazf1* and *Thada* were 60-70% lower in the high fat diet-fed mice. *Lgr5* also tended to be decreased (not shown). In fasted versus non-fasted chow-fed mice, *Cdkal1* expression was decreased by approximately 50% (Figure 3). No significant differences were found for *Cdc123*, the *Arf* isoform of *Cdkn2a*, *Ext2*, *Hhex*, *Ide*, *Lgr5*, or *Tspan8*.

In the remainder of the brain outside the hypothalamus none of the genes were significantly affected by the consumption of the high fat diet, but strikingly many of them were regulated by feeding/fasting status (Figure 4). *Cdkn2b* expression was increased ~3-fold by both conditions, but neither reached statistical significance. Only four genes expressed in the brain, *Cdc123*, *Cdkal1*, *Ide*, and *Thada*, were not affected by either experimental condition. *Camk1d* expression was increased over 7-fold in non-fasted versus fasted chow-fed mice. *Jazf1* and *Lgr5* expression were increased 3.5 and 4.5-fold, respectively, in non-fasted mice. *Ext2* and *Hhex* had a 2 to 2.5-fold increase in expression in fasted versus non-fasted mice. *Tspan8* on the other hand, had a significant 60% decrease in expression in non-fasted versus fasted mice. Like in the liver, the expression of the *Arf* isoform of *Cdkn2a* was low but detectable in both chow-fed groups, but not in the mice consuming the high fat diet. *Adam30* expression was only detected in the brains of non-fasted chow-fed mice. Together, these results illustrate that the expression of several type 2 diabetes susceptibility genes can be metabolically regulated in the brain and point to the potential importance of neuronal function in type 2 diabetes susceptibility.

Regulation of GWAS diabetes genes by glucose in pancreatic islets

Many of the recently discovered type 2 diabetes genes have been suggested to affect the development and/or function of pancreatic islets [6]. The function, growth and survival of β -cells can be regulated acutely and chronically by glucose [34]. Thus, we examined whether the new type 2 diabetes susceptibility genes are regulated by overnight incubation in low (5 mM) or high (25 mM)

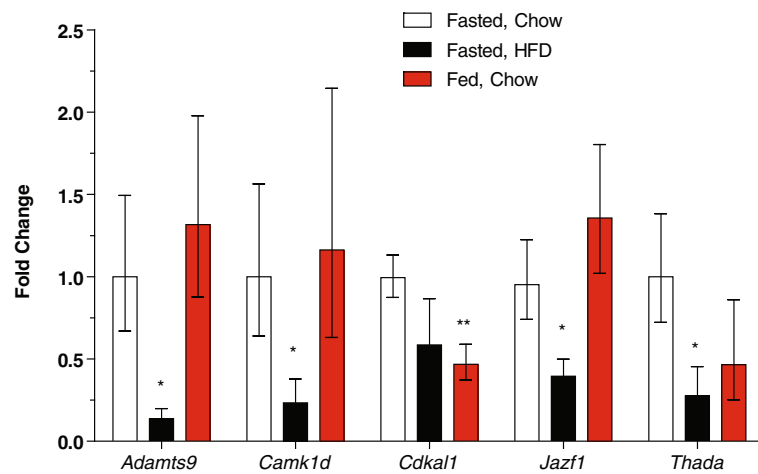


Figure 3 Regulation of new diabetes genes by nutritional status in the hypothalamus. Data are shown as fold-change relative to those observed in the fasted chow-fed mice. The number of samples per group for the fasted chow-fed mice, fasted HFD-fed mice and non-fasted (fed) chow-fed mice, respectively are *Adamts9* (7, 5, 4), *Camk1d* (7, 5, 5), *Cdkal1* (10, 5, 9), *Jazf1* (10, 8, 7), *Thada* (7, 5, 5). P-values are shown for the indicated group compared to the fasted chow-fed controls. Data are shown as the fold change ($2^{\Delta\Delta Ct} \pm 2^{\Delta\Delta Ct \pm SE}$) [87]. * $P < 0.05$, ** $P < 0.01$.

glucose (Figure 5). Most genes were significantly or tended to be downregulated under conditions of high glucose. *Cdkal1*, *Cdkn2a* (*Arf*, $P = 0.07$), *Ide*, *Jazf1*, *Camk1d*, and *Tspan8* ($P = 0.06$) expression levels were decreased ~50-60%. Meanwhile, the expression of *Cdkn2b*, *Hhex* ($P = 0.10$), *Cdc123*, *Adamts9* ($P = 0.09$), and *Thada* were reduced 30-40%. To ensure the islets incubated in high glucose did not have globally decreased expression, we examined the expression of

Txnip, which has been shown to be highly upregulated by glucose [35] and found that its expression was still significantly elevated in the islets cultured in high glucose (Figure 5). Mouse islets consist of β -cells and other cell types. Thus, the MIN6 β -cell line was also examined. We found that all the genes were expressed in this cell line (not shown), although this does not preclude that they also are expressed in other cell types within the islet.

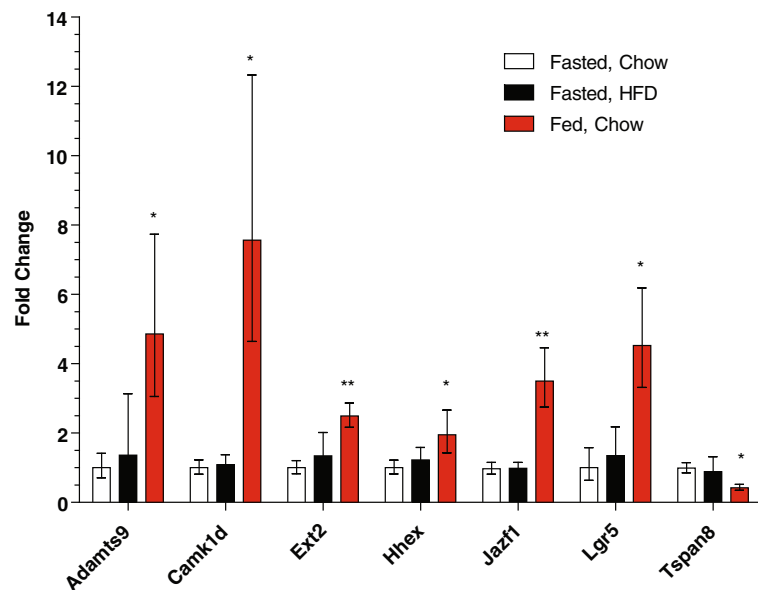


Figure 4 Regulation of new diabetes genes by nutritional status in the remainder of the brain. Data are shown as fold-change relative to those observed in the fasted chow-fed mice. HFD = high fat diet. The number of samples per group for the fasted chow-fed mice, fasted HFD-fed mice and non-fasted (fed) chow-fed mice, respectively are (6, 5, 10) except for *Adamts9* (6, 4, 10). P-values are shown for the indicated group compared to the fasted chow-fed controls. Data are shown as the fold change ($2^{\Delta\Delta Ct} \pm 2^{\Delta\Delta Ct \pm SE}$) [87]. * $P < 0.05$, ** $P < 0.01$.

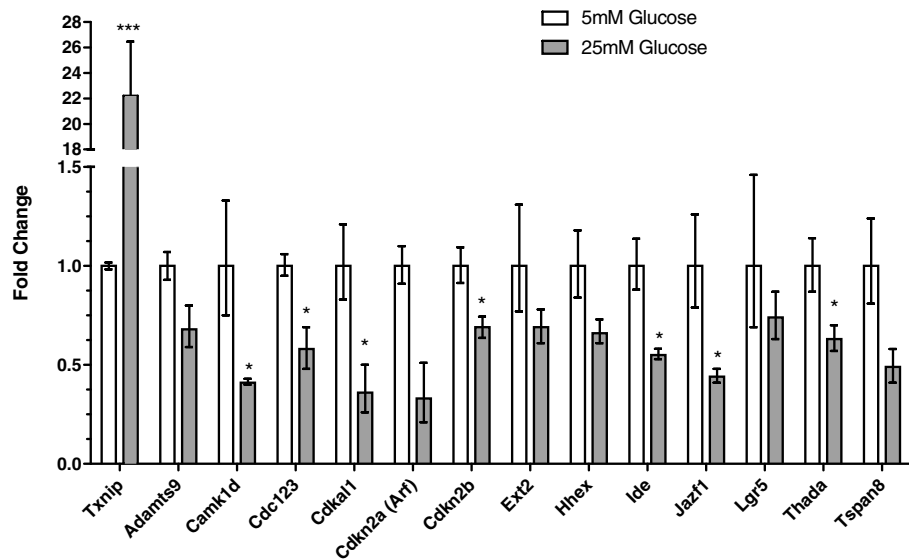


Figure 5 Regulation of new diabetes genes by glucose levels in pancreatic islets. Data are shown as fold-change, $(2^{\Delta\Delta Ct}) \pm 2^{\Delta\Delta Ct \pm SE}$ [87], relative to those observed in the islets incubated in low (5 mM) glucose. Each group is the average of three replicates, each of which was comprised of pooled islets from two mice. * $P < 0.05$, *** $P < 0.001$.

Discussion

The goal of the present study was to understand whether metabolic factors affect the expression of the genes recently implicated in the development of type 2 diabetes for which there was little prior evidence of their potential role(s) in this disease. Although many additional SNPs have been identified in subsequent GWAS and meta-analyses [18], we focussed these studies on the genes identified in the first waves of GWAS, as these have been the subject of most follow-up studies to date. Specifically, we examined acute changes in expression of these genes in response to feeding and fasting and longer term changes in the expression of these genes in response to a diet high in fat and sugar, recognized as a critical environmental risk factor for type 2 diabetes.

It has been hypothesized that most of the new genetic variants affect β -cell function, development or survival but not insulin sensitivity [6]. Consistent with this, we found all of the genes except *Adam30* and *Cdkn2a* were expressed in pancreatic islets. These genes were expressed, however in the transformed β -cell line, MIN6. The expression of all the genes except *Lgr5* decreased following incubation of the islets in high glucose concentrations. It can thus be hypothesized that these genes may normally play a beneficial role in islet function, and a reduction in the expression of these genes could contribute to glucotoxic β -cell dysfunction or survival. However, we also found evidence that most of the genes could have potential roles in other metabolically-relevant tissues. Genes affecting insulin sensitivity may be expected to be expressed in peripheral

insulin sensitive tissues, such as liver and adipose tissue, and be responsive to metabolic status. Consumption of a high fat diet was associated with a tendency for the expression of several of these genes to be decreased. Similarly, many of the genes were regulated by feeding and fasting. Only the two splice isoforms of *Cdkn2a* had no evidence of metabolic regulation in any of the other tissues examined.

Jazf1, also known as *Tip27*, encodes a transcriptional repressor of *Nr2c2*, an orphan nuclear receptor of the steroid receptor family also known as TR4 and TAK1. *Nr2c2* has been reported to modulate apoptosis [42,43] and its loss in mice is associated with reduced mitochondrial function and increased oxidative stress, and conversely with reduced adipose tissue inflammation, hepatic steatosis and insulin resistance [44-46]. *Jazf1*-mediated alterations in *Nr2c2* could thus affect both insulin sensitivity and β -cell function. Genetic variation in *Jazf1* has been variably associated with measures of insulin sensitivity and β -cell function [47-52], and our expression data support roles for this gene in both. *Jazf1* was expressed in nearly all tissues examined and its expression in islets was decreased following culture in high glucose-containing media. Consistent with a pathogenic role in islets, it has recently been shown that *JAZF1* expression is reduced in individuals with type 2 diabetes or hyperglycemia, and that *JAZF1* expression was correlated with insulin secretion [53]. However, our findings suggest the reduced expression may be a consequence of their hyperglycemia, not the underlying cause. These data are consistent, however, with a role of *Jazf1* in

further accelerating β -cell dysfunction once individuals develop hyperglycemia or perhaps impaired glucose tolerance. *Jazf1* expression in the liver and hypothalamus was decreased in mice fed the high fat diet, with the same tendency in adipose tissue. The GWAS SNPs may affect the expression of *Jazf1* in adipose tissue, suggesting that its function in this tissue may be important for its role in type 2 diabetes [54]. That we also observed changes in the expression of *Jazf1* in the brain and hypothalamus, suggests further potentially important sites of action.

Adamts9 is an anti-angiogenic factor known to be expressed in vascular endothelial cells [55] and is implicated in endoplasmic reticulum to Golgi transport [56]. Although some studies have found associations between SNPs in this gene and various measures of insulin sensitivity or secretion [49,50], many others have not [47,48,52,57]. Microvascular structure affects both insulin secretion and sensitivity [58]. *Adamts9* expression tended to be downregulated by high glucose in islets and was decreased in the hypothalamus and liver of high fat diet-fed mice. These data suggest that this gene may play a role in the neural regulation of metabolism in addition to having effects, perhaps on insulin sensitivity, in the liver and also in islets that may be related to its role in vascularization [59].

Hhex encodes a homeobox transcription factor known to be involved in pancreatic and liver development [60,61]. SNPs in *HHEX* have been associated with decreased insulin secretion perhaps due to alterations in vesicle docking [57,62-66]. Most studies have failed to find associations between *HHEX* SNPs and insulin sensitivity [52,57,62-64,67], although associations with insulin clearance and hepatic insulin sensitivity have been reported [52,64]. We found *Hhex* to be expressed in several tissues besides the pancreas, with evidence of decreased expression in the liver in response to high fat feeding and increased expression in the brain in non-fasted mice. These findings provide potential support for roles of *Hhex* in metabolism outside the pancreas. The GWAS SNPs associated with type 2 diabetes are located between *Hhex* and *Ide*. There was prior evidence for a role of *Ide* in type 2 diabetes [21,22,25,26]. We found *Ide* expression to be increased by high fat feeding in adipose tissue and to be decreased by the incubation of islets in high glucose, consistent with a role of *Ide* in β -cell function [63]. Combined, these data suggest potential roles for both *Hhex* and *Ide* in type 2 diabetes susceptibility.

Little is known about the potential roles of *Thada* in metabolic disease. It is a gene associated with a common chromosomal breakpoint in thyroid cancers, that may affect cell death receptors [68]. We found widespread expression of *Thada* and noted its decreased expression in islets following culture in high glucose. *Thada* expression was also reduced in response to high fat feeding in

both adipose tissue and the hypothalamus. No evidence for association of SNPs in this gene with insulin sensitivity have been found [48,49,52,57], and while most studies have not found associations with insulin secretion [47-49,57], an association with reduced insulin secretion in response to non-nutrient secretagogues and potentially β -cell mass has been reported [50]. Our data are consistent with a primary role of this gene in the pancreas in determining type 2 diabetes susceptibility, although indicate it may also have effects on the regulation of energy balance and metabolism.

Although *Adam30* (*A disintegrin and matrix metalloprotease 30*) has been reported to be expressed only in the testis [69], we found evidence of expression in several metabolically relevant tissues including the brain, adipose tissue, heart and stomach. It was not expressed in islets. We observed a marked reduction of its expression in adipose tissue collected from non-fasting animals, providing a potential site of action whereby this gene may affect metabolism and thus type 2 diabetes risk.

In contrast to the above genes, the expression of other genes were not widely altered aside from within pancreatic islets, consistent with the primary mechanism by which they are associated with diabetes being through alterations in islet biology. The mechanisms by which SNPs at the *CAMKID-CDC123* locus affect diabetes susceptibility are unknown, and it is unclear which of these two genes is affected by the causative genetic variation. Some, but not all, studies have found associations of SNPs at this locus with insulin secretion, while no associations with insulin sensitivity have been found [47-50,52,57]. There is evidence that genetic variation near *CAMKID* can affect its expression, at least in lymphocytes [54]. The expression of both genes was similarly reduced in islets cultured in high glucose, suggesting the possibility that they are under common regulatory control in these cells. We found decreased expression of *Camk1d* in the hypothalamus of high fat-fed mice and increased expression in other regions of the brain in non-fasted mice, suggesting it may affect the neuronal control of metabolism or islet function. In contrast, no substantial changes in *Cdc123* expression in response to feeding and fasting or high fat diet consumption were observed. As the SNPs at this locus are primarily associated with insulin secretion and the expression of both genes in islets was altered, these data cannot distinguish which may be the causative gene.

SNPs within *CDKAL1* have been associated with insulin secretion and not insulin sensitivity [13,47,52,57,63,66,67,70]. *Cdkal1* is a tRNA modification enzyme. Specifically, this protein is a methyltransferase that modifies tRNA^{Lys}, stabilizing interactions between the tRNA and mRNA, decreasing misreading of its cognate codon [70]. Mice deficient in *Cdkal1* have impaired glucose tolerance and insulin secretion, and

evidence of β -cell ER stress [71,72]. We found that *Cdkal1* expression in pancreatic islets was decreased following incubation in high glucose, which could contribute to β -cell dysfunction in settings of hyperglycemia. Interestingly, we also found that *Cdkal1* expression was reduced in the fed state in the hypothalamus, suggesting it may have metabolic functions in addition to those in insulin synthesis and secretion.

The GWAS have identified SNPs at the *Cdkn2a-Cdkn2b* locus. The *Cdkn2a* (cyclin dependent kinase inhibitor 2a) gene has two alternative splice isoforms that encode distinct proteins, Cdkn2a and Arf. The Arf isoform is generated by the use of an upstream alternative first exon. Both are involved in cell cycle control. We found expression of the *Arf* isoform to be very low in chow-fed mice and not detectable in most tissues from high fat diet-fed mice. We found higher levels of expression of this gene in islets, and potential downregulation of its expression by high glucose concentrations. In contrast, the *Cdkn2a* isoform was found in a limited number of tissues and not in islets. These data suggest that Arf might be the relevant isoform of *Cdkn2a*, affecting diabetes by affecting β -cell mass. Given the loss of expression of this gene in high fat diet-fed mice it is tempting to speculate that this may be a mechanism affecting high fat diet-induced metabolic dysfunction. The expression of *Cdkn2b* was decreased in adipose tissue of high fat-fed mice. As this gene encodes a cell cycle inhibitor, this reduced expression may reflect increased proliferation of adipocyte precursors or perhaps infiltrating inflammatory cells. Interestingly, *Cdkn2b* expression was also decreased in islets incubated in high glucose, consistent with a role in the regulation of β -cell mass and glucose-induced β -cell proliferation [73]. Thus, as with the *Hhex-Ide* locus, these studies cannot distinguish whether *Cdkn2a* or *Cdkn2b* is the causative gene, and actually suggest a role for both in the development of type 2 diabetes.

Ext2 is a glycosyltransferase involved in the synthesis of heparin sulphate, and mutations in this gene are associated with abnormal bone growths (exostoses) [74]. This gene may also be involved in neural development [75]. The association between SNPs in this gene and type 2 diabetes has not been as well replicated [10,12,13,62]. We found increased expression of this gene in brain, suggesting a possible site of action as to where this gene could affect diabetes risk.

Lgr5 is a seven transmembrane receptor and a member of the rhodopsin family [76]. It is a marker of mitotically active intestinal stem cells and potentiates Wnt/ β -catenin signalling [76,77]. This is the only gene for which we did not find a significant decrease in expression in islets cultured in high glucose, although this certainly does not preclude it from having a role in pancreatic and β -cell development. Its expression was increased in the brains of non-fasted mice, suggesting

another potential site of action through which it may mediate type 2 diabetes susceptibility.

Tspan8, also known as Co-029, is a cell surface protein implicated in pancreatic, colon and liver tumors and their metastasis, possibly through interaction with integrins [78]. Although some studies have found associations between SNPs in this gene and insulin sensitivity or secretion [47,57], others have not [48-50,52]. Loss of this gene is associated with decreased body weight, although there were no detectable effects on glucose tolerance or insulin sensitivity [79]. In contrast to that study which did not detect expression of this gene in mouse pancreas [79], we found expression of this gene in isolated pancreatic islets and suggested regulation of its expression by glucose. *Tspan8* expression was significantly decreased in brains of fed compared to fasted chow-fed mice, suggesting it may also have a role in the neural control of metabolism.

In summary, we have identified nutritional regulation of many of the newly found type 2 diabetes-associated genes. As these studies were performed with a relatively small number of samples, it should be noted that smaller changes in expression may also exist that we had insufficient power to detect. These data provide support for the involvement of these newly identified type 2 diabetes susceptibility genes in β -cell function and also suggest potential roles for many of them in peripheral tissues, notably in the brain and hypothalamus, highlighting the potential importance of neuronal regulation of metabolism and islet function to type 2 diabetes [38-41]. Our study also highlights the tissue-specific regulation of these genes (changes in one or more tissues where the gene is expressed but not in all tissues), suggesting that the SNPs identified in the GWAS studies may need to be examined in the appropriate tissues and under several metabolic contexts [37]. Indeed, recent studies aimed at identifying genetic variants that affect gene expression (eQTLs) have found varying effects of these SNPs on gene expression in different tissues, particularly for SNPs located within not between genes, and notably that the SNPs were more associated with expression of diabetes-associated genes in metabolically relevant tissues such as liver, adipose and muscle than in lymphocytes, which are sometimes used as a surrogate because they are easily accessible [80-82]. The abundant regulation of these genes by nutritional status found in our study also suggests there are likely gene-diet interactions involving these SNPs [83] that may be a complicating factor in future human studies to assess the functional implications of the associated SNPs.

Conclusions

As SNPs may affect the regulation of genes up to 1 Mb away [84], future studies should examine the regulation

of other genes in the region of the associated SNPs to identify other possible candidates. Future studies should also examine the regulation of the remaining newly discovered type 2 diabetes-associated genes and their neighbours. Studies to discover how the type 2 diabetes-associated GWAS SNPs affect the regulation of nearby genes to promote diabetes will be important to realize the value of the GWAS studies, however our findings suggest that such studies will need to be carried out in the appropriate tissues and under controlled environmental conditions.

Methods

Animals

Female C57BL/6 J mice were housed in an environmentally controlled facility with 14 hour light cycles (7 am - 9 pm) with unlimited water and were fed either a standard rodent chow (LabDiet 5010, Jamieson's Pet Food Distributors, Delta, BC, Canada) or a diet containing 60% calories from fat (primarily lard) and 20% calories from sugar (sucrose and maltodextrin; D12492, Research Diets, New Brunswick, NJ) from weaning. Mice were sacrificed at 8 weeks of age by CO₂ asphyxiation. Mice were sacrificed either after a physiological 4 hour fast (9 am - 1 pm) or at 9 am without fasting, as indicated. Tissues were rapidly collected and flash frozen in liquid nitrogen. All procedures were approved by the UBC Committee on Animal Care and were performed according to Canadian Council on Animal Care guidelines.

Pancreatic islet isolation

Islets were isolated from chow-fed female C57BL/6 J mice by collagenase digestion, using previously described modifications [32] of the filtration method reported by Salvalaggio et al. [85]. We handpicked islets into dishes of RPMI media (Invitrogen, Burlington, ON, Canada) containing either 5 mM glucose or 25 mM glucose. Although the mice were not fasted prior to islet isolation, the islets were incubated in these conditions overnight at 37°C and 5% CO₂ prior to RNA isolation. Experiments were performed in triplicate; each replicate comprised of 50 islets from each of 2 mice, for a total of 100 islets per replicate.

Measurement of gene expression

RNA extraction and cDNA synthesis were performed as described [37]. Primers were designed for all the genes from the first waves of type 2 diabetes GWAS for which there was no other evidence of their potential role in type 2 diabetes. Primers were designed to span an intron and to be located in exons common to all isoforms for any genes with alternatively spliced forms, except for *Cdkn2a* which has two well characterized isoforms, *Arf* and *Cdkn2a*, for which specific primers were generated.

The primer sequences used for each gene are provided in Additional file 1: Table S1. Because these studies were performed over a span of several months, and in conjunction with other studies [37], the sample numbers varied between genes (e.g. due to the addition of new samples or when samples were completely used).

Gene expression was assessed by real-time quantitative reverse transcription PCR (qPCR) using SYBR Green I-based detection (PerfeCTa SYBR Green FastMix, Quanta Biosciences, Gaithersburg, MD), as previously described [37]. We confirmed that only a single product was amplified from all samples through melt-curve analysis in addition to the direct visualization of PCR amplification products on an agarose gel prior to real-time analysis. *Gapdh* was selected as the reference gene as its expression was more consistent than β -actin (*Actb*), Cyclophilin (*Ppib*), *Arbp*, and *18S* RNA across the different experimental conditions and tissues.

Delta Ct (Δ Ct) values were calculated by subtracting the cycle threshold (Ct) value for each gene from the Ct value of the control gene amplified contemporaneously. Delta delta Ct ($\Delta\Delta$ Ct) values for each sample were calculated by subtracting the Δ Ct of each sample from the average Δ Ct of the fasted mice consuming chow (control) group or the islets incubated in 5 mM glucose. For each gene, negative controls included both a no reverse transcriptase (no RT) and no template control (water).

For the tissue distribution, genes were considered expressed if the Ct value was at least 2 cycles lower (i.e. higher expression) than the lowest value in the negative controls, which was typically undetected. To examine the relative expression levels of each gene across the tissues in which it is expressed, we calculated the average Ct value for all the tissues that each gene is expressed in. Tissues with at least a 5-fold higher (2.3 cycles lower) expression than the average across tissues are shown as relatively "high" expression of that gene, while tissues where expression was more than 5-fold lower (Ct values > 2.3 cycles higher) than the average are indicated as having relatively "low" expression of that gene. For each gene, these calculations permit the comparisons of the same gene between tissues, however due to likely variation in primer efficiency, comparisons cannot be made between genes.

Statistical analysis

The study was comprised of three groups: two experimental groups (non-fasted chow-fed and fasted high fat diet-fed) each compared to a single control group (fasted chow-fed). Changes in gene expression within liver, adipose tissue, brain and hypothalamus, were compared using non-parametric Mann-Whitney U-tests for each of the experimental groups compared to the controls

because of the small sample sizes [37]. This approach was chosen over the Kruskal-Wallis test because the comparison between the fasted high fat diet-fed and non-fasted chow-fed mice was not meaningful, as both fasting and diet conditions differed between these two groups. Unadjusted P-values are presented [86]. Comparisons between islets incubated in low and high glucose were performed by Student's *t*-test. Statistics were performed on the $\Delta\Delta C_t$ values, prior to conversion to fold-change [37,87] using Prism (GraphPad Software). Data are shown as fold change, calculated as $2^{\Delta\Delta C_t}$, with the upper and lower limits calculated as $2^{\Delta\Delta C_t \pm \text{its standard error}}$, respectively [87].

Additional file

Additional file 1: Table S1. Primer sequences.

Abbreviations

Ct: Cycle threshold; GWAS: Genome-wide association; HFD: High fat diet; qPCR: Quantitative reverse transcription real time PCR; SNP: Single nucleotide polymorphism.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

MH, PY, KC and SK performed the experiments. SC conceived of and designed the study. MH and SC analyzed and interpreted the data. Some of the experiments were performed in the laboratory of JJ. All authors contributed to the writing of the manuscript and approved its final version.

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