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Identification of Differentially Expressed Genes and Processes in Myocardial Tissue of Liver-Specific *Gck* Knockout Mice

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Abstract

Background:

Diabetic cardiomyopathy is a ventricular disease caused by diabetes mellitus. Abnormalities in the function of the glucokinase (GCK) play an important role in the development of diabetes. The present study is aimed at exploring changes in gene expression and related molecular mechanisms of diabetic myocardial injury in *Gck* knockout mice.

Methods:

Liver-specific glucokinase gene knockout mice (*Gck*^{w/-}) and wide type (*Gck*^{w/w}) mice generated using the Cre-loxP gene targeting strategy of 30- and 60-weeks of age were used in these studies. Determination of liver glucokinase enzyme activity, liver glycogen content and serum biochemistry parameters reflect the metabolic disorder in these mice. Echocardiography and surface electrocardiographs were used to evaluate cardiac function. Superoxide dismutase activity and malondialdehyde levels reflect oxidative stress in the myocardium. RNAseq, GO enrichment analysis and qPCR were used to detect differences in the myocardial gene expression profiles of *Gck*^{w/-} and *Gck*^{w/w} mice.

Results:

Hyperglycemia and insulin resistance induced by decreased liver glucokinase expression and enzyme activity throughout the life of heterozygous *Gck* knockout mice do not yield body weight significant difference. However, prolonged PR interval and QRS duration, decreased left ventricular diameter and increased thickness of the posterior wall of the left ventricle were accompanied by increase of PAS and Masson positive substances in the myocardium of 60-week-old *Gck* knockout mice. RNAseq analysis showed that genes related to the myosin heavy and light chains, insulin signaling pathway and oxidative phosphorylation were significantly differentially expressed between 60-week-old *Gck*^{w/-} and *Gck*^{w/w} mice. Phosphorylation of AMPK β 1 and ACC in 60-week-old *Gck* knockout mice was decreased.

Conclusions:

Liver-specific *Gck* knock-out can induce myocardial fibrosis at an early stage and diabetic myocardial injury at late stage. Through this process, the proportion of myosin heavy chain and light chain falls out of balance, the insulin signal pathway becomes down regulated and mitochondrial oxidative stress is up regulated, leading to myocardial disease.

Keywords: glucokinase, Diabetic cardiomyopathy, myosin, insulin signaling pathway, oxidative phosphorylation

1. Background

Diabetic cardiomyopathy (DCM) is a ventricular disease caused by diabetes mellitus, which leads to extensive focal necrosis of the myocardium due to microvascular lesions and metabolic disorders[1]. The structural changes of diabetic cardiomyopathy are characterized by increased left ventricular weight and wall thickness, myocardial hypertrophy and fibrosis, and cardiac myocyte fat deposition[2]. The functional changes are characterized by impaired diastolic function and inconspicuous systolic function, and decreased ventricular wall elasticity[3]. Many factors play important roles in the pathogenesis of DCM, which is very complex. Hyperglycemia, hyperlipidemia and hyperinsulinemia lead to the changes in the function of downstream transcription factors leading to changes in gene expression[4]. The changes are not mutually exclusive and may affect the development of cardiomyopathy through synergistic effects, including changes in effect energy metabolism, electrophysiological properties of the myocardium, and local hormone activation [5]. To date, the complete pathogenesis of DCM has not been thoroughly described.

Abnormalities in the function of glucokinase(GCK) play an important role in the development of diabetes[6]. Glucokinase catalyzes the conversion of glucose to glucose 6-phosphate in the liver cells, which is the rate limiting step in glucose metabolism in these cells, thus, a decrease in glucokinase activity leads to decreased insulin action and increased glucose levels in the blood[7]. The expression and activity of *Gck* is also reported to be decreased in spontaneously diabetic mice [8], and changes in the *GCK* gene and activity play important roles in the pathogenesis of diabetes in humans[9].

The present study was designed to identify differentially expressed genes, and molecular mechanisms, associated with diabetic myocardial injury in the cardiomyocytes of GCK knockout mice. Here, we used liver-specific glucokinase gene knockout (*Gck*^{w/-})mice that were previously generated using a Cre-loxP gene targeting strategy[10]. RNAseq was used to characterize changes in gene expression in myocardial cells of *Gck*^{w/-} mice. Analysis of this data revealed several key

genes and networks among the transcripts influenced by *Gck* knock out. In addition, by profiling selected important genes, and their functions, we identified specific cardiac gene expression pattern features of the *Gck*^{w/-} mice. Overall, these approaches uncover a new gene expression pattern that is induced in the liver-specific *Gck* knockout, as well as its influence on DCM, which should provide theoretical support for early screening for the diagnosis of diabetic cardiomyopathy, as well as a new target for drug therapy.

2. Methods

2.1 Animals

Liver-specific glucokinase gene knockout mice were previously generated using a Cre-loxP gene targeting strategy by our lab[10]. Liver-specific glucokinase gene knockout (*Gck*^{w/-}) mice were obtained by cross breeding mice, whose glucokinase gene was flanked by loxP sites, with the *Alb-Cre* transgenic mice. *Gck*^{w/-} knockout and wide type (*Gck*^{w/w}) mice at 30 and 60 weeks of age were used in these studies (n = 3-6 as stated in the figure legends). A protocol for these experiments, following the “Guidelines for Animal Experiments”, was approved by the Peking University Health Science Center.

2.2 Glucokinase enzyme activity

100 mg of mouse liver tissue was homogenized in pre-cooled buffer solution containing 100 mM KCl, 25 mM HEPES, 7.5mM MgCl₂, and 4 mM dithiothreitol (pH 7.4) and the enzyme was released from the extract after being left at 4 °C for 5 hours. After centrifugation at 4 °C for 5 min at 3,000 rpm, the supernatant was carefully extracted to form the crude extract. A equal volume of 100 or 0.5 mM glucose reaction buffer was then added and preheated at 30 °C for 15 minutes. To measure GCK enzyme activity 0.2 unit of glucose-6-phosphate 1-dehydrogenase was immediately mixed and the absorbance of the extract was measured at 340 nm for 10 minutes. Enzyme activity is determined by measuring the increase of absorbance at 340nm in a unit of time, and is expressed as mU/mg protein.

2.3 Liver glycogen content

The anthrone method was used to determine liver glycogen content[11]. Liver tissue was prepared and washed with 0.9% NaCl. After weighing, about 100 mg liver tissue was treated by boiling for 30 min in alkaline solution and cooled on ice. A detection buffer containing concentrated sulfuric acid and anthrone was then added followed by boiling for 5 min. Blue compounds generated were then assayed with a spectrophotometer at 620 nm.

2.4 Serum biochemistry parameters

Before measurement of serum biochemistry, mice were fasted for 8h. Blood samples, 100 μ l, were collected from the eyes. Fasting blood glucose levels were measured with a Roche blood glucose monitor (Glucotrend 2, Roche, Germany), while plasma triglyceride (TG) and total cholesterol (TC) (Nanjing Jiancheng Bioengineering Institute, CHN) levels were assayed by enzymatic methods.

To determine intraperitoneal glucose tolerance (IPGTT), blood was taken from the tail vein of mice 8 hours after fasting, and blood glucose levels were measured by a Roche blood glucose monitor (Glucotrend 2, Roche, Germany). Mice were then injected with 2 g/kg glucose intraperitoneally. Blood glucose levels were measured at 30, 60 and 120 min after injection, and the IPGTT blood glucose values were plotted and the area under the curve (AUC) was calculated.

Fasting insulin levels were quantified using a commercially available radio immune assay kit (China Institute of Atomic Energy, CHN). Fasting blood glucose and serum insulin were measured, and the homeostasis model assessment (HOMA) index[12] was calculated to evaluate peripheral insulin resistance and the function of β -cells.

The following formula was used:

$$[\text{HOMA-IR}] = \text{fasting blood glucose (mmol/l)} \times \text{fasting serum insulin (mIU/l)} / 22.5.$$

$$[\text{HOMA-}\beta\text{-cell}] = 20 \times \text{fasting blood insulin (mIU/l)} / (\text{fasting serum glucose (mmol/l)} - 3.5).$$

2.5 Echocardiography

Transthoracic echocardiography was performed on anesthetized mice using a high-resolution

imaging system for small animals (Vevo 770, VisualSonics, CAN), equipped with a high-frequent ultrasound probe (RMV-707B). Under the guidance of long-axis images of the left ventricle, the maximum left ventricular diameter (the level of papillary muscle) displayed an M-mode image, the left ventricular diameter and wall thickness were measured, and left ventricular function was analyzed. Ejection fraction(EF) and fractional shortening (FS) were calculated using the Vevo770 software. The final data represent the averaged values of 3–6 cardiac cycles.

2.6 Electrocardiographic recordings

Surface electrocardiographic (ECG) recordings were obtained from conscious mice. The onset and the offset times of P, Q, R, S, and T waves were measured, and the ECG parameters were analyzed from lead II. Since the length of the QT interval is affected by the heart rate, corrected QT (QTc) intervals were calculated using the following formula[13].

$$QTc(sec)=QT(sec) / RR^{1/2}(sec) \text{ (RR, R-R interval)}$$

2.7 Cardiac histological analysis

Hematoxylin and eosin (HE), trichrome Masson, and periodic acid-schiff (PAS)stains were used to evaluate the heart pathology of the mice. After euthanasia, the heart was immediately removed, and a portion of each heart was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. These sections were stained with HE, Masson and PAS to visualize tissue fibrosis. The extent of fibrosis in the myocardial tissue sections was quantified using Image-Pro Plus 6 (Media Cybernetics, USA)as the relative area of positive stained area (PAS: purple red-stained glycogen; Masson: blue–green fibrosis) normalized to the total tissue area[14].

2.8RNAseq

RNAseq was used to determine whether there were differences in the levels of gene expression in different samples. mRNA was purified with poly-T oligo-attached magnetic beads from 3μg total RNA per sample (60-week-old *Gck^{w/-}* and *Gck^{w/w}* mice). Single-stranded cDNA was synthesized using random hexamers as primers with reverse-transcriptase, and then double-stranded cDNA was synthesized using DNA polymerase I. AMPure XP beads were used to

select the fragment size, and the cDNA library was enriched by PCR. RNAseq was performed on an IlluminaHiseq 2000 platform. Clean reads were obtained after removing reads with adapters, removing reads with an N ratio greater than 10%and removing low quality reads. The threshold for differential gene screening was set to $|\log_2(\text{Fold change})|>0.58$ with a corrected P value <0.001 .

2.9 GO enrichment and PPI analysis of differentially expressed genes

Gene Ontology(GO) terms of cellular component, molecular function and biological process in DAVID[<http://david.abcc.ncifcrf.gov/home.jsp>] were employed to categorize the enriched biological themes of the differential expression genes. GO terms with corrected P value less than 0.05 were considered significantly enriched in the differential expressed genes.PPI analysis of the differentially expressed genes was based on the STRING database(<http://string-db.org/>), which contains known and predicted Protein-Protein Interactions(PPI). The networks were imported into Cytoscape for further analysis and visualization.

2.10Quantitative RTPCR

Total RNA was extracted from mouse myocardium tissues using Trizol Reagent (Invitrogen, USA) and reverse-transcribed into cDNA by using Prime Script 1st Strand cDNA Synthesis Kit (Takara, CHN). Quantitative RT PCR was performed with the StepOne™ System with PowerUp™ SYBR® Green Master Mix (ABI, USA). Amplifications were performed using the following conditions with the primer list in Table1: initial denaturation at 95°C for 10 min followed by 39 cycles performed at 95°C for 15 s and 54°C, 57°C or 60°C for 1 min. Transcription levels were normalized to those of β actin.

2.11Western Blot

Total protein was extracted from mouse liver and myocardium tissue homogenate and its concentration was determined by the Bradford method. Sixty micrograms of protein was separated on SDS-PAGE and transferred by electroelution onto PVDF paper (Millipore, USA).Blots were probed with antibodies to insulin receptor β (INSR β), protein kinase B(AKT), adenosine 5'-monophosphate-activated protein kinase α (AMPK α), Phospho-AMPK α (Thr172),AMPK β 1/2,

Phospho-AMPK β 1 (Ser108), acetyl-CoA carboxylase (ACC), Phospho-ACC (Ser79)(the above antibodies were purchased from Cell Signaling Technology (Beverly, USA)) and myosin light chain 2(MYL2)(Santa Cruz Biotechnology, USA) with incubation overnight at 4°C. Antibody binding was detected after incubation with an appropriate secondary antibody conjugated with horseradish peroxidase (HRP), with the membrane-bound antibodies visualized by luminal chemiluminescence ChemiDoc XRS(Bio-Rad, USA).

2.12 Myocardial tissue SOD activity and MDA levels

Frozen left ventricular myocardium samples were weighed and homogenized (1:10, w/v) in 50 mmol/l phosphate buffer (pH 7.4) and kept in an ice-bath. Superoxide dismutase (SOD) activities and malondialdehyde(MDA) levels in the myocardial tissues were determined using commercially available kits (NanjingJiancheng Bioengineering Institute, China).

2.12 Statistical analysis

Differences between the $Gck^{w/-}$ and $Gck^{w/w}$ groups were determined by analyses with one-way ANOVA with SPSS 13.0 software. P values less than 0.05 were considered to be statistically significant. Experimental data are expressed as means $\pm$ SD.

3. Results

3.1 GCK activity and glycogen content is decreased in the liver of $Gck^{w/-}$ mice

To confirm the effect of the liver-specific deletion of glucokinase, we measured liver glucokinase protein expression, glucokinase activity and glycogen content, which is an approximate measure of glucokinase function. Results of our western blots show decreased expression(Figure1a and b) and activity of glucokinase (Figure1c) in the liver of $Gck^{w/-}$ mice and indicate that the Gck knockout mice were heterozygous and the glucokinase alleles in the liver were only partially deleted.GCK catalyzes the conversion of glucose to glucose-6-phosphate, which is then the substrate for glycogen synthesis. When liver tissue lysates were assayed for glycogen content, the Gck gene knockout resulted in 43% (30weeks) and 33% (60weeks)

decreases in glycogen levels (Figure 1d). This result can be explained by the reduction in glucose-6-phosphate levels caused by the *Gck* knockout.

Compared with *Gck*^{w/-} mice at 30 weeks of age, the liver GCK protein expression, GCK activity and the content of glycogen of the livers of 60-week old *Gck*^{w/-} mice were not significantly different, but were significantly lower than those of wild-type (*Gck*^{w/w}) mice of the same age. This shows that the effect of the *Gck* gene knockout in the liver of mice persists with an increase in age.

3.2 Metabolic disorders and insulin resistance in *Gck*^{w/-} mice

To determine the physiological consequences of liver-specific *Gck* knockout, glucose metabolism, body weight, serum glucose, insulin, total cholesterol and triglyceride were investigated. To investigate long-term changes due to *Gck* gene knockout on body weight and serum glucose, we compared the weights and fasting glucose levels of *Gck*^{w/-} mice to *Gck*^{w/w} maintained on normal chow diets. In the *Gck*^{w/-} mice, fasting glucose was elevated at 6 weeks compared to control littermate wild-type (*Gck*^{w/w}) mice, and this was maintained until 70 weeks (Figure 2a). Despite this, changes in body weights were not statistically different in the *Gck*^{w/-} mice, compared to age matched *Gck*^{w/w} mice, from 2 to 70 weeks of age (Figure 2b).

Glucose tolerance in the 60-week-old mice was also significantly worse than in the *Gck*^{w/-} mice compared to their *Gck*^{w/w} littermate controls. Fasting blood glucose levels and the 30, 60, and 120 minute blood glucose levels after a glucose load were significantly higher in the *Gck*^{w/-} mice than in *Gck*^{w/w} littermate controls, however, no significant difference in blood glucose levels was seen among the wild-type mice of different ages, or among the *Gck*^{w/-} mice of different ages (Figure 2c and d). With the results of the IPGTTs, we calculated the area under the blood glucose curves. A significant increase was seen in the *Gck* knock-out group compared with their littermate control group in the 30- and 60-week-old mice (Figure 2e).

Insulin is an anabolic hormone and its secretion is stimulated by hyperglycemia. To determine whether long-term changes in glucose metabolism affect insulin secretion and induce insulin resistance, fasting insulin levels were measured and HOMA-IR and HOMA- β -cell values were calculated. There was no significant change in serum insulin levels in the mice of different ages or different genotypes (Figure 2f). In the 60-week-old mice, HOMA-IR levels were significantly

higher and HOMA- β -cell levels significantly lower in the $Gck^{w/-}$ compared to $Gck^{w/w}$ mice. In the 30-week-old mice, only the HOMA- β -cell levels were significantly lower in the $Gck^{w/-}$ mice (Figure 2g-h). These results indicated that the long-term Gck gene deletion and hyperglycemia gradually cause insulin resistance in peripheral tissues.

Taking this into account, we conclude that glucose homeostasis and insulin sensitivity were affected by the Gck gene deletion, while total cholesterol and triglyceride display no significant changes (Figure 2i,j). These data demonstrate that the $Gck^{w/-}$ mice had a glucose metabolism disorder and insulin resistance.

3.3 Age-dependent cardiac hypertrophy occurs in $Gck^{w/-}$ mice

To determine the effect of Gck knockout on the heart in mice, we examined Gck liver-specific knockout ($Gck^{w/-}$) and wild-type ($Gck^{w/w}$) mice by echocardiography, electrocardiogram and cardiac histological analysis. At 30 weeks of age, only the left ventricle (LV) internal dimension during systole (LVID;s) was significantly decreased in the $Gck^{w/-}$ mice, compared to $Gck^{w/w}$ mice. At an age of 60 weeks, the LV posterior wall thickness during systole (LVPW;s) has also significantly increased in $Gck^{w/-}$ mice, compared to wild-type littermates ($P < 0.001$). Similarly, significant reduction of LVID and an increase of LVPW during diastole were observed (Figure 3a-g). Myocardial hypertrophy was followed by electrophysiological disorders at the age of 60 weeks, with the $Gck^{w/-}$ mice developing remarkably longer PR and QRS intervals compared to $Gck^{w/w}$ mice (Figure 3h-k). In $Gck^{w/-}$ mice, significantly increased levels of collagen (Masson positive material) and glycoproteins (PAS positive material) were detectable, as assessed by tissue structural analyses in 60-week-old mice. In contrast, only increased levels of glycoproteins could be detected in the 30-week-old $Gck^{w/-}$ mice (Figure 3l-n). Thus, we conclude that Gck deletion in the liver causes age-dependent heart dysfunction with a disease course going from glycoprotein aggregation and left ventricular cavity reduction to significant hypertrophy and fibrosis.

3.4 Analysis of the differences in gene expression in the myocardium of $Gck^{w/-}$ and $Gck^{w/w}$ mice by RNAseq

To elucidate the cellular mechanisms underlying age-dependent heart dysfunction in $Gck^{w/-}$

mice, we applied high-throughput RNAseq to identify the myocardium gene expression profiles in 60-week-old *Gck^{w/w}* and *Gck^{w/-}* mice. Expression was detected for 12,680 unique genes, with fold change in expression defined as the ratio of *Gck^{w/-}* FPKM to *Gck^{w/w}* FPKM. Based on a threshold of total fold change of >1.5 and a *P* value <0.001 , we identified 68 up-regulated and 141 down-regulated genes in *Gck^{w/-}* compared to *Gck^{w/w}* mouse myocardium tissue (Figure 4a). A total of 143 proteins encoded by the differentially expressed genes interact closely with each other, of which MT-ND1, INSR, SRF, VEGFA, RPS3, B2M, ASB2, RPL13A, MT-CO3, MYH6, SLC4A1, TTN, NPPA, MYL7, EDNRA, FURIN, HBA-A1, OBSCN, MT-ATP6 and NDUFA4 are in the top 20 genes in Betweenness Centrality (Table 2).

To analyze the functional significance of the proteins with changed expression corresponding to the *Gck* knockout, we used the STRING programs to identify functional networks, which were visualized with Cytoscape. Figure 4b provides a graphical overview of the STRING results and indicates that the changed behavior in response to *Gck* knockout is observed for different groups of proteins. Using the GeneMANIA Cytoscape plugin gene function prediction, the protein interaction network can be seen as mainly divided into four subgroups, which are related to (1) myocardial cell development and function, (2) material metabolism, (3) mitochondria oxidative phosphorylation and (4) ribosomal function (Figure 4b). This phenotype is supported by the GO analysis.

To further categorize biological processes, we classified the differentially expressed genes using the Functional Annotation Cluster (FAC) tool available in DAVID. GO analysis of the 68 up-regulated genes revealed a focus on 4 biological processes, 1 molecular function and 7 pathways as defined by DAVID bioinformatics (*P* values <0.05). From these results, it can be seen that the GO terms are enriched in genes with functions necessary for mitochondria oxidative phosphorylation such as electron transport chain, cytochrome-c oxidase activity, oxidative phosphorylation and respiratory electron transport ATP synthesis, all of which were up-regulated (Figure 4c). Similarly, 18 biological processes, 7 molecular functions and 6 pathways were enriched by the 141 down-regulated genes. The major processes enriched by lower levels of gene expression in the *Gck^{w/-}* mice include GO terms related to myocardial cell development and function (such as muscle structure development, muscle contraction, and cardiac muscle contraction) and material metabolism (such as insulin receptor substrate binding, hexokinase

activity, insulin signaling pathway, and type II diabetes mellitus)(Figure4d-e).

3.5 Conformation of the abnormality of myocardial expression profiles in *Gck^{w/-}* mice

Changes in the expression profiles of genes related to myocardial cells were confirmed by qPCR. qPCR results show that the gene expression levels of *Myh7* (Myosin heavy chain 7), *Myh6* (Myosin heavy chain 6), *Popdc2* (Popeye domain containing 2), *Myom1* (Myomesin 1), *Cmya5* (Cardiomyopathy associated 5), *Myo18b* (Myosin XVIIIb), *Xirp1* (Xin actin-binding repeat containing 1, also named *Cmya1*(Cardiomyopathy associated 1)), *Mypn* (Myopalladin) and *Myom2* (Myomesin 2) were significantly decreased in the 60-week-old *Gck^{w/-}* mice. In contrast, the gene expression levels of *Myl2* (Myosin light chain 2) and *Myl6* (Myosin light chain 6) were significantly increased in the 60-week-old *Gck^{w/-}* mice compared to wild-type mice (Figure5b). These qPCR results are consistent with the RNAseq results.

Western blot analysis showed that the expression of MYC2 was significantly increased in 60-week-old *Gck^{w/-}* mice compared to the control group, and that MYC2 was increased in 30-week-old *Gck^{w/-}* mice, however, there was no significant difference with *Gck^{w/w}* mice of the same age (Figure5c and d). The trends in MYC2 protein gene changes are consistent.

3.6 Metabolic and insulin pathways are impaired in *Gck^{w/-}* mice

Genes for confirmation were selected based on their perceived importance and likely functions for material metabolism. qPCR results show that the gene expression levels of *Snca* (Alpha-synuclein), *Gys1* (Glycogen Synthase 1), *Slc4a1* (Solute carrier family 4member 1), *Hk1* (Hexokinase1), *Hk2* (Hexokinase2), *Pik3r1* (Phosphatidylinositol3-kinaseregulatory subunit1 (p85alpha)), *Insr* (Insulin receptor), *Bpgm* (2,3-bisphosphoglycerate mutase), *Vegfa* (Vascular endothelial growth factor A) and *Ednra* (Endothelin receptor type A) were significantly decreased in the 60-week-old *Gck^{w/-}* mice compared to wild-type mice (Figure6b). These qPCR results are consistent with the RNAseq results. Among these genes, the Betweenness Centrality of *Insr* (0.171432) ranked first, so we examined the expression levels of proteins upstream and downstream of INSR and studied the state of the insulin signing pathway, which plays an important role in the pathogenesis of type 2 diabetes mellitus. At 60 weeks of age, IR β and Akt

expression was robustly decreased in the *Gck*^{w/-} mice compared to wild-type mice, which was paralleled by significant decreases in ACC and AMPK β 1 phosphorylation. In contrast, no significant changes in the levels of PI3K, mTOR or AMPK α phosphorylated proteins were observed. Interestingly, in the 30week-old mice, the above-mentioned proteins of the insulin signaling pathway were found at comparable levels in both *Gck*^{w/-} and wild-type mice, suggesting that normal insulin signaling existed in 30-week-old *Gck*^{w/-} mouse myocardial tissue. (Figure6 c-e).

3.7 Mitochondrial dysfunction and oxidative stress occur in *Gck*^{w/-} mice

Genes related to mitochondrial function were also confirmed by qPCR. qPCR results show that the gene expression levels of *Ndufa3* (NADH dehydrogenase1 alpha subcomplex3), *Ndufa4* (NADH dehydrogenase1 alpha subcomplex 4), *Ndufb4* (NADH dehydrogenase1 beta subcomplex 4) and *Tomm7* (Translocase of outer mitochondrial membrane 7 homolog) were significantly increased in the 60-week-old *Gck*^{w/-} mice compared to wild-type mice (Figure7b). These qPCR results are consistent with the RNAseq results. In the 60-week old mice, MDA concentrations were significantly higher and SOD activity and TAOC content significantly lower in the *Gck*^{w/-} compared to the *Gck*^{w/w} mice. In 30-week-old *Gck*^{w/-} mice, only TAOC content was significantly lower (Figure7c-e).

4. Discussion

4.1 *Gck* knockout induced metabolic disturbances and cardiac dysfunction

Glucokinase is mainly expressed in β cells of pancreatic islets and hepatocytes and catalyzes the phosphorylation of glucose to glucose 6 phosphate, the initial and rate-limiting enzymatic step in glucose metabolism. In mammals, it is a key component of the glucose sensing system and plays a very important role in maintaining the stability of blood glucose levels[15]. The primary role of glucokinase in the liver is to increase the use of glucose by this tissue, especially in postprandial and hyperglycemic conditions, to reduce blood glucose levels and increase glycogen synthesis[16].

We used a Cre-loxP gene targeting strategy to generate liver-specific *Gck* knockout mouse[10]

where liver GCK activation is profoundly impaired (30–50%) and blood glucose homeostasis is disrupted. Western blot analysis showed that GCK expression in the liver is reduced in *Gck*^{w/-} mice. At the same time, enzymatic activity of GCK is also decreased in these *Gck* gene knockout mice. Results from our *Gck* knockout mice showed that heterozygous disruption of *Gck* results in a 42% and 31% reduction in liver GCK activity at 30 and 60 weeks, respectively. Additionally, it is interesting that the reduction in hepatic GCK activity is substantially greater than the reduction in the GCK protein levels. This suggests that some of the synthesized GCK proteins, despite retaining immunogenicity, exhibit abnormal function with loss of enzymatic activity in the *Gck*^{w/-} mice. Glucokinase, a rate-limiting enzyme in glycogenesis, plays a key role in glucose metabolism. Hepatic glycogen assay results showed that the glycogen content of livers is significantly decreased in *Gck*^{w/-} mice due to the glucokinase deficiencies. This indicates that glucokinase plays an important role in glucose homeostasis and its dysfunction can lead to impaired glycogenesis followed by high levels of blood glucose[17].

The *in vivo* knockout of *Gck* yields hyperglycemia in mice aged 6 to 70 weeks but does not result in a significant body weight change. However, the induced hyperglycemia selectively impaired the pancreatic islet cells, causing insulin resistance with an increased HOMA-IR and a decreased HOMA- β cell. Based on these results we speculate that the *Gck* knockout in the hepatocytes leads to higher plasma glucose concentrations and insulin resistance throughout the life of the heterozygous *Gck* knockout mice.

The current study found strong support for an association between HOMA-IR and diabetic cardiomyopathy[18], and that insulin resistance is associated with diastolic dysfunction and the risk of heart failure[19]. Abnormal HOMA index and peripheral insulin resistance in the 60-week-old *Gck* knockout mice may lead to myocardial damage and cardiac dysfunction. The cardiac function and state of the *Gck* knockout mice were studied by surface electrocardiogram (ECG), cardiac M-mode echocardiography and morphological methods. P wave of the surface electrocardiogram reflects atrial depolarization, PR interval reflects the time from atrial depolarization to ventricular depolarization, and QRS waves reflect the process of ventricular depolarization. Prolongation of the PR interval time and QRS duration is associated with left ventricular hypertrophy[20]. The PR interval and QRS duration of the 60-week-old *Gck* mice were significantly longer than those of the same aged wild-type mice, suggesting that left ventricular

hypertrophy may be present in the 60-week-old *Gck* knockout mice. The results of the M-mode echocardiography showed that the left ventricular diameter decreased and the thickness of the posterior wall of the left ventricle increased in the 60-week-old *Gck* knock-out mice. In the 30-week-old *Gck* knockout mice, only the left ventricular diameter during systole was shortened. The 60-week-old *Gck* knockout mice had hypertrophy and disorder of cardiac myocytes and increased deposition of PAS and Masson positive substances in cardiac tissues. PAS positive material deposition was observed in the 30-week-old *Gck* knockout mice, but at a lower level than seen in the 60-week-old *Gck* knockout mice. Our results demonstrate that there is fibrosis in the 60-week-old *Gck* knockout mice, which further supports the existence of DCM that develops with age in this *Gck* knockout mice model.

4.2 *Gck* knockout induces changes in the myocardial protein expression profile

Genes associated with myofibrils are involved in myocardial contraction and affect the severity of heart disease[21]. Sarcomeres are the basic units of striated muscle and make up the myofibril, which is a highly ordered assembly of three components: actin, myosin, and skeletal proteins[22]. Among these proteins, myosin is a large molecular dynamic protein, which through interactions with actin participates in many cellular processes including myocardial contraction[23]. Myosin is a multimeric protein consisting of two myosin heavy chain subunits and four myosin light chain subunits. Studies have shown that the proportion of light and heavy chains of myosin affects the assembly and function of myosin[24]. Our RNAseq analysis indicates that an abnormal proportion and remodeling of the myosin heavy and light chains occurred with the diabetic cardiomyopathy seen in the *Gck* knockout mice.

Myosin light chain2 (MYL2) is the regulatory light chain of myosin, which induce structural changes and affects myocardial contraction through phosphorylation. It has been shown that *Myh2* expression in the myocardium of diabetic rats is upregulated[25], which is consistent with our observations in mice. Although the mechanism is not clear, we speculate that the increase in MYL2 levels in 60-week-old *Gck* knockout mice affects myocardial fiber assembly leading to myocardial hypertrophy. Increased production of advanced glycation end products (AGEs) secondary to hyperglycemia in diabetic cardiomyopathy can lead to increased myocardial stiffness[4]. AGEs can lead to the cross-linking of collagen molecules, which inhibits the

degradation of collagen, thus increasing fibrosis and an increase in myocardial stiffness and impairment of cardiac diastolic function. In addition, the oxidative stress of diabetes leads to increased expression of the receptor for AGEs in the heart, which in turn increases the stiffness of the myocardium[4].

MYH6 and MYH7 are the two main myosin heavy chain proteins, with MYH6 mainly involved in the rapid contraction of myocardium, and MYH7 in the maintenance of myocardial tone[26]. As a structural protein, MYH7 is the main component protein of the thick filament and plays an important role in myocardial contraction. Mutations in *Myh7* gene are known to increase left ventricular wall thickness in hypertrophic cardiomyopathy[27]. The MYOM protein, located in the thick muscle filaments, is an important structural and regulatory protein. MYOM cross-links with myosin to form antiparallel dimers, which promote the elasticity of the thick filaments. During myofibro genesis, MYOM interacts with myosin through the my1 and my2 domains to promote myosin aggregation and participate in the correct localization of myosin and MYOM[26]. CMYA1 and CMYA5 belong to cardiomyopathy associated gene families, and CMYA1 plays an important role in cardiac development, cardiac remodeling and myocardial injury. *Cmya* encoded proteins are located in the intercalated disc, a structure specific to the heart muscle, and are involved in cell-to-cell communication and signaling[28]. Abnormal expression of *Cmya1* may be associated with cardiac hypoplasia and primary heart disease[29]. As our results show, the downregulation of *Myh*, *Myom* and *Cmya* may lead to disorder of the myocardial fiber structure and lead to the thickening of the left ventricular wall.

The *Popdc* gene family members have similar structures and functions, as they encode a class of membrane-bound cyclic AMP effector proteins. Both *Popdc1* and *Popdc2* are highly expressed in the myocardium, and *Popdc1* is also expressed in skeletal muscle[30]. *Popdc1* and *Popdc2* deletion variants were found to be associated with arrhythmias such as long QT syndrome, sinus bradycardia and atrio ventricular block in animal models such as mice and zebrafish, as well as in human patients[31]. Null mutations in *Popdc1* increase the sensitivity to oxidative stress and ischemia-reperfusion injury in myocytes[32], suggesting that POPDC is an essential protein for the maintenance of cardiac function and has a protective effect on the myocardium. The prolongation of the PR interval and QRS interval in 60-week-old *Gck* knockout mice may be related to the downregulation of *Popdc* expression in myocardium. These results suggested that

changes in the myocardial related gene expression profiles cause cardiac dysfunction in diabetic cardiomyopathy.

4.3 Metabolic abnormalities and mitochondrial dysfunction are associated with DCM induced by the *Gck* knockout

The presence of INSR and INSR substrates (IRS) in cardiac myocytes, along with their downstream signaling proteins, constitutes a complex signal transduction pathway that regulates insulin action and glucose utilization[33]. Glucose is an important source of energy for the heart muscle[34]. Normally, blood glucose and insulin levels rise, and INSR activation causes the translocation of GLUT4 to the membrane, increasing glucose uptake within the cell. Glucose and fat are the main energy sources of myocardium. Insulin resistance leads to the decrease of glucose utilization, myocardial energy deficiency and cardiac dysfunction[35]. At the same time, insulin resistance is closely related to myocardial hypertrophy and myocardial fibrosis[36].

Damage to any part of the insulin signaling pathway can lead to insulin resistance. Our study found that factors related to the insulin signaling pathway including *Insr*, phosphatidylinositol 3-kinase regulatory subunit (*Pik3r1*), glycogen synthase (*Gys1*), hexokinase 1(*Hk1*) and hexokinase 2(*Hk2*) were down-regulated in the myocardium of *Gck* knockout mice. INSR and AKT protein expression levels were significantly reduced in the 60-week-old *Gck*^{w/-} mice. AMPK is an energy sensor that regulates glucose and fatty acid metabolism in mammalian cells[37]. AMPK is a heterotrimer that includes catalytic α and regulatory β and γ subunits, with phosphorylation of the $\beta 1$ subunit necessary for AMPK activation. Phosphorylated AMPK inhibits fatty acids and glycogen synthesis and promotes the uptake of glucose. Acetyl CoA carboxylase (ACC) catalyzes the carboxylation of acetyl CoA to form malonyl CoA, a key step in fatty acid synthesis. AMPK phosphorylates ACC, thus inhibiting the role of ACC and fatty acid synthesis[38]. The phosphorylation of AMPK $\beta 1$ and ACC in our 60-week-old *Gck* knockout mice was decreased. These results suggest that the down regulation of the insulin signaling pathway and the up regulation of NADH dehydrogenase, combined with changes in blood glucose and insulin levels, leads to abnormal metabolism in the myocardium, which is mainly seen as a decrease in glucose uptake and increase in fatty acid synthesis, leading to myocardial toxicity and cardiac dysfunction in 60-week-old *Gck* knockout mice. These results indicate that the abnormalities of

insulin resistance and the insulin signaling pathway have important significance in the occurrence and development of diabetic cardiomyopathy.

Mitochondria account for 35-40% of the volume of mammalian cardiomyocytes[39]. The primary role of mitochondria in cardiomyocytes is to generate ATP, which provides energy for cardiac exercise and is accompanied by the formation of reactive oxygen species (ROS) and participates in the metabolism of glucose and free fatty acids in the myocardium. Our RNAseq results show that the genes related to mitochondrial function are upregulated in the course of diabetic cardiomyopathy, of which *Ndufa3*, *Ndufa4*, *Ndufb4* and *Tomm7* were verified by qPCR. NDUF A3, NDUF A4 and NDUF B4 are subunits of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I)[40], which functions in the transfer of electrons from NADH in the respiratory chain[41]. GO functional enrichment analysis of the RNAseq data showed that ATP biosynthesis and oxidative phosphorylation were up-regulated significantly in 60-week-old *Gck* knockout mice. Partially consistent with gene expression, oxidative stress is increased by deletion of *Gck*, as reflected by an increased MDA content and decreased SOD and TAOC activity. During ATP synthesis and oxidative phosphorylation, superoxide anion and peroxide are produced[42]. There is increasing evidence that diabetic cardiomyopathy (DCM) is associated with mitochondrial dysfunction and increased production of ROS [43]. Impaired insulin signaling, high glucose and hypoxia can affect the function of mitochondria and lead to the development of oxidative stress in the myocardium[44]. Cardiac dysfunction may be due to abnormal oxidative phosphorylation in heart muscle cells induced by *Gck* knockout, where the associated genes are strongly expressed and produce greater numbers of oxygen free radicals. Oxygen free radicals can damage proteins, phospholipids, and DNA, either directly or indirectly, by oxidation of lipids to lipid peroxidation or by converting nitric oxide to reactive nitrogen[45]. In this process, the calcium induced opening of mitochondrial membrane permeability transporters is more sensitive, which results in damage to the mitochondrial electron transport chain and affects energy supply.

5. Conclusions

Liver specific *Gck* knock-out can induce myocardial fibrosis at an early stage and diabetic myocardial injury at a late stage. Decreased glucokinase activity leads to disorders of glucose

metabolism and induces hyperglycemia. Long-term hyperglycemia damages the insulin signaling pathway, AMPK and ACC function decline, and causes cardiac myocyte energy metabolism disorders. The genes associated with mitochondrial oxidative phosphorylation are upregulated, and oxidative stress is increased, which induces oxidative damage to cardiomyocytes. In this process, the proportion of myosin heavy chain and light chain falls out of balance. Myosin imbalance leads to hypertrophy and contraction of the heart cavity, which is related to cardiac function damage in diabetic cardiomyopathy (Fig 8).

Abbreviations

ACC: Acetyl-CoA carboxylase; AKT: Protein kinase B; AMPK: Adenosine 5'-monophosphate-activated protein kinase; BPGM: 2,3-bisphosphoglycerate mutase; CMYA1: Cardiomyopathy associated 1; CMYA5: Cardiomyopathy associated 5; DCM: Diabetic cardiomyopathy; EDNRA: Endothelin receptor type A; EF: Ejection fraction; FS: Fractional shortening; GCK: Glucokinase; GYS1: Glycogen Synthase 1; HK: Hexokinase; HOMA: Homeostasis model assessment index; INSR: Insulin receptor; IPGTT: Intraperitoneal glucose tolerance; LVID;s: Left ventricle internal dimension during systole; LVPW;s: Left ventricle posterior wall thickness during systole; MDA: Malondialdehyde; MYH: Myosin heavy chain; MYL: Myosin light chain; MYO18B: Myosin XVIIIb; MYOM: Myomesin; MYPN: Myopalladin; NDUF: NADH dehydrogenase 1; PIK3R1: Phosphatidylinositol3-kinaseregulatory subunit1 (p85alpha); POPDC2: Popeye domain containing 2; SLC4A1: Solute carrier family 4member 1; SNCA: Alpha-synuclein; SOD: Superoxide dismutase; TC: Total cholesterol; TG: Triglyceride; TOMM7: Translocase of outer mitochondrial membrane 7 homolog; VEGFA: Vascular endothelial growth factor A; XIRP1: Xin actin-binding repeat containing 1.

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Authors' contributions

Huanran Tan, Gang Niu and David M Irwin are the guarantors of this work, and as such, had full access to all of the data in this study and take full responsibility for the integrity of the data

and accuracy of the data analysis. Hui Li researched the data and wrote the manuscript; DMI reviewed and edited the manuscript; Wei Xu, Yiqing Mao, Xi Wang, Ruoxuan Zhang, Binghua Li and Zhen Bai researched data. All authors read and approved the final manuscript.

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Availability of data and materials

Datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experiments were performed in accordance with the guidelines and study protocols of the Peking University Health Science Center.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Table 1.List of primers used in this study

Genes	Sense Primers	Antisense Primers
ALAS1	TCCCGATGGCGGATGACTA	TCCAGCACCATGCTGTTTCAC
ASB2	TCACCCCTTTGTTTGTGGCT	ATGCTCATTCTTGCTGGCCT
ATP5E	GCTGGACTCAGCTACATCCG	CGTTCGCTTTGAACTCGGTC
BPGM	CTGCTCACGGGAATAGCAGTAGA	TTGCCCAGGAACTGGTGAG
CLU	CCACAGGATGCCTGAAGATGAA	AGGTTAGCCTGGGCAGGATTG
CMYA5	CTCTCCAGATCTCCGCGAAC	GTGCAGATGCCGAGTCGATA
COX6B1	CCGCTGTGAGAAGGCAATGA	CAGCTATGCGGTCATCCCAG
COX8B	GCGAAGTTCACAGTGGTTCC	CGACTATGGCTGAGATCCCC
EDNRA	CATCGGCATTAACCTGGCAAC	GGACTGGTGACAACAGCAACAGA
GPD1	GATCATCAACACTCAGCACGAGAA	GCCCTTGAGCTGGTCACAG
GYS1	TCAGAGCAAAGCACGAATCCAG	CATAGCGGCCAGCGATAAAGA
HK1	ATGATCGCCGCGCAACTAC	AGAGCCGCATGGCATAACAGA
HK2	CCGTGGACTGGACAACATCA	CGTCACATTTCGGAGCCAGA
INSR	CAGCTCGAACTGCATGGTTG	GGTGACATCCACCTCACAGGAA
LIPIN	AAGCCGTGTCATATCAGCAAT	GCATCGCCAGAAGTAGAGG
MYH6	ACACCAACCTGTCCAAGTTCC	GCTGGAGAGGTTATTCCTCGT
MYH7	TTACTTGCTACCCTCAGGTGG	CTCCTTCTCAGACTTCCGCA
MYL1	ATGAGCCGTGAAGAAGCTGG	AGAGCAGTGTGACTCGCTTG
MYL2	GTCCACATCATTACCCACGG	CCAAGACTTCCTGTTTATTTGCG

MYL6	AGAGCTCAGCAGCCAAGATG	CAGCTGGAAAGCCTCCTTGA
MYL7	GCTCGGGAGGGTAAGTGTTT	GTCCCATTTAGCTTCTCCCC
MYO18B	CCCTTTGTAACTCGGGG	AAGCCAGACACACTGGTGG
MYOM1	CAAAGCTGCTATCGCCGTG	CCTGGTACCTCACCAACCAC
MYOM2	TGGAGCAAAAGCGTGGGAAC	ATGACTGGGTGGTTGCTTGC
MYPN	GGCTACTCAATGGGCAACCT	ACTGGCGCCTTCTTTACCTC
NDUFA3	AGCCCCTACACCAAGTATGC	AGGCATGTTCCCGTCATCTC
NDUFA4	AGCCATGGAACAACTGGGT	TGGAAAATTGTGCGGATGGC
NDUFB4	GCTTCAGTACAACGACCCCA	CCCTGCCACAGCTCCTAAAA
NPPA	TCGGAGCCTACGAAGATCCA	GTGGCAATGTGACCAAGCTG
OBSCN	AACAGATTGTGGCTGCCGTA	TGTGCCATGTCCCCTACTTG
PIK3R1	CCCATGGGACAACATTCCAA	CATGGCGACAAGCTCGGTA
POPDC2	GCGATGGGGAGTACATGAGG	GGCTAAGAGGAGGGGTGAGA
PPAR	GTTGTCAGGCTGGAGTGTA	CAGAAGCAATGTCACCAAGAA
SLC4A1	GATGATGAACGTGTCCAAGATTCC	ATGCAGCAATTCCGATTCCA
SNCA	TGATCGGCGTCCTGTACCT	TGTGAAGCCACAACAATATCCA
TOMM7	CAGGTGCAGTTCCCTTTTGC	TAGGCACAATCCCCAACAC
VEGFA	TATTCAGCGGACTCACCAGC	GAGCCCAGAAGTTGGACGAA
XIRP1	CTGCCACTGGGGTTACTCAA	ATGGTGGATTCCGCTTTGGA

Table2.Top 20 genes in Betweenness Centrality

Genes	Betweenness Centrality	AverageShortest PathLength	Closeness Centrality	Clustering Coefficient	Degree	Neighborhood Connectivity
MT-ND1	0.226518	2.818182	0.354839	0.410256	13	12.00000
INSR	0.171432	3.027972	0.330254	0.155556	10	6.100000
SRF	0.162151	3.160839	0.316372	0.200000	11	7.727273
VEGFA	0.154251	3.118881	0.320628	0.131868	14	5.642857
RPS3	0.141073	3.363636	0.297297	0.760684	27	21.88889
B2M	0.107467	3.244755	0.308190	0.214286	8	9.000000
ASB2	0.096458	3.734266	0.267790	0.166667	4	10.50000
RPL13A	0.091793	3.524476	0.283730	0.824615	26	22.53846
MT-CO3	0.084512	3.160839	0.316372	0.282051	13	8.846154
MYH6	0.077820	3.412587	0.293033	0.366667	16	8.875000
SLC4A1	0.075137	4.000000	0.250000	0.095238	7	3.714286
TTN	0.074700	3.671329	0.272381	0.323810	15	8.066667
NPPA	0.074280	3.475524	0.287726	0.422222	10	9.600000
MYL7	0.073879	3.692308	0.270833	0.316667	16	7.875000
EDNRA	0.072892	3.363636	0.297297	0.200000	5	6.800000
FURIN	0.068551	3.909091	0.255814	0.166667	4	5.250000
HBA-A1	0.063230	3.342657	0.299163	0.500000	8	8.500000
OBSCN	0.061809	3.545455	0.282051	0.384615	13	8.615385
MT-ATP6	0.061342	3.076923	0.325000	0.363636	11	13.00000
NDUFA4	0.058561	3.111888	0.321348	0.477124	18	12.11111

Figure 1. GCK activity and glycogen content are decreased in the liver of *Gck^{w/-}* mice. (a) Representative western blots of GCK from the livers of *Gck^{w/-}* and *Gck^{w/w}* mice. (b) Semiquantitative measurement of GCK protein levels in the livers of *Gck^{w/-}* and *Gck^{w/w}* mice. The relative levels of GCK protein were normalized with β actin. (c) GCK enzyme activity and (d) glycogen content in the livers of *Gck^{w/w}* and *Gck^{w/-}* mice. Data are expressed as the mean $\pm$ SD and statistical comparison was through a one-way ANOVA, ** $P < 0.005$, * $P < 0.05$ (n=3 mice per group).

Figure2. *Gck^{w/-}* mice display abnormal glucose metabolism. (a)Fasting serum glucose levels and (b)body weight were followed for 70 weeks in *Gck^{w/-}* and *Gck^{w/w}* mice. (c) Serum glucose concentrations after IPGTT in 30-week-old *Gck^{w/-}* and *Gck^{w/w}* mice. (d) Serum glucose concentration after IPGTT in 60-week-old *Gck^{w/-}* and *Gck^{w/w}* mice. (e) Area under the curve (AUC) calculated from the results of IPGTT used in (b) and (c). (f) Fasting serum insulin levels after an 8-hour fast in *Gck^{w/-}* and *Gck^{w/w}* mice. (g) Homeostasis model assessment index-insulin resistance (HOMA-IR) calculated from fasting serum glucose and insulin levels. (h) Homeostasis model assessment index- β cell (HOMA- β cell) calculated from fasting serum glucose and insulin levels.(i) Serum total cholesterol(TC) levels after an 8-hour fast in *Gck^{w/-}* and *Gck^{w/w}* mice. (j)Serum triglyceride (TG) levels after an 8-hour fast in *Gck^{w/-}* and *Gck^{w/w}* mice. Data are expressed as mean $\pm$ SD and statistical comparison was through a one-way ANOVA, * $P < 0.05$ (n=6 mice per group).

Figure 3. *Gck* deletion in the liver causes age-dependent heart dysfunction. (a) Representative echocardiography images (M-mode) of 30- and 60-week-old mice. (b) Ejection fraction (EF) and (c) shortening fraction (FS) were calculated by echocardiography. (d) Diastole left ventricular posterior wall thickness (LVPW;d), (e) diastole left ventricular interior diameter (LVID;d), (f) systolic left ventricular posterior wall thickness (LVPW;s) and (g) systolic left ventricular interior diameter (LVID;s) were measured by echocardiography. (h) HR, (i) PR interval, (j) QRS interval and (k) QTc interval were measured in ECG recordings. (l) Paraffin-embedded cardiac sections were subjected to hematoxylin-eosin staining (HE), periodic acid-schiff staining (PAS) and Masson trichrome staining (scale=100μm). (m) Cardiac interstitial fibrosis was evaluated by Masson staining. (n) Glycogen deposition was determined based on PAS staining. Data are expressed as the mean±SD and the statistical comparison was through a one-way ANOVA, * statistically different from *Gck*^{w/w} littermate controls. ****P*<0.001, ***P*<0.005, **P*< 0.05 (n=3 mice per group).

Figure 4. Analysis of differences in gene expression and GO enrichment in myocardium due to liver-specific *Gck* knockout. (a) Volcano plot showing $\log_2$ (fold change, FC) against $-\log_{10}$ (p-value) of transcripts identified by RNASeq analysis of 60-week-old $Gck^{w/-}$ and $Gck^{w/w}$ mouse myocardium. Genes with 1.5-fold change and $P < 0.001$ were considered differentially expressed. Dots represent different genes, with the gray dots being genes that do not display significantly different expression levels. Red dots are significantly up-regulated genes, and green dots are significantly down-regulated genes in the $Gck^{w/-}$ mice. The total number of genes with significant changes in expression is displayed above the plot. (b) Network of differentially expressed proteins in heart tissues between $Gck^{w/-}$ and $Gck^{w/w}$ mice, analyzed by STRING and displayed using Cytoscape software. Protein interaction networks were generated by STRING, then analyzed and visualized by Cytoscape. Nodes connect proteins within the network. Nodes are colored based on the fold change in expression ($Gck^{w/-}$ vs. $Gck^{w/w}$). The solid triangles indicate the top 20 proteins in the Betweenness Centrality. GO term enrichment analysis for biological processes (c), molecular function (d) and pathway (e) of the up- and down-regulated genes. Significantly enriched GO terms are shown as histograms. The X-axes is enrichment score ($-\log_{10}$ P value) for each GO term. The significance of each GO term was estimated based on P values ($P < 0.05$).

Figure5. Analysis of the myosin expression profile in the myocardium caused by the liver-specific *Gck* knockout. (a) Network representation of Cluster-1(myocardial cell development and function) using the “organic” style in Cytoscape. All nodes relevant to myocardial signaling are arranged using the “circular” style in Cytoscape. (b) Gene expression log2 fold change in heart of *Gck*^{w/w} relative to *Gck*^{w/w} mice measure by RNAseq and qPCR are shown as abar chart. Expression of gene mRNA and *Gapdh* mRNA were analyzed by qRT-PCR. Relative levels of genes were normalized with *Gapdh*. (c) Representative western blot images with quantification of MYL2 from heart homogenates of 30- and 60-week-old *Gck*^{w/w} and *Gck*^{w/-} mice. (d)Quantification of MYL2 protein levels normalized by β actin.Data are expressed as the mean $\pm$ SD and the statistical comparison was through a one-way ANOVA, * statistically different from *Gck*^{w/w} littermate controls. **P* < 0.05 (n=3 mice per group).

Figure6. Analysis of impairment of the insulin receptor signaling pathway in *Gck*^{w/-} mice. (a) Network representation of Cluster-2(material metabolism) using the “organic” style in Cytoscape. All nodes relevant to material metabolism signaling are arranged using the “circular” style in Cytoscape. (b) Gene expression log2 fold changes in the heart of *Gck*^{w/-} compared to *Gck*^{w/w} mice measure by RNAseq and qPCR are shown as abar chart. Expression of gene mRNA and *Gapdh* mRNA were analyzed by qRT-PCR. Relative levels of genes were normalized by *Gapdh*. (c) Representative western blot images with quantification of IRβ, PI3K, mTOR and Akt from heart homogenates from 30- and 60-week-old *Gck*^{w/w} and *Gck*^{w/-} mice. (d)Representative western blot

images with quantification of pACC, ACC, pAMPK α , AMPK α , pAMPK β and AMPK β from heart homogenates of 30- and 60-week-old *Gck*^{w/w} and *Gck*^{w/-} mice are shown. (e) Quantification of IR β , PI3K, mTOR and Akt protein levels normalized by β actin. Ratio for pACC/ACC, pAMPK α /AMPK α and pAMPK β /AMPK β were calculated. Data are expressed as the mean $\pm$ SD and statistical comparison was through a one-way ANOVA, * statistically different from *Gck*^{w/w} littermate controls. **P* < 0.05 (n=3 mice per group).

Figure 7. Analysis of changes in mitochondrial function induced by *Gck* knockout. (a) Network representation of Cluster-3 (mitochondrial function) using the “organic” style in Cytoscape. All nodes relevant to mitochondrial function signaling are arranged using the “circular” style in Cytoscape. (b) Gene expression log2 fold change in the hearts of *Gck*^{w/-} compared to *Gck*^{w/w} mice measure by RNAseq and qPCR are shown as a bar chart. Expression of gene mRNA and *Gapdh* mRNA were analyzed by qRT-PCR. Relative levels of genes were normalized by *Gapdh*.

Figure8.A schematic model of the molecular mechanisms associated with Diabetic cardiomyopathy induced by liver-specific *Gck* knockout. Liver-specific *Gck* knock-out can induce metabolic disorders that worsen with age. Through this process, the proportion of myosin heavy chain and light chain proteins fall out of balance, the insulin signal pathway becomes down regulated and mitochondrial oxidative stress is up regulated. These factors contribute to the thickening of the ventricular wall and worsen cardiac function.

Figures

Figure 1

GCK activity and glycogen content are decreased in the liver of $Gckw^{-/-}$ mice. (a) Representative western blots of GCK from the livers of $Gckw^{-/-}$ and $Gckw^{w/w}$ mice. (b) Semiquantitative measurement of GCK protein levels in the livers of $Gckw^{-/-}$ and $Gckw^{w/w}$ mice. The relative levels of GCK protein were normalized with β actin. (c) GCK enzyme activity and (d) glycogen content in the livers of $Gckw^{w/w}$ and $Gckw^{-/-}$ mice. Data are expressed as the mean $\pm$ SD and statistical comparison was through a one-way ANOVA, ** $P < 0.005$, * $P < 0.05$ ($n = 3$ mice per group).

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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.pdf](#)
- [Table2.pdf](#)