

Mechanistic exploration of hexokinase 2 and metabolism in diabetic cardiomyopathy

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Abstract. The pathogenesis of diabetic cardiomyopathy (DCM) remains incompletely understood. The present study employed weighted gene co-expression network analysis to analyze the DCM transcriptome dataset from the Gene Expression Omnibus (GEO) database to identify genes associated with this disease. Subsequently, both internal and external validation of the expression of the characterized genes was performed using additional GEO datasets. Key DCM genes were validated at both the *in vitro* and *in vivo* levels by Western blot and immunohistochemistry, IHC). Furthermore, the mechanisms of gene and metabolite co-expression in DCM were investigated through transcriptome sequencing of cells overexpressing disease-associated genes, combined with quantitative measurements of metabolites. Notably, hexokinase 2 (HK2) was downregulated in both DCM cell and db/db mouse models. Low expression of HK2 was implicated in the disruption of organic acids and their derivatives, as well as the receptor for advanced glycation end-products pathway.

Introduction

Diabetes mellitus (DM) is a prevalent chronic disease worldwide, with type 2 diabetes (T2D) accounting for >90% of cases. The International Diabetes Federation estimates that by 2040, 624 million individuals worldwide will be affected by T2D (1). Diabetic cardiomyopathy (DCM), a severe cardiovascular complication of DM, accounts for 50-60% of DM-associated mortality, representing the leading cause of death in diabetic populations (2). DCM is associated with immune-inflammatory infiltration, dysregulation of glucolipid metabolism (2), mitochondrial energy metabolism disorder, oxidative stress (3), focal myocardial cell death (4) and programmed necrosis (5). Metabolic remodeling is a manifestation of DCM, characterized by the loss of cardiomyocyte capacity for carbohydrate and fatty acid metabolism, which is supplanted by mitochondrial fatty acid β -oxidation. This shift leads to cardiac steatosis, lipotoxicity and apoptosis of cardiomyocytes (6).

DCM is a pathophysiological condition associated with DM that manifests as cardiac decompensation or heart failure (3). It typically presents with thickening of the left ventricular wall, impaired left ventricular diastolic function (7) and heart failure resulting from non-coronary artery disease, hypertension and valvular heart disease. Decreased cardiomyocyte function is an important contributor to the development of heart failure in DCM (8), with early signs including abnormal diastolic relaxation (9). Chronic hyperglycemia (HG) and HG-induced oxidative stress lead to ultrastructural changes in cardiomyocytes, characterized by mitochondrial swelling, a decreased number of mitochondria and defective collagen fibers (10). These alterations may result in myocardial fibrosis, mitochondrial dysfunction, myocyte hypertrophy and myogenic fibrosis disorder, all of which disrupt the homeostasis of cardiomyocytes and may ultimately lead to heart failure (10).

Metabolic disorders induced by HG and insulin resistance are associated with the development of DCM (9). Protein glycosylation resulting from HG leads to an increase in advanced glycation end products (AGEs), which promotes

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collagen cross-linking, myocardial fibrosis and impaired diastolic function, triggering the onset of DCM (9). HG, insulin resistance, AGEs, collagen cross-linking, myocardial fibrosis and impaired diastolic function serve key roles in the progression of DCM (9). Emerging evidence highlights the potential of bariatric surgery, particularly Roux-en-Y gastric bypass and sleeve gastrectomy, in ameliorating DCM (11). Insulin resistance is a key pathogenic driver of DCM, leading to impaired myocardial insulin signaling, mitochondrial dysfunction and endoplasmic reticulum (ER) stress. In insulin resistance or hyperinsulinemia, the heart experiences notable metabolic dysregulation, characterized by diminished glucose uptake and use alongside heightened dependence on fatty acid oxidation. These metabolic shifts exacerbate cardiac dysfunction by fostering oxidative stress, chronic inflammation and cell injury, collectively contributing to the progression of DCM (12). The energy and lipid metabolism associated with DCM, along with its regulatory mechanisms, are complex and not fully understood. Therefore, it is important to investigate the pathogenesis of DCM through a combined analysis of genomics and metabolomics, as the underlying mechanisms of DCM at the metabolic level.

Materials and methods

Ethics approval. All experimental procedures involving db/db and db/m mice were conducted in accordance with the ARRIVE guidelines and were approved by the Institutional Animal Care and Use Committee of Hubei University of Medicine (Shiyan, China; approval no. 2024S071). Humane endpoints were as follows: i) Body weight loss $\geq 20\%$ within 48 h (relative to peak body weight); ii) severe hypoglycemia (<40 mg/dl) or hyperglycemia (>600 mg/dl) refractory to clinical stabilization (for example, dietary adjustment or insulin administration); iii) functional impairment (inability to access food/water independently or lethargy persisting >24 h) and iv) overt signs of distress (labored breathing, hunched posture lasting >12 h or ulcerative lesions affecting $>5\%$ body surface area). No animals met criteria for early euthanasia. Animals were humanely euthanized via intraperitoneal injection of sodium pentobarbital at a dose of 150 mg/kg body weight. Animal death was confirmed by the absence of heartbeat, breathing and corneal reflex. These methods align with the American Veterinary Medical Association guidelines to ensure minimal animal suffering (13).

Data source. Key genes associated with DCM were investigated using The National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (ncbi.nlm.nih.gov/geo/). A total of three rat transcriptome expression profiling datasets (accession nos. GSE4745, GSE5606 and GSE6880) were retrieved from the GEO database (Table I). To eliminate batch effects and other sources of variance, surrogate variable analysis (14) and limma packages (version 3.54.2, <http://bioconductor.org/packages/release/bioc/html/limma.html>) were employed in R (version 4.2.3; <https://cran.r-project.org/bin/windows/base/old/4.2.3/>) to merge the GSE4745 and GSE6880 datasets, and the resulting merged dataset served as the internal training set. For independent validation, GSE5606 dataset as an external validation

cohort. This strategic approach of combining GSE4745 and GSE6880 not only increased statistical power but also maintained platform diversity within the training set. Importantly, all three datasets investigated identical species and disease models, ensuring biological consistency while allowing rigorous assessment of model generalizability across different experimental platforms.

Screening for differentially expressed genes (DEGs). GEO datasets were corrected using the limma package. The expression data files of the combined dataset were investigated for deviation analysis on the basis of $\log_2$ (fold-change) ≥ 1 and adjusted P-value <0.05 . DEGs were visualized using heatmaps generated by the heatmap package (version 1.0.12; cran.r-project.org/web/packages/heatmap/index.html) and volcano plots created with the ggplot2 package (version 3.5.1; <https://ggplot2.tidyverse.org/>).

Functional enrichment analysis. Gene Ontology (GO) (15) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (16) pathway enrichment analyses were performed using the clusterProfiler R package (version 4.7.1; bioconductor.org/packages/release/bioc/html/clusterProfiler.html). GO enrichment analysis provides a comprehensive description of the gene and protein functions associated with biological processes (BPs), molecular functions and cellular components. Adjusted P <0.05 was considered to indicate a statistically significant difference. For the Gene Set Enrichment Analysis (GSEA), the clusterProfiler package (version 4.7.1; bioconductor.org/packages/release/bioc/html/clusterProfiler.html) was utilized with adjusted P <0.05 to indicate statistically significant enrichment. The results of enrichment analyses were visualized using the ggplot2 package (version 3.5.1; ggplot2.tidyverse.org/), and chord plots were generated using the Xian Tao Academic Online Analysis platform (xiantaozi.com/products/apply/c0b6febb-52dd-4525-970a-61bbe9e263ff/analyse/f9496965-0e1a-4249-b123-e6a00c924021).

Weighted gene co-expression network analysis (WGCNA)-mediated core gene screening. WGCNA is a bioinformatics tool that calculates gene expression levels and clusters genes with similar expression patterns into modules. These functional gene clusters are used to explore interactions between genes and biological phenotypes within the modules (17). The present study employed the WGCNA package (version 1.72; cran.r-project.org/web/packages/WGCNA/index.html) to identify gene modules with similar expression patterns by constructing a gene co-expression network using the mean chained hierarchical clustering algorithm. These modules were further distinguished through hierarchical clustering based on proximity. Additionally, the association of each module was inferred by calculating the association between different modules and biological phenotype. Finally, core genes from key modules were identified. Diagnostic potential was evaluated by ROC curve analysis (AUC >0.7), with differential expression visualized through boxplots (Wilcoxon test, P <0.05).

Least absolute shrinkage and selection operator (LASSO) regression screening for key DCM genes. Expression of DEGs intersecting with modal genes significantly associated

Table I. Gene Expression Omnibus datasets of mRNA expression in rat heart tissue.

Accession no.	Platform	Control, n	Diabetic cardiomyopathy, n
GSE4745	GPL85	12	12
GSE5606	GPL1355	6	7
GSE6880	GPL341	3	3

with DCM, as identified through WGCNA, were analyzed. Intersection analysis was performed using the Venn package (version 1.12; cran.r-project.org/web/packages/venn/index.html) The GLMNET package (version 4.1; cran.r-project.org/web/packages/glmnet/index.html) was employed to construct a LASSO regression model for the intersected genes and DCM feature genes were identified through cross-validation. The genes corresponding to the points with the lowest cross-validation error were designated key genes associated with DCM.

Validation of key genes. To analyze the expression of key genes in both internal and external datasets, the Limma package was used. $P < 0.05$ was considered to indicate a statistically significant difference. Additionally, the ggpvr package (version 0.6.0; <https://cran.r-project.org/web/packages/ggpvr/index.html>) was employed to create a diverging box plot to visualize significant differences in key gene expression levels between the disease and control groups.

Receiver operating characteristic (ROC) curve. ROC curves were used to validate the accuracy of key genes in diagnosing DCM. R software (version 4.2.3; cran.r-project.org/bin/windows/base/old/4.2.3/) was used to analyze key gene expression and the pROC (version 1.18.5; <https://xrobin.github.io/pROC/>) package was used to calculate and plot the area under the curve (AUC), with a larger AUC indicating a greater diagnostic value of the key gene.

Cell culture and model. The HL-1 murine cardiomyocyte cell line (Unico) was maintained at 37°C in a humidified atmosphere of 5% CO₂, 21% O₂ and 74% N₂. The cells were maintained in high-glucose DMEM (cat. no. SH30022.01; Cytiva) supplemented with 10% fetal bovine serum (FBS; Cat. No. SA101.02, Cellmax, Beijing, China). The medium was changed every 2 days, and cells in mid-logarithmic growth phase were collected for experiments. Cultured HL-1 cells were divided into low- (5.5 mM) and high-glucose (33.3 mM) groups, and incubated at 37°C for 48 h (18,19).

Animals. All experimental protocols were approved by the Animal Ethics Committee of Hubei University of Medicine (Shiyan, Hubei, China). A total of six male db/db diabetic mice (age, 6 weeks; body weight 27.5±1.6 g at study initiation; Certificate No. 202341294-202341295, Jiangsu Huachuang Sino Pharmaceutical Technology Co., Ltd, China) were used to establish a model of DCM, with six db/m mice serving as controls. Mice were maintained under a 12/12-h light/dark

cycle at a controlled temperature of 22±1°C and 50±10% relative humidity, with ad libitum access to food and water. All animals were acclimatized for 2 weeks prior to experiments. Body weight and blood glucose levels were measured. Blood glucose levels were monitored via tail vein sampling. Briefly, the distal 1-2 mm of the tail tip was sterilized with 70% ethanol and air-dried. A sterile lancet was used to make an incision, and a single blood droplet (~1 µl) was collected. Glucose concentration was analyzed using a Sinocare Blood Glucose Monitoring System (Sinocare Inc., Changsha). Triplicate measurements were averaged per mouse to ensure reliability. All measurements were performed between 9:00 and 11:00 AM under non-fasting conditions to minimize circadian variation. In longitudinal studies, sequential sampling was restricted to the same tail region to avoid tissue damage. The animals were monitored in SPF-grade animal laboratories for 24 weeks, with echocardiograms performed every 4 weeks. Sampling was conducted at 32 weeks of age.

Lentivirus transfection and hexokinase 2 (HK2) overexpression. HL-1 cardiomyocytes were transduced with recombinant lentiviral particles carrying the human HK2 gene (Lenti-HK2; Vigene Biosciences, cat. no. FC-6073) following the manufacturer's recommended protocol. HL-1 cells were seeded in 12-well plates at 1×10⁵ cells/well and cultured at 37°C until reaching 40-50% confluence. Cells were maintained at 37°C in medium, which was replaced with 420 µl fresh complete DMEM supplemented with 10% FBS [cat. no. SA101.02; CellTech (Beijing) Co., Ltd.], supplemented with 20 µl LV-Enhance (50X, Vigene Biosciences) and 60 µl/ml lentivirus overexpressing human HK2 (LV-hHK2-OE) (Vigene Biosciences, cat. no. FBE0001, Shandong, China; <https://www.wzbio.com.cn/>). LV-ZsGreen-Puro-CON (Vigene Biosciences) served as negative controls. Subsequent experiments were performed 48 h post-transfection. The transfection was conducted at 37°C in a 5% CO₂ atmosphere for 18-24 h, followed by the addition of 500 µl complete DMEM (supplemented with 10% FBS [cat. no. SA101.02; CellTech (Beijing) Co., Ltd.]) for an additional 24 h under the same culture conditions. Green fluorescence was monitored for 24-48 h post-transfection to assess transfection efficiency. Once green fluorescence was detected in the cells, puromycin (3 g/ml; cat. no. HY-K1057; MedChemExpress) was added to each well, and the same concentration of puromycin was maintained for 7 days at 37°C with 5% CO. The cells were collected for further experiments and cryopreserved (-196°C) in liquid nitrogen.

Western blotting. Total protein was extracted from heart tissue and HL-1 cells using RIPA lysis buffer (Servicebio Technology Co., Wuhan, China; cat. no. G2002) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA-2Na, 1% sodium deoxycholate, 0.1% SDS and 1% Triton X-100. Protein concentration was determined using the BCA method. Equal amounts of protein (20 µg/lane) were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Immobilon-PSQ Blotting Membranes; 0.2 µm; Merck KGaA). Following 1-hour blocking at room temperature with 5% skimmed milk, the membranes were incubated overnight at 4°C with the following antibodies: Anti-GAPDH mouse monoclonal (cat. no. 60004-1-Ig; 1:5,000; Proteintech Group,

Inc.), anti-microsomal glutathione S-transferase 1 (MGST1) rabbit monoclonal (cat. no. A0880; 1:500; ABclonal Biotech Co., Ltd.), anti-HK2 rabbit polyclonal (1:1,000; cat. no. A0994; ABclonal Biotech Co., Ltd.) and anti-Phosphatidylinositol-4-Phosphate 3-Kinase C2 Domain-Containing Subunit Gamma (PIK3C2G) polyclonal antibody (cat. no. 25904-1-AP; 1:2,000; Proteintech Group, Inc.). Following three washes with TBST (0.05% Tween-20), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; Beyotime Institute of Biotechnology, cat. no. A0208) at room temperature for 1 h. The membranes were washed three additional times with TBST, and chemiluminescent signals were detected using an ultra-sensitive ECL substrate (cat. no. BL520B, Biosharp, China) and imaged with a gel imaging system (Bio-Rad ChemiDoc XRS+; Bio-Rad Laboratories, Inc.). Data were analyzed using Image Lab 6.0 software (Bio-Rad), and semi-quantification of band intensities was performed with ImageJ 1.54d (National Institutes of Health, USA).

Echocardiography. Echocardiography was performed using a Visualsonic Vevo 2100 (VisualSonics, Inc.) equipped with a 30 MHz linear array ultrasound transducer (20). Echocardiography was performed under inhalant anesthesia using isoflurane delivered via a nose cone connected to an anesthetic vaporizer. Induction was achieved with 4% isoflurane and maintenance was performed using 1.5-2.0% isoflurane. The internal diameter of the left ventricle (LV) was measured in M-mode over ≥ 3 cardiac cycles, and the mean value was subsequently calculated. The left ventricular end-diastolic inner diameter (LVEDd) and left ventricular end-systolic inner diameter (LVESd) were measured at the maximum and minimum LV areas, respectively. The left ventricular shortening fraction (LVFS) and left ventricular ejection fraction (LVEF) were calculated as follows: $LVFS\% = (LVEDd - LVESd) / LVEDd \times 100\%$.

Immunohistochemical staining. The myocardial tissues were fixed in 4% paraformaldehyde (PFA, cat. no. BL539A, Biosharp, China) at 4°C for 24-48 h, processed through graded ethanol, cleared in xylene, and embedded in paraffin. Samples were sliced into 5- μ m-thick serial sections were prepared, dewaxed in xylene at room temperature (25°C) for 30 min and hydrated with graded alcohol. Antigens were retrieved using microwave heating at 95-100°C with sodium citrate buffer (pH 6.0; cat. no. 36311-B, Yeasen, China) for 30 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature. Next, 5% bovine serum albumin (cat. no. HY-D0842; MedChemExpress) was applied at 37°C for 1 h. The sections were incubated overnight at 4°C with anti-MGST1 rabbit monoclonal (1:100) and anti-HK2 rabbit polyclonal antibody (1:200), followed by incubation with liquid 3,3'-diaminobenzidine (cat. no. 36311-H and 36311-I, Yeasen) substrate for color development according to the manufacturer's instructions. Sections were stained with hematoxylin at room temperature for 3 min to visualize nuclei, differentiated using 1% hydrochloric acid alcohol, dehydrated through ascending ethanol series (70, 95, and 100% ethanol; 2 min each at room temperature) and cleared

in xylene (two changes, 3 min each at room temperature) prior to mounting with neutral balsam.

Stained sections were visualized and digitized using an Aperio CS2 digital slide scanner (Leica Biosystems, Germany) under bright-field microscopy.

Hematoxylin and eosin (H&E) and Masson's trichrome staining. Cardiac tissue was fixed in 4% paraformaldehyde (PFA, cat. no. BL520B, Biosharp, China) at 4°C for 24 h. Following fixation, the samples were washed in phosphate-buffered saline (PBS) and processed through a graded ethanol series (70, 85, 95, and 100%), cleared in xylene, and embedded in paraffin using an automated tissue processor (Leica TP1020; Leica Biosystems). Serial sections (5 μ m thickness) were cut using a rotary microtome (Leica RM2235; Leica Biosystems) and mounted on poly-L-lysine-coated glass slides for subsequent histological staining. The sections were stained with H&E following deparaffinization and dehydration as previously described (21). Following deparaffinization and rehydration, the sections were stained with hematoxylin solution for 5 min, rinsed in running tap water for 5 min, differentiated in 1% acid alcohol for 30 sec, and blued in 0.2% ammonia water. Subsequently, the sections were counterstained with eosin for 3 min, dehydrated through a graded ethanol series (70, 95, and 100%), cleared in xylene, and mounted with neutral balsam. Additionally, sections were stained using a modified Masson's Trichrome Stain kit (cat. no. G1340; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. Sections were visualized using an Aperio CS2 digital slide scanner (Leica Biosystems, Germany) under bright-field microscopy.

Transcriptomic assay. To investigate the regulatory effect of HK2 overexpression on downstream gene expression in HL-1 cells, cultured wild-type (WT) HL-1 cells (HK2-WT) and lentivirus-transfected HK2 overexpressing HL-1 cells (HK2-OE) were washed with a sterile PBS solution, lysed by adding TRIzol (cat. no. 15596026CN; Thermo Fisher Scientific, USA) lysis solution and incubated for 5 min at room temperature. The lysed cells were mixed and transferred to 1.5 ml sterile Eppendorf tubes for storage at -80°C. The samples were preserved and transported on dry ice to Metware Biotechnology Co., Ltd. (Wuhan, China) for analysis. Total RNA was processed with the NEBNext Ultra II RNA Library Prep kit (Cat. No. E7770; New England Biolabs) for Illumina sequencing. RNA integrity was verified using the Qsep400 Bioanalyzer (Bioptic Inc.) with all samples exhibiting RNA Integrity Number >7.0 . Sequencing was performed on the Illumina NovaSeq 6000 platform using the NovaSeq 6000 SP Reagent Kit (300 cycles; Cat. No. 20027464; Illumina Inc.) for paired-end 150 bp sequencing. Final libraries were quantified by qPCR using the KAPA Library Quantification Kit (Cat. No. KK4824; Roche) and loaded at 12.5 pM concentration. Unsupervised principal component analysis was performed by statistics function pcomp within R (version 3.5.1; r-project.org). The data was unit variance scaled before unsupervised PCA.

Quantitative analysis of energy metabolism and lipid metabolism. To investigate the effects of HK2 gene expression on energy and lipid metabolism in HL-1 cells, targeted quantification of metabolites was performed using liquid

chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (22). All metabolites were detected through MetWare (metware.cn/) using the AB Sciex QTRAP 6500 LC-MS/MS platform. The analysis was performed in both positive and negative ionization modes, with the following parameters: Ion spray voltage set at 5,500 V (positive) and -4,500 V (negative), curtain gas at 35 psi, and source temperature maintained at 550°C. Multiple reaction monitoring (MRM) transitions were optimized for each metabolite, with specific precursor and product ion pairs (*m/z*) selected for quantification. The nebulizer gas flow rate was maintained at 1.5 l/min, and the collision gas pressure was set to medium. Pearson correlation analysis was performed using quantitative values of genes and metabolites from all samples. Fold-change differences in correlation coefficients with absolute values >0.8 and *P*<0.05 were analyzed.

Sample preparation and extraction. The cells were pelleted by centrifugation at 1,000 × *g* for 10 min at 4°C, and the resulting cell pellets were stored at -80°C until metabolomic analysis. The collected cell pellet samples for metabolomic analysis were thawed on ice and 100 µl ultrapure water was added to resuspend the cell pellet. A 50-µl aliquot of the cell suspension was added to 200 µl methanol (precooled to -20°C). The mixture was vortexed for 2 min at 1,000 × *g* at 4°C. The sample was flash-frozen in liquid nitrogen (-196°C) for 5 min, thawed on ice (0-4°C) for 5 min and vortexed at maximum speed (700 × *g*) at room temperature for 2 min. The freeze-thaw-vortex cycle was repeated three times. The sample was centrifuged at 12,000 × *g* for 10 min at 4°C and 200 µl supernatant was transferred to a new centrifuge tube and stored at -20°C for 30 min. The supernatant was centrifuged again at 12,000 × *g* for 10 min at 4°C. A total of 180 µl supernatant was filtered through a protein precipitation plate for LC-MS analysis, while the 50 µl cell suspension underwent three freeze-thaw cycles, followed by centrifugation at 12,000 × *g* for 10 min at 4°C. The protein concentration in the supernatant was determined using a BCA protein assay kit.

Quantitative PCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and reverse-transcribed into cDNA using HiScript IV RT SuperMix for qPCR (+gDNA wiper) (cat. no. R423-01; Vazyme Biotech Co., Ltd., Nanjing, China). Quantitative PCR was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with Taq Pro Universal SYBR qPCR Master Mix (cat. no. Q712; Vazyme Biotech Co., Ltd.). The thermal cycling conditions consisted of an initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Gene expression levels were normalized to GAPDH reference genes and calculated using the 2-ΔΔC_q method (23). All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China; Table SI).

Protein-protein interaction (PPI) network analysis. PPI networks were constructed using the STRING database (version 12.0; minimum required interaction score, 0.4; <https://cn.string-db.org/>) with the differentially expressed

genes as input. The networks were visualized and analyzed using Cytoscape software (version 3.9.0; National Institute of General Medical Sciences), with the CytoHubba plugin (version 0.1, <https://apps.cytoscape.org/apps/cytohubba>) used to identify hub genes based on topological parameters (degree centrality and betweenness centrality).

Statistical analysis. All data are presented as the mean ± standard deviation from three independent experimental repeats. All analyses were conducted using GraphPad Prism 9.0 software (Dotmatics). Two samples were compared using unpaired two-tailed Student's *t*-test. For >2 groups, one-way ANOVA followed by Tukey's post hoc test was used if the data met the assumptions of normality (assessed by the Shapiro-Wilk test) and homogeneity of variance (verified by Levene's test). If these assumptions were violated, the non-parametric Kruskal-Wallis test, followed by Dunn's post hoc test, was applied. *P*<0.05 was considered to indicate a statistically significant difference.

Results

Identification of DEGs. To identify DEGs, a fold-change threshold of log₂ (fold-change) ≥ 1 and adjusted *P*<0.05 were employed. In the merged dataset of GSE4745 and GSE6880, 47 DEGs were identified, comprising 25 up- and 22 downregulated genes (Fig. 1A and B). To explore the biological functions of the DEGs and the potential biological pathways associated with DCM, GO functional and KEGG pathway enrichment analysis were performed (Tables SII and SIII).

Functional characterization of DEGs. GO enrichment analysis indicated DEGs were involved in BPs, including 'fatty acid metabolic process', 'pyruvate metabolic process', 'small molecule catabolic process', 'response to toxic substance', 'response to starvation', 'response to hypoxia' and 'response to insulin' (Fig. 1D). Additionally, the DEGs were localized to cellular components such as 'banded collagen fibril', 'fibrillar collagen trimer', 'organelle outer membrane', 'sarcolemma', 'organelle inner membrane', 'mitochondrial inner membrane' and 'mitochondrial matrix'. Furthermore, DEGs associated with 'hexose transmembrane transporter activity', 'glucose transmembrane transporter activity', 'neuropeptide hormone activity', 'CoA hydrolase activity', 'acyl-CoA hydrolase activity', 'glutathione peroxidase activity', 'platelet-derived growth factor binding', 'palmitoyl-CoA hydrolase activity' and 'phospholipid binding' were significantly enriched (Fig. 1C-E).

Pathway enrichment analysis. KEGG enrichment analyses indicated that the DEGs were significantly enriched in 'fatty acid elongation', 'drug metabolism-other enzymes', 'butanoate metabolism', 'arachidonic acid metabolism', 'metabolism of xenobiotics by cytochrome P450', 'drug metabolism-cytochrome P450', 'adipocytokine signaling pathway', 'glycolysis/gluconeogenesis', 'diabetic cardiomyopathy', 'carbon metabolism', 'linoleic acid metabolism', 'biosynthesis of amino acids' and 'HIF-1 signaling pathway' (Fig. 1F and G). Additionally, GSEA demonstrated enrichment of DCM-associated genes in 'collagen biosynthesis and modifying enzymes' and 'metabolism of lipids' (Fig. 1H).

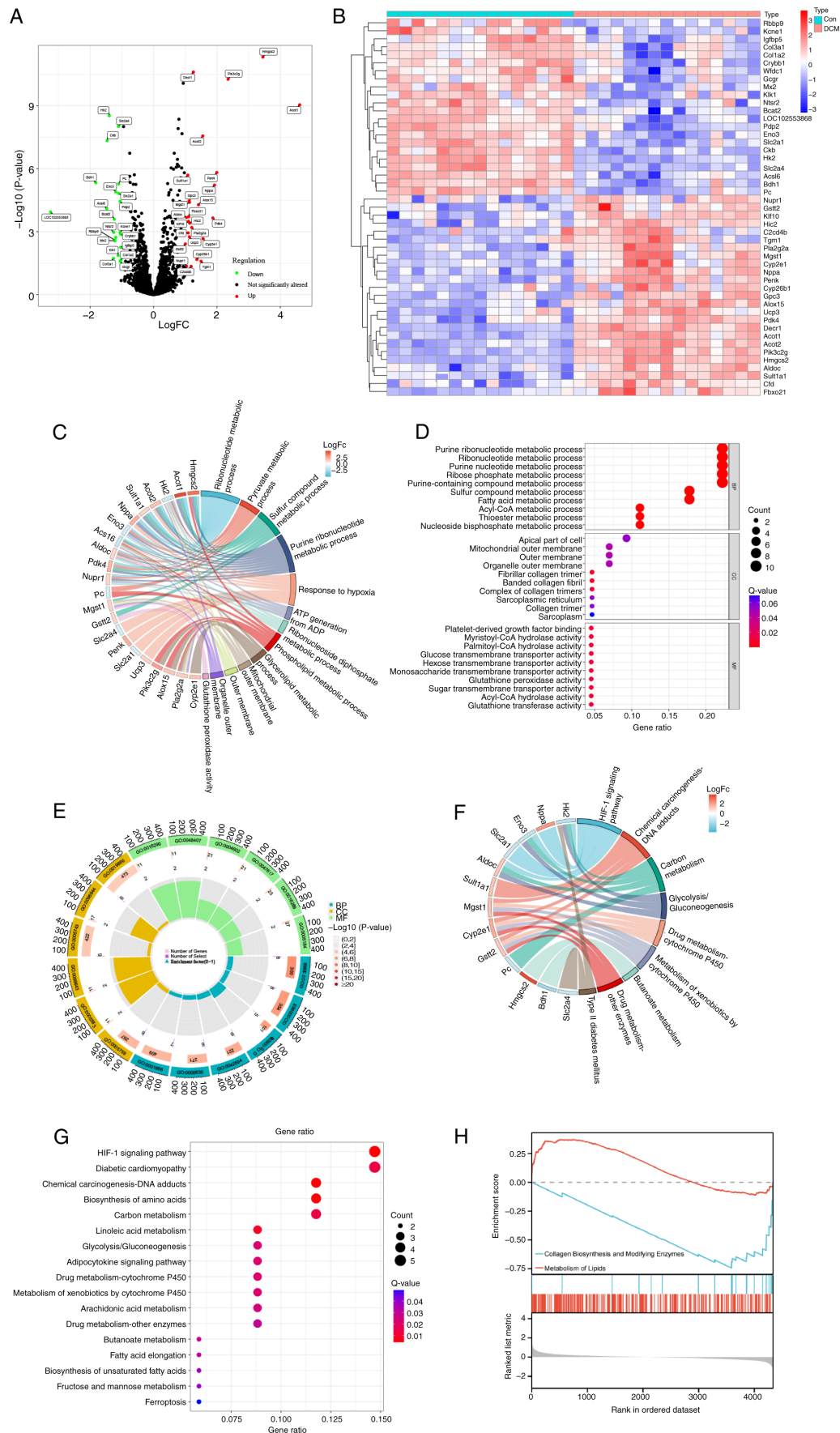


Figure 1. Identification and enrichment analysis of DEGs. (A) Volcano plot of DEGs. (B) DEG expression heatmap. (C) Chord, (D) bubble and (E) Circos plot of the GO enrichment analysis results. (F) Chord and (G) bubble plot of the KEGG enrichment analysis results. (H) Gene Set Enrichment Analysis of gene sets. Red curve indicates that the gene set is upregulated, while the blue color indicates that the gene set is downregulated. DEG, differentially expressed gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; Con, control; DCM, diabetic cardiomyopathy; FC, fold change; BP, biological process; CC, cellular component; MF, molecular function.

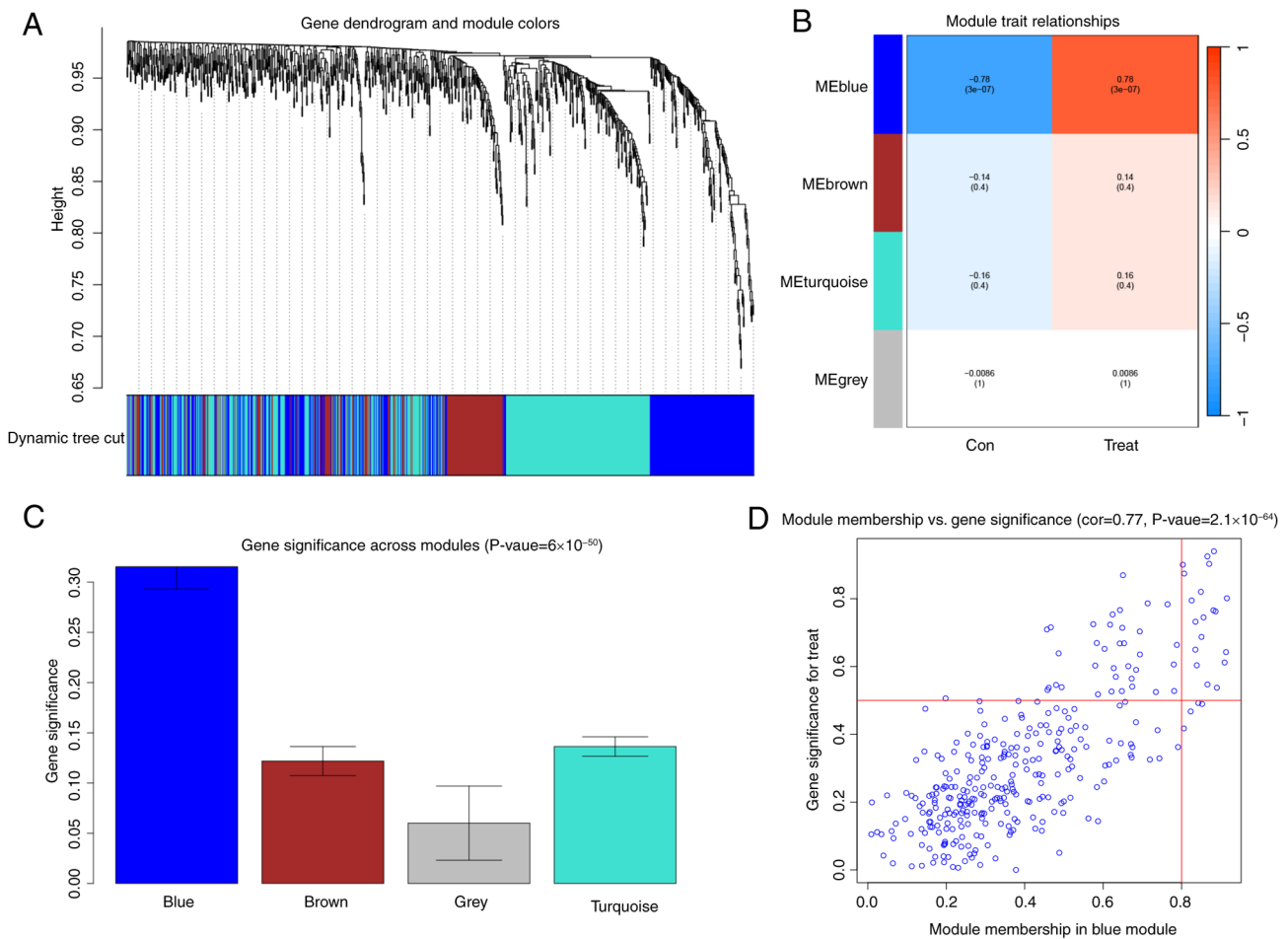


Figure 2. Weighted gene co-expression network analysis plot. (A) Gene dendrograms were obtained via the mean chained hierarchical clustering algorithm. The colored rows indicate module assignments determined via the dynamic tree slicing technique. (B) Modular correlation analysis heatmap with clinical features. The module with the smallest P-value is the most relevant gene clustering module for the disease. (C) Genetic significance. The blue module had the highest tie score and thus being the most relevant disease module. (D) Protein-protein interaction plot of blue module genes. The blue module genes are positively associated with diabetic cardiomyopathy traits. Con, control; Treat, treated; ME, module eigengene; cor, correlation coefficient.

Identification of the most relevant module genes to DCM through WGCNA. After conducting a WGCNA, the blue module was the most relevant to DCM (Fig. 2A-D). This module contained 19 functionally diverse hub genes that represent critical pathways in cardiac pathophysiology. The metabolic regulators included acyl-CoA thioesterases (ACOT1 and ACOT2) and acyl-CoA synthetase long-chain family member 6 (ACSL6), which coordinate lipid metabolism, along with β -hydroxybutyrate dehydrogenase (BDH1) and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), key enzymes in ketone body metabolism. Extracellular matrix remodeling was represented by collagen type I α 2 chain (COL1A2) and collagen type III α 1 chain (COL3A1), while immune modulation involved CD74 molecule (CD74) and cathepsin K. Metabolic enzymes included cytochrome P450 2E1 (CYP2E1), hexokinase 2 (HK2), and microsomal glutathione S-transferase 1 (MGST1). Additional significant genes were natriuretic peptide A, pyruvate dehydrogenase phosphatase catalytic subunit 2 (PDP2), proenkephalin (PENK), phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 gamma (PIK3C2G), phospholipase A2 group IIA (PLA2G2A), WAP four-disulfide core domain 1 (WFDC1), and the uncharacterized protein LOC102553868. These

molecular networks provide crucial insights into the complex interplay of metabolic dysregulation, structural remodeling, and inflammatory responses in DCM development.

Identification and characterization of DCM signature genes. Using Venn analysis of DEGs and WGCNA module genes, 17 overlapping genes were identified (Fig. 3A). A LASSO regression model was constructed based on the expression matrix of these genes. Using the GLMNET package, the cross-validation curves (Fig. 3B) and LASSO coefficient path diagrams (Fig. 3C) of the model were visualized. Ultimately, four disease signature genes (HMGCS2, PIK3C2G, HK2 and MGST1) were identified. A comparative analysis of the expression of these genes was conducted in both the internal and external datasets using the limma package. Next, the ggpubr package was employed to visualize the differences in gene expression between the disease and control groups using boxplots (Fig. 3D and F). Compared with the control group, HK2 expression was downregulated in the DCM group, while the expression of HMGCS2, PIK3C2G and MGST1 was upregulated. Finally, the diagnostic value of signature genes for DCM was assessed using the pROC package. All four genes demonstrated high diagnostic value for DCM, with AUC >0.9 (Fig. 3E and G).

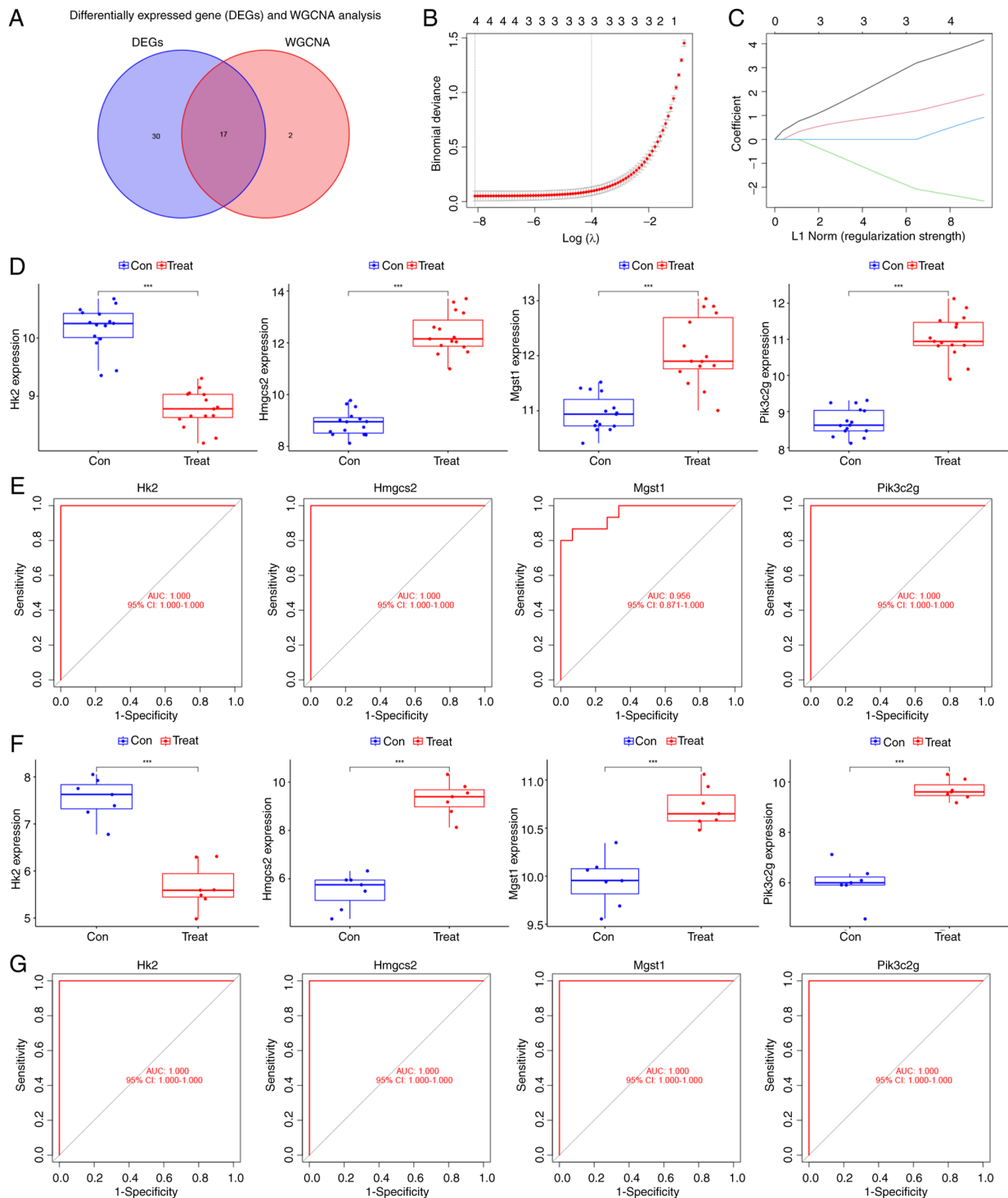


Figure 3. LASSO regression analysis and validation of intersecting genes in DCM. (A) Venn diagram of intersecting genes in the differentially expressed genes and WGCNA modules. (B) Cross-validation curves of the LASSO regression analysis. (C) LASSO coefficient path plots of feature genes, with each curve representing the coefficient trajectory of a variable gene. LASSO regression employed an L1 norm penalty, defined as the sum of absolute coefficient values ($\sum|\beta_i|$), to enforce sparsity by driving non-essential feature coefficients to zero. (D) Differential expression box plots of characteristic genes in the internal dataset (training dataset). (E) ROC curves of the feature genes in the internal dataset and area under the curve response genes for DCM diagnostic value. (F) Differential expression box plots of the feature genes in the external dataset (validation dataset). (G) ROC curves of genes in the external dataset. *** $P < 0.001$. LASSO, least absolute shrinkage and selection operator; DCM, diabetic cardiomyopathy; ROC, receiver operating characteristic; Con, control; Treat, treated; WGCNA, weighted gene co-expression network analysis; AUC, area under the curve; CI, confidence interval; HK2, hexokinase 2; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; MGST1, microsomal glutathione S-transferase 1; PIK3C2G, phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 γ .

Signature gene expression and validation in HL-1 cardiomyocytes. The surface area of HL-1 cardiomyocytes cultured in a high-glucose environment was notably increased compared

with that of cardiomyocytes cultured in a low-glucose environment (Fig. 4A). A significant increase in cell surface area was confirmed by quantitative analysis following 48 h high-glucose

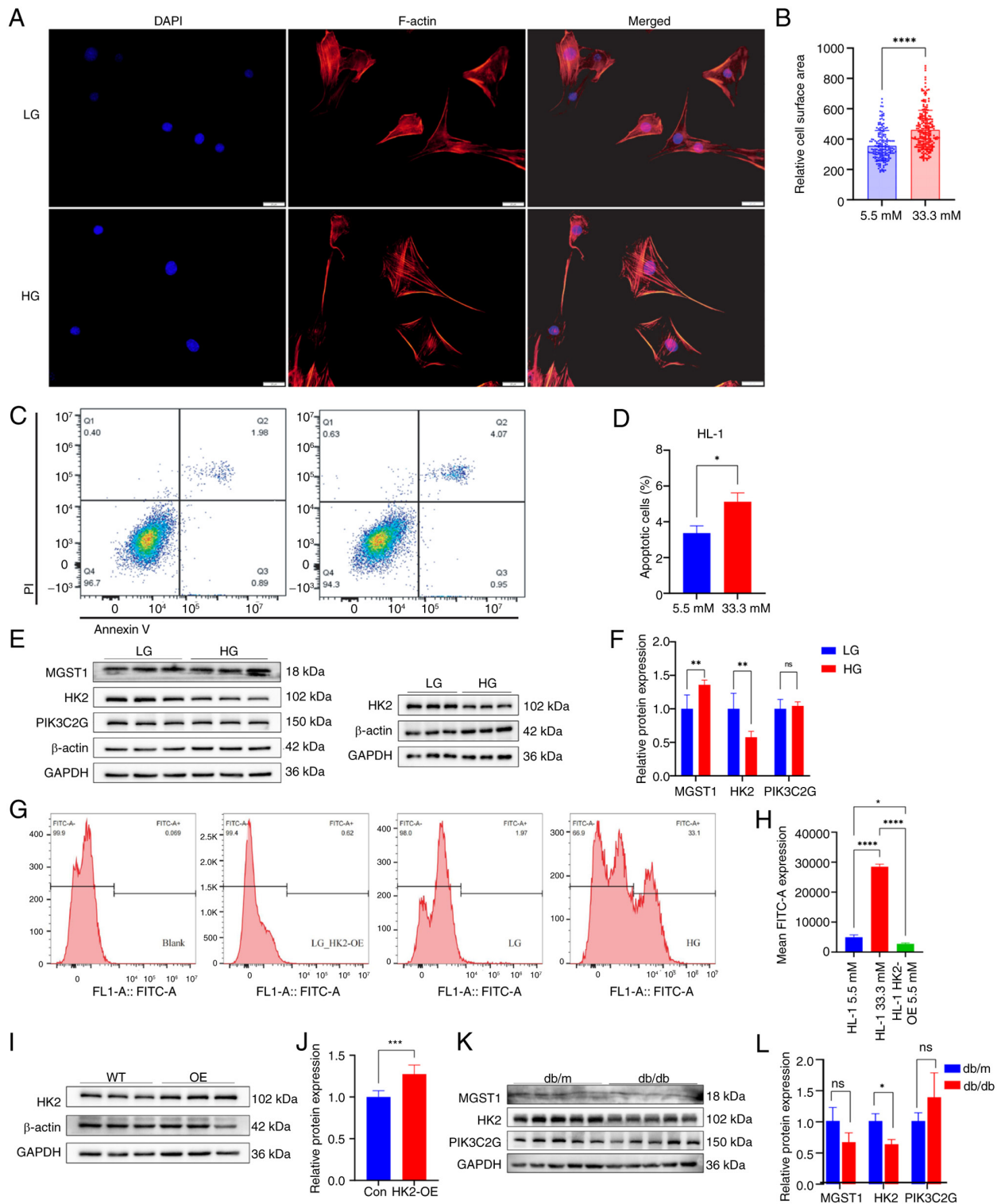


Figure 4. Validation of the HL-1 cardiomyocyte model and western blot analysis of myocardial tissue. (A) F-actin in HL-1 cardiomyocytes treated with HG or LG. (B) Cell surface area. (C) HL-1 cardiomyocytes treated for 48 h were subjected to apoptosis analysis via flow cytometry. (D) Proportion of apoptotic cells. (E) Western blot analysis of HL-1 cardiomyocytes. (F) Semi-quantitative results of western blot analysis. (G) Representative flow cytometry and (H) quantification of ROS levels revealed high ROS levels in the HG group and a significant decrease in ROS levels in response to HK2-OE. (I) Representative western blotting images and (J) semi-quantification of HK2-OE and WT HL-1 cardiomyocytes. (K) Representative western blot and (L) semi-quantification of db/db and db/m mouse cardiomyocytes. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$. ROS, reactive oxygen species; HK2, hexokinase 2; OE, overexpression; LG, low glucose; HG, high glucose; WT, wild-type; QQ, quantile-quantile; ns, not significant; F, filamentous; MGST1, microsomal glutathione S-transferase 1; PIK3C2G, phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 gamma; con, control.

treatment (Fig. 4B). The number of apoptotic cells was significantly higher following high-glucose compared with low-glucose treatment (Fig. 4D and E).

Western blot analysis revealed that MGST1 protein expression increased, while HK2 protein expression decreased in HL-1 cells treated with high- compared with low-glucose

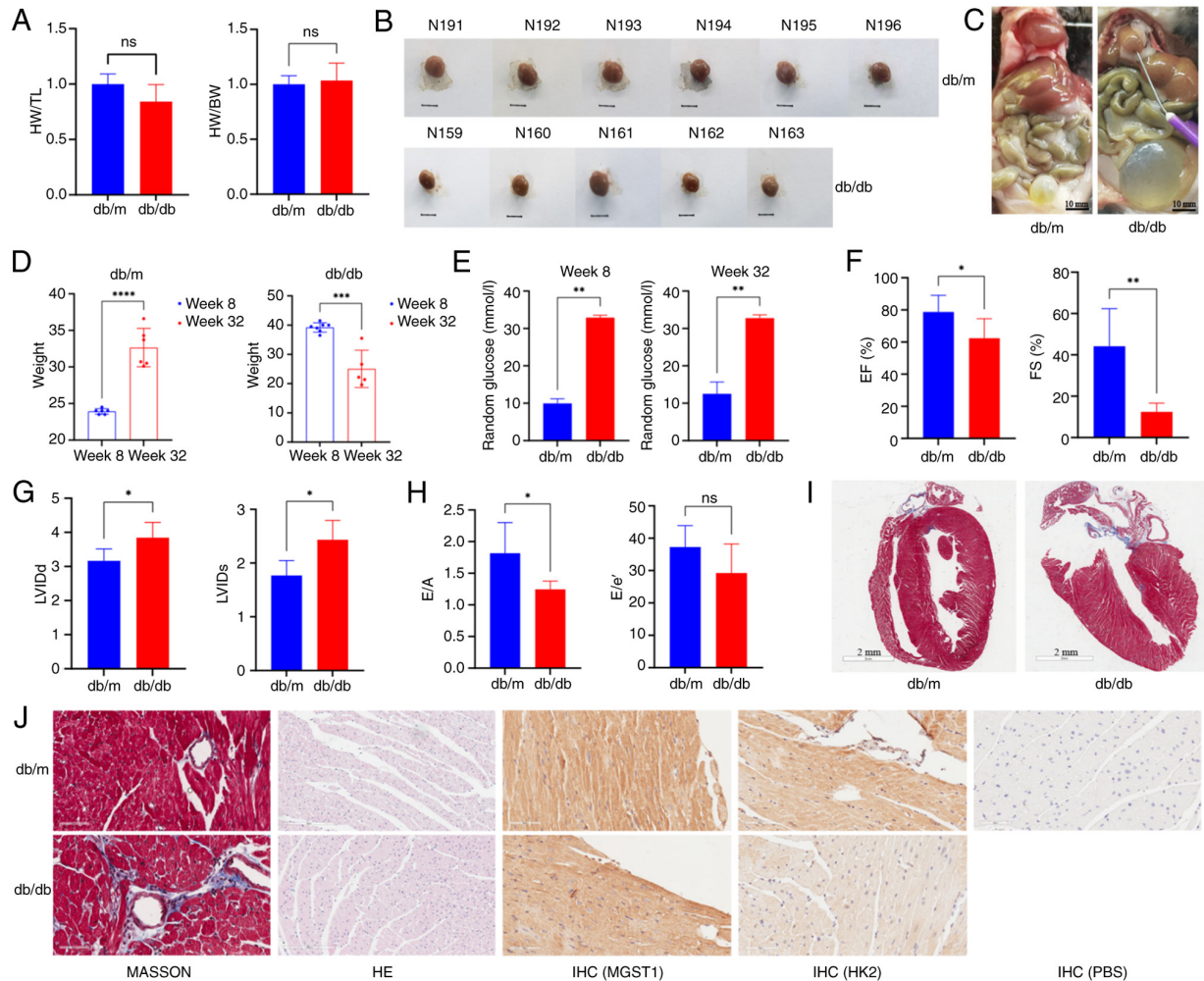


Figure 5. Indicators of metabolic and cardiac dysfunction, cardiac structure, and function in mice. (A) HW/TL and HW/BW ratio. (B) Mouse hearts. N191-N196 are db/m mouse hearts, while N159-N163 are db/db mouse hearts. Scale bar, 5 mm. (C) Mouse anatomy. db/db mice exhibited large white fat deposits on the heart surface and overfilled bladders. (D) Mouse body weight change. (E) Blood glucose levels. (F) EF. (G) LVIDd and LVIDs. (H) E/A and E/e' ratios reflecting cardiac diastolic function. (I) Masson staining of left ventricular long axis sections. (J) Masson, H&E and immunohistochemical staining of cardiac tissue. Compared with db/m mice, db/db mice presented severe fibrosis, decreased cardiomyocyte hypertrophy, reduced tissue gap and low hexokinase 2 expression. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. ns, not significant; IHC, immunohistochemistry; H&E, hematoxylin and eosin; EF, ejection fraction; LVIDd, diastolic left ventricular internal diameter; LVIDs, systolic left ventricular internal diameter; HW, heart weight; TL, tibia length; FS, fractional shortening; BW, body weight; E/A, early-to-late diastolic mitral inflow ratio; E/e', early diastolic mitral annular velocity to early diastolic mitral inflow ratio; MGST1, microsomal glutathione S-transferase; HK2, hexokinase 2.

(Fig. 4E and F). PIK3C2G expression did not significantly differ between low-glucose and high-glucose treatment groups (Fig. 4E and F).

ROS production was significantly elevated in HL-1 cells treated with high-glucose, whereas ROS production was significantly decreased in HL-1 cells following HK2-OE (Fig. 4G and H). The increase in HK2 protein expression after HK2-OE was validated by a lentiviral transfection assay (Fig. 4I and J).

The present study found that HK2 was abundantly expressed in the nuclei of HL-1 cells cultured under high-glucose conditions (Fig. S1), suggesting it may influence the regulation of disease-associated genes in DCM. LLPS was detected in both HK2-WT and HK2-OE HL-1 cardiomyocytes by immunofluorescence analysis (Fig. S2).

Mouse model validation and histological testing. In the present study, db/db mice were selected as a model for T2D,

whereas db/m mice served as the control group. Western blot analysis of cardiac tissue revealed that HK2 was expressed at lower levels in the cardiac tissue of db/db compared with db/m mice. The difference in MGST1 and PIK3C2G cardiac tissue expression between the two groups was not significant (Fig. 4K and L). The heart-to-body weight ratio and the heart weight-to-tibia length ratio did not significantly differ between the two groups (Fig. 5A). Gross examination of the cardiac tissue indicated that the hearts of db/m mice were larger than those of db/db mice (Fig. 5B). A total of one db/db mouse died at ~32 weeks of age, and four of the surviving db/db mice exhibited significant frontal bladder overfill (4/5), suggesting the possibility of neurogenic cystitis with urinary retention (Fig. 5C).

To evaluate the effects of the intervention, blood glucose levels and body weight were measured at 8 and 32 weeks of age. At 8 weeks, the body weight of the db/db mice was significantly greater than that of the control mice, with a mean

body weight ranging from 35 to 40 g. However, at 32 weeks, the db/db mice exhibited a significant decrease in body weight, and their mean weight was notably lower compared with that of the control mice (Fig. 5D). Blood glucose levels were significantly elevated in db/db mice compared with control mice at 8 and 32 weeks (Fig. 5E). Echocardiography data indicated that the LVEF and LVFS were significantly lower in db/db compared with db/m mice at 32 weeks (Fig. 5F). Additionally, the left intraventricular diameter was greater in db/db mice (Figs. 5G and S3), and diastolic function was impaired in these mice (Fig. 5H). Histological staining revealed increased cardiac fibrosis and cardiomyocyte hypertrophy in db/db mice (Fig. 5I and J).

Immunohistochemistry staining revealed no notable difference in MGST1 expression. By contrast with db/db mice, expression of HK2 was higher in the cardiac tissue of db/m mice. Similarly, the expression levels of HK2 in both db/m and db/db mice were greater than those in the PBS-treated controls (Fig. 5J).

Transcriptomic analysis and metabolic data. Transcriptomic sequencing analysis was conducted on HK2-WT and HK2-OE cells. Energy and lipid metabolisms were quantitatively analyzed in six samples from each group. Transcriptomic sequencing was performed using Illumina high-throughput sequencing platform, which included three HK2-WT and three HK2-OE samples. Similarities between the samples were assessed through principal component analysis (PCA) (Fig. 6A). Gene expression was quantified using fragments per kilobase of transcript per million fragments mapped as a measure of gene expression levels (Fig. 6B). DEGs were identified with $\log_2$ (fold-change) ≥ 1 and a false discovery rate < 0.05 .

A total of 1,292 DEGs were identified, comprising 432 up- and 860 downregulated genes (Fig. 6C). Energy metabolomics analysis was performed using a LC-MS/MS detection platform in conjunction with the MetWare database, resulting in the detection of 57 metabolites (Fig. 6D; Table SIV). Differences in metabolites within sample groups were analyzed using PCA (24) (Fig. 6E). In total, 11 differentially expressed metabolites were identified based on fold-change ≥ 2 and ≤ 0.5 (Fig. 6F and G; Table SV). Lipid metabolomics was used to quantify 1,269 lipids, and PCA was used to elucidate the overall metabolic differences and variability between samples within groups (Fig. 6H). Lipid cluster analysis revealed significant differential expression of genes associated with lipid metabolism (Fig. 6I). After screening for variable importance in projection value > 1 and $P < 0.05$, 471 differential lipids were identified (Fig. 6J; Table SVI).

Co-analysis of transcriptomics and energy metabolism. KEGG pathway enrichment was compared to identify co-enriched KEGG pathways (Fig. 7A). DEGs and metabolites were enriched in 'purine metabolism', 'nucleotide metabolism' and 'biosynthesis of cofactors'. KEGG enrichment analysis was conducted using the P-values from the transcriptome to identify common pathways within the top 50 most significantly enriched pathways. Transcriptomic analysis revealed significant enrichment of pathways associated with 'glutathione metabolism', 'cholinergic synapse',

'HIF-1 signaling pathway', 'terpenoid backbone biosynthesis', 'insulin resistance', 'alcoholic liver disease' and 'purine metabolism' ($P < 0.05$). Additionally, metabolites involved in 'nucleotide metabolism' were significantly enriched (Fig. 7B). Both histologies exhibited significant enrichment in 'purine metabolism' (Fig. 7C).

Gene and metabolite expression patterns showed consistent differential regulation in quadrants 3 and 7, but opposing patterns in quadrants 1 and 9 (demarcated by black dashed lines), which visualizes gene-metabolite fold-change relationships 1 and 9 (Fig. 7D). DEGs and metabolites enriched in 'purine metabolism' were selected for correlation analysis. A total of four energy-associated metabolites were significantly associated with 14 genes involved in 'purine metabolism' (Fig. 7E). Finally, by constructing a correlation network of genes and metabolites, 114 genes associated with seven energy metabolites were identified (Fig. 7F).

Co-analysis of transcriptomic and lipid metabolism data.

Transcriptomics and lipid metabolomics of the co-enriched KEGG pathways were compared. Metabolites associated with 'glycerolipid metabolism', 'lipid and atherosclerosis', 'sphingolipid signaling pathway', 'insulin resistance', 'ether lipid metabolism' and 'glycerophospholipid metabolism' were enriched (Fig. 8A). Transcriptomic data revealed enrichment in 'vascular smooth muscle contraction', 'Rap1 signaling pathway', 'human cytomegalovirus infection', 'cAMP signaling pathway', 'MAPK signaling pathway', 'amoebiasis' and 'calcium signaling pathway' (Fig. 8A). Downstream genes of HK2, which are involved in various BPs, were significantly enriched in 'calcium signaling pathway', 'thyroid hormone synthesis', 'vascular smooth muscle contraction', 'insulin secretion', 'MAPK signaling pathway', 'regulation of lipolysis in adipocytes', 'aldosterone synthesis and secretion', 'Rap1 signaling pathway', 'cAMP signaling pathway', 'inflammatory mediator regulation of TRP channels' and 'AGE-RAGE signaling pathway in diabetic complications'. Additionally, metabolites associated with 'AGE-RAGE signaling pathway in diabetic complications' and 'sphingolipid signaling pathway' were also significantly enriched (Fig. 8B and C). Numerous metabolites correlated with specific genes (Fig. 8D). Based on this analysis, regulatory network maps for genes and metabolites associated with 'AGE-RAGE signaling pathway in diabetic complications' and 'sphingolipid signaling pathway' were constructed (Fig. 8E and F).

'AGE-RAGE signaling pathway in diabetic complications'.

A total of 14 genes [I16, I11A, thrombomodulin (THBD), angiotensinogen (AGT), COL1A1, COL3A1, endothelin-1 (EDN1), phospholipase C (Plc)b1 (PLCB1), COL4A2, CCL2, TGFB2, SERPINE1, PIK3CD and protein kinase C β (PRKCB)] and 32 lipid metabolites were identified by combined analysis of transcriptomics and lipid metabolomics within 'AGE-RAGE signaling pathway in diabetic complications' and significantly enriched (Fig. 8E). Of these, I16 and I11A were excluded due to the absence of specific primers (Table SI). Quantitative PCR analysis of the mRNAs of the remaining 12 genes in HL-1 cardiomyocytes from the WT and HK2-OE strains indicated that the difference between the

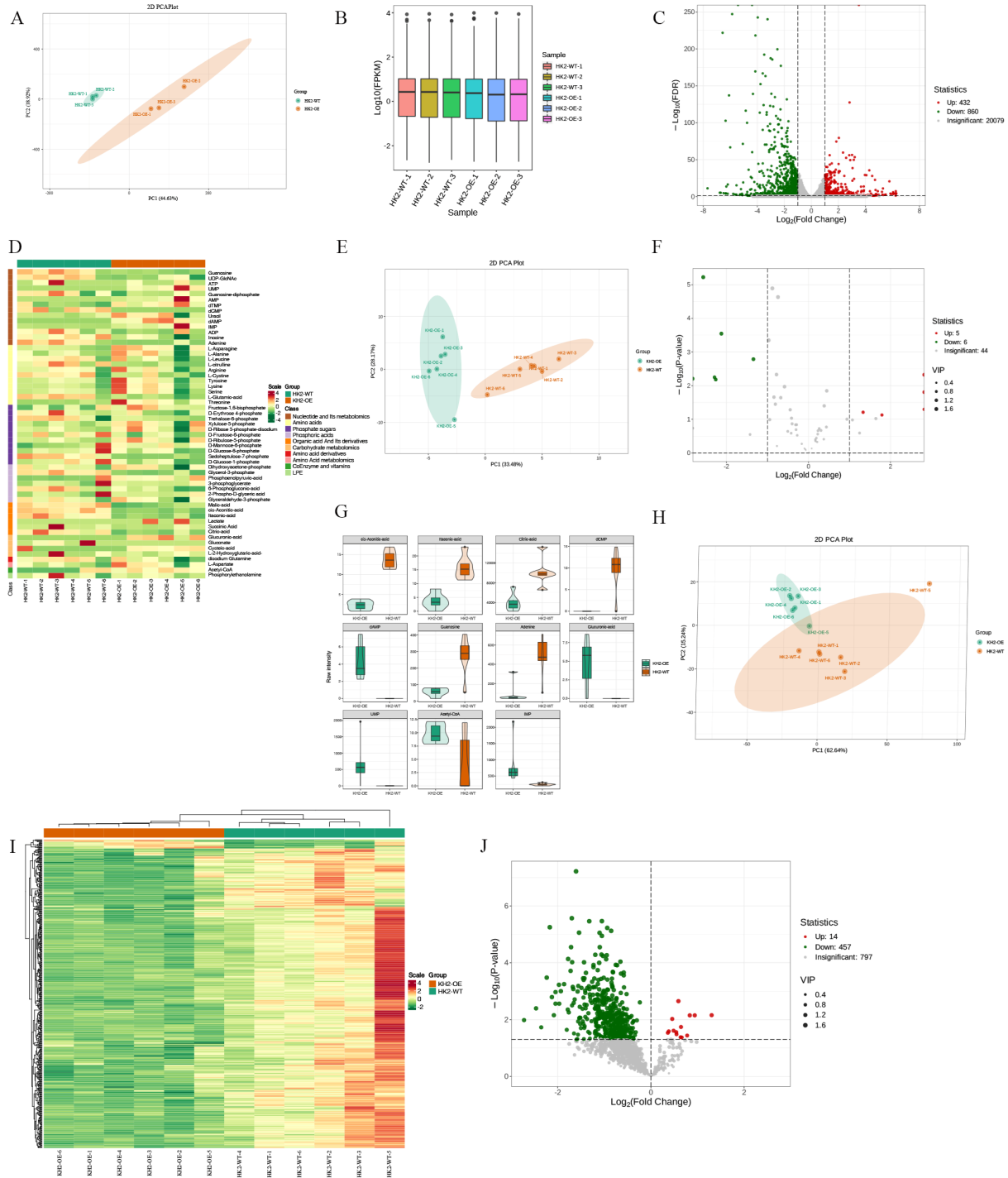


Figure 6. Quantitative transcriptomics and metabolomics analysis of HL-1 cardiomyocytes. (A) PCA plot of transcriptomic data demonstrating differences in gene expression between groups and overall differences between samples. (B) Box plot of gene expression. (C) Differential gene volcano plot. (D) Cluster heatmap of energy-associated metabolites. (E) PCA plot of quantitative analysis of energy metabolites. (F) Volcano plot of differential energy metabolites. In total, 11 different metabolites were identified. (G) Differentially abundant metabolite violin plot comparing the expression of 11 energy-related metabolites among subgroups. (H) Quantitative analysis of lipid metabolites. (I) Cluster heatmap and (J) volcano plot of differential lipid metabolites. PCA, principal component analysis; WT, wild-type; OE, overexpression; FDR, false discovery rate; FPKM, fragments per kilobase per million; HK2, hexokinase 2; VIP, variable importance in projection.

subgroups was not significant for COL3A1. PRKCB exhibited high expression levels in HL-1 cardiomyocytes from the HK2-OE strain, while the other genes were expressed at low

levels (Fig. 9A-C). Additionally, protein-protein interaction network maps were constructed for the remaining 12 genotypes (Fig. 9D).

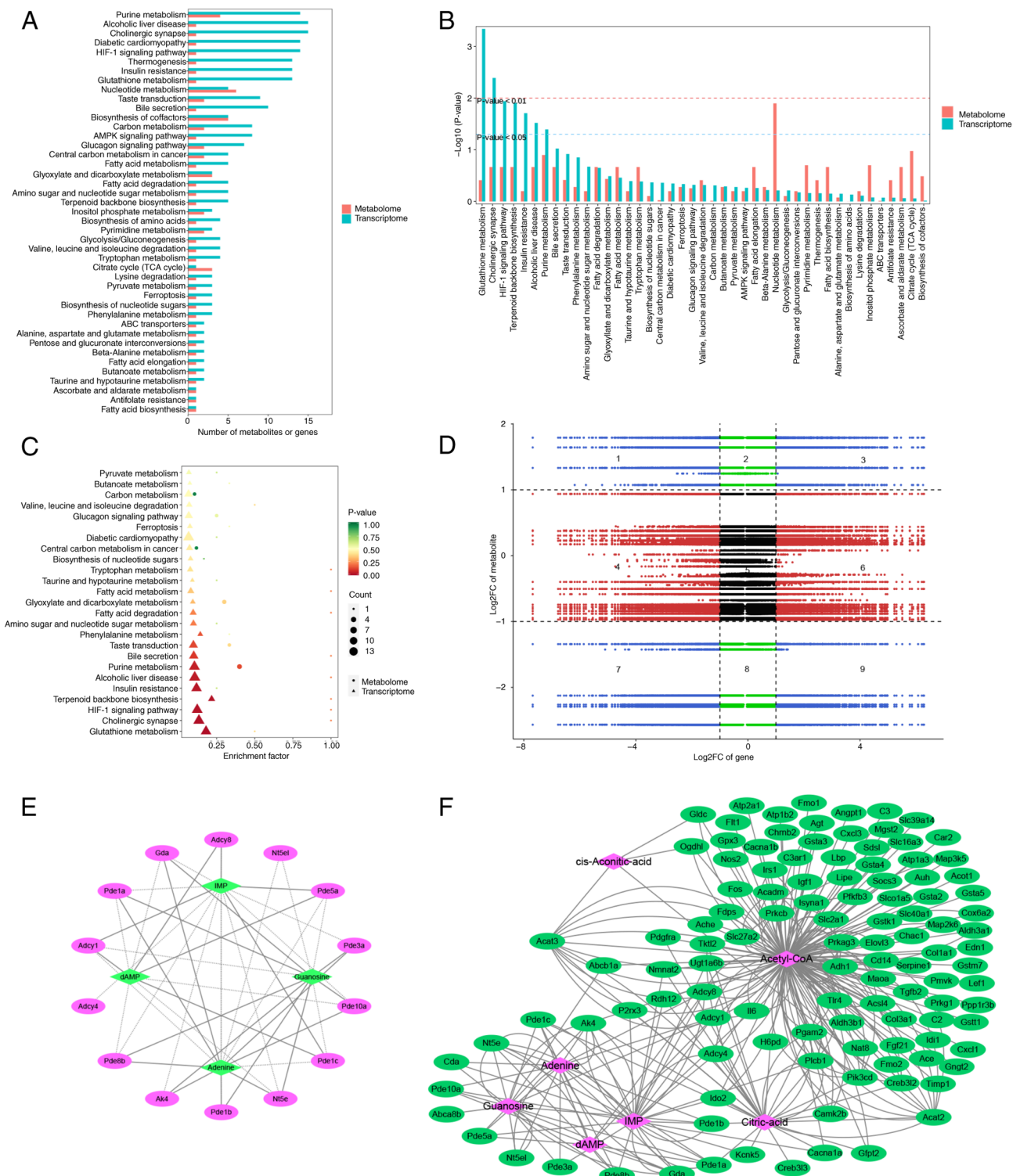


Figure 7. Combined transcriptomics and energy metabolism analysis. (A) KEGG enrichment analysis. (B) KEGG pathway enrichment analysis of the transcriptome (green) and metabolome (red). (C) Bubble plot of the KEGG enrichment analysis. (D) Nine-quadrant plot for correlation analysis. Each point represents a pair of correlations. (E) Correlation network diagram of the purine metabolic pathway. Green, metabolites, whereas the pink ovals represent the corresponding genes. Solid line, positive correlations, while the dashed lines represent negative correlations. (F) Correlation network plot of energy metabolism. Pink, metabolites; green, gene. KEGG, Kyoto Encyclopedia of Genes and Genomes; FC, fold-change.

Discussion

DCM is a distinct form of cardiomyopathy associated with diabetes, characterized by ventricular dysfunction in the absence of traditional cardiovascular risk factors such as

coronary atherosclerosis, hypertension or valvular disease (1). DCM typically presents with early diastolic dysfunction, which can progress to late systolic dysfunction accompanied by fibrosis. This multifaceted disease involves various underlying mechanisms, including mitochondrial dysfunction,

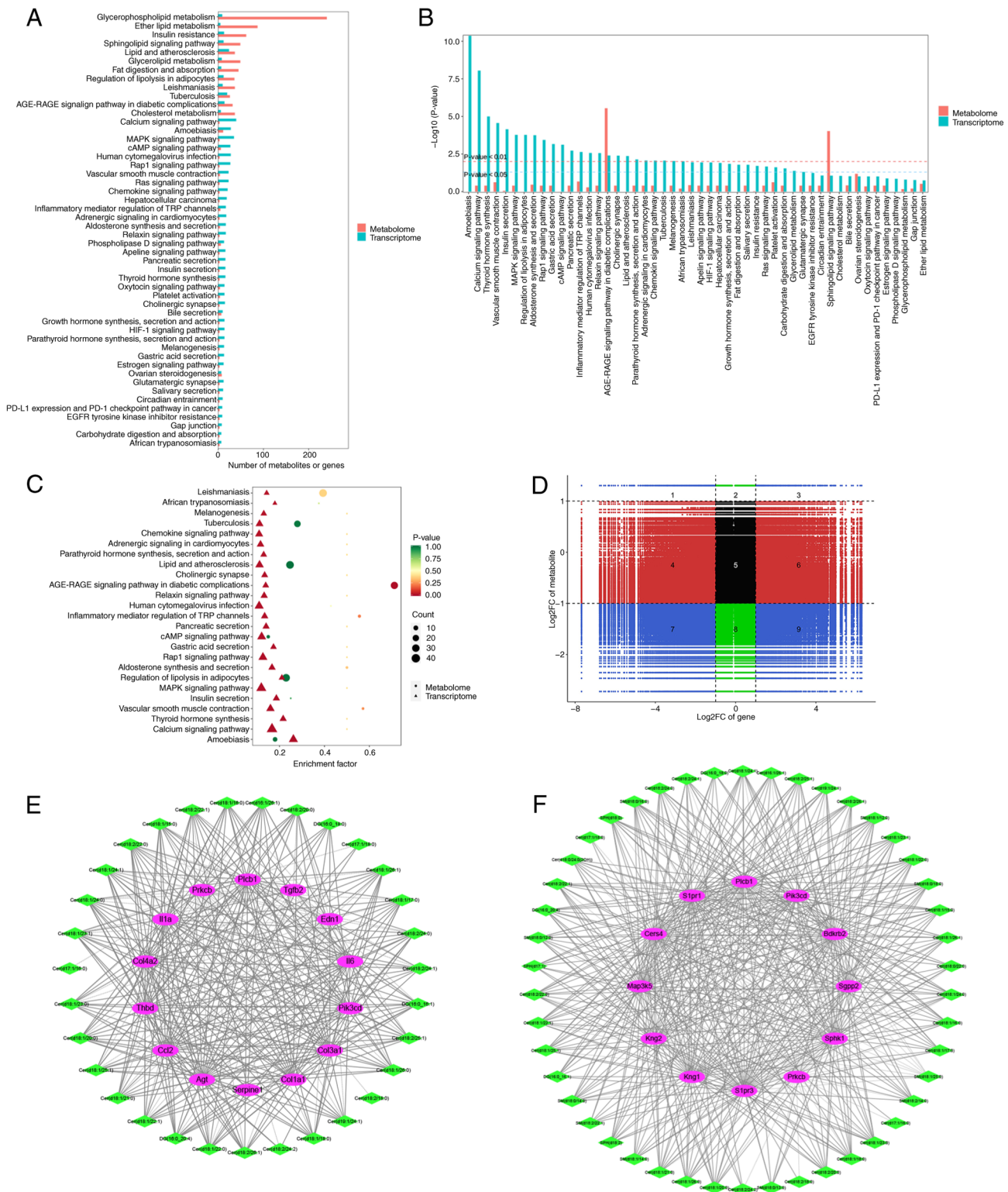


Figure 8. KEGG pathway analysis of transcriptomics and lipid metabolomics co-enrichment. (A) KEGG enrichment analysis. (B) KEGG enrichment analysis column plot. (C) KEGG enrichment analysis bubble plot. (D) Nine-quadrant plot for correlation analysis. Each point represents a pair of correlations. Correlation network plots of (E) advanced glycation end products-receptor for advanced glycation end products and (F) sphingolipid signaling pathways. Green, metabolite; pink, gene. KEGG, Kyoto Encyclopedia of Genes and Genomes; FC, fold-change.

ER and oxidative stress, alterations in the extracellular matrix, disruptions in the insulin signaling pathway, inflammation, microvascular dysfunction and cardiometabolic abnormality (7). Despite extensive research (6,8), the underlying mechanisms of DCM remain poorly understood, and,

to the best of our knowledge, there are currently no targeted treatments or preventive strategies available.

In the present study, bioinformatics analysis revealed that HK2, HMGSC2, MGST1 and PIK3C2G were characteristic genes associated with DCM. Both *in vitro* and *in vivo*

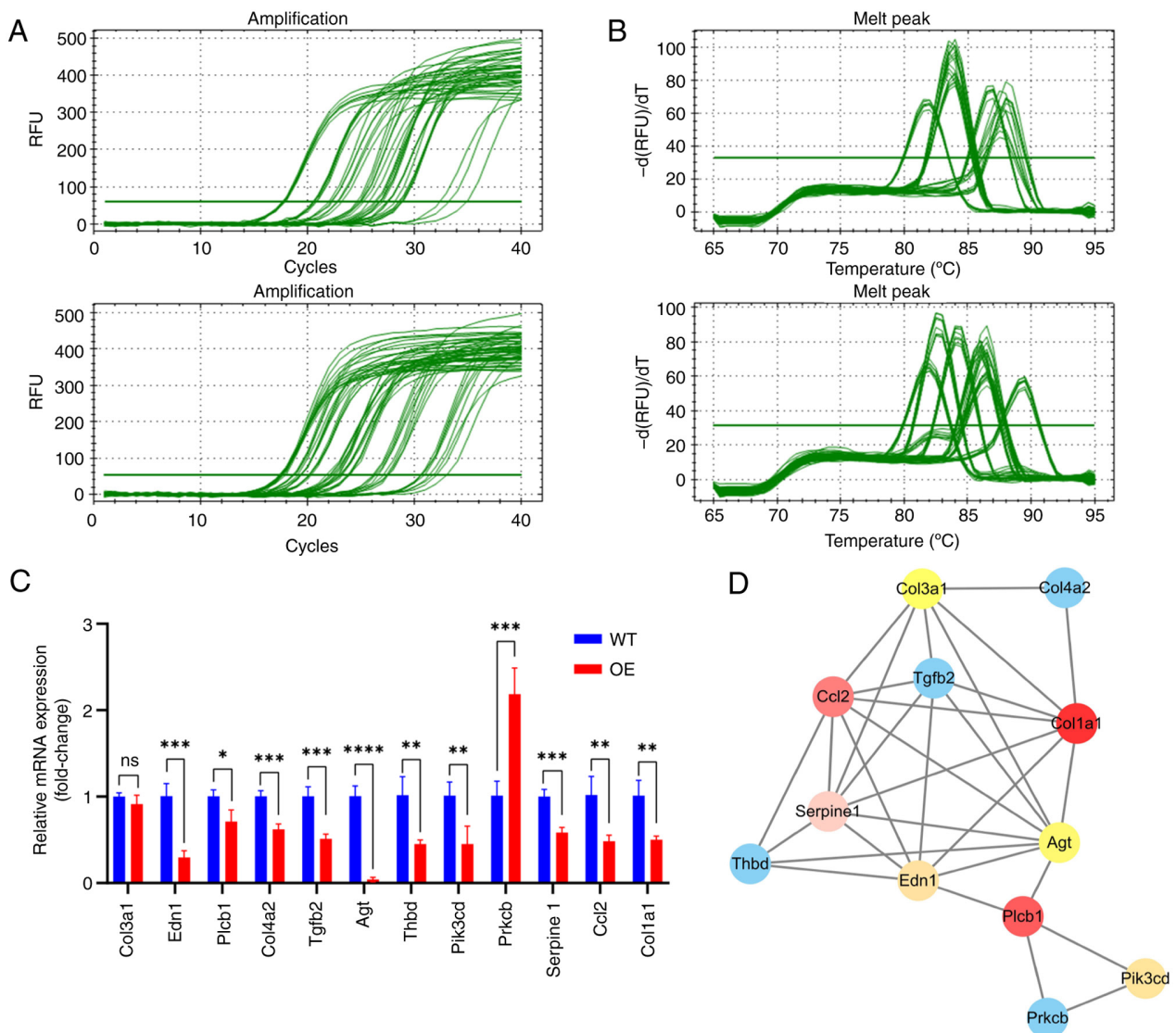


Figure 9. mRNA qPCR data for 12 HK2 downstream genes in 'AGE-RAGE signaling pathway in diabetic complications'. qPCR (A) amplification and (B) melt curve and (C) quantification. (D) Protein-protein interaction network diagram of 12 HK2 downstream genes. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. q, quantitative; HK2, hexokinase 2. WT, wild-type; OE, overexpression; COL3A1, collagen type III alpha 1 chain; EDN1, endothelin-1; PLCB1, phospholipase C beta 1; TGFB2, transforming growth factor β 2; THBD, thrombomodulin; PIK3CD, phosphoinositide 3-kinase catalytic delta; PRKCB, protein kinase C beta; RAGE, receptor for advanced glycation end-products; RFU, relative fluorescence units; ns, ; CCL2, C-C motif chemokine ligand 2.

experiments confirmed that HK2 was associated with DCM, exhibiting significantly decreased expression levels in affected tissues. Apoptosis and ROS levels were notably elevated in HL-1 cardiomyocytes treated with high-glucose. HK2-OE resulted in a significant decrease in ROS production and apoptosis in HL-1 cardiomyocytes.

Oxidative stress serves a key role in the progression of DCM. ROS interact with protein, lipids and DNA, leading to detrimental remodeling of cardiac tissue (25). Mitochondrial ROS production is a key factor in the pathogenic mechanisms triggered by elevated blood sugar levels; excessive ROS can cause DNA damage as well as oxidative modifications of proteins and lipid (26). The upregulation of heme oxygenase induced by diabetes contributes to oxidative stress in the myocardium (27). The activation of acidic sphingomyelinase (ASMase) in cardiomyocytes due to a high-fat diet may exacerbate oxidative stress and apoptosis by upregulating the expression of NADPH oxidase 4, leading to DCM (28).

The present analysis revealed that the HK2 gene regulates the expression of 12 genes in 'sphingolipid signaling pathway' (including sphingosine-1-phosphate receptors 1/S1PR3, kinases SPHK1/PIK3CD/PRKCB, metabolic enzymes CERS4/SGPP1, and kallikrein-kinin system components KNG1/KNG2/BDKRB2) and 49 lipid metabolites, including glycerolipids, ceramides and sphingomyelins (SMs). SM serves as a precursor to ceramide, a metabolite generated by the cleavage of SM by neutral sphingomyelinase or ASMase (29). SM is a key component of cell membranes, while ceramide functions as a second messenger of lipids, regulating phosphatase, kinases and transcription factors (30), and is associated with cell proliferation and apoptosis. Lower concentrations of SM and ceramide promote cell proliferation and function, whereas elevated levels lead to cell dysfunction and death (31). Diacylglycerol (DG) and SM are produced by SM synthase 1 (SMS1) from phosphatidylcholine and ceramide as substrates. SMS1 catalyzes the biosynthesis of DG, which modulates

SMS1 activity (32). Therefore, HK2 may influence the function of cardiomyocytes in DCM through the metabolism of ceramide and SM.

In the present study, db/db mice revealed a notable accumulation of white adipose tissue on the epicardial surface. The bladder of these mice exhibited excessive urinary storage, suggesting that the 32-week-old db/db mice may have experienced severe diabetic neurogenic cystitis and abnormal lipid accumulation as comorbidities. By contrast with Li *et al* (33), db/db mice in the present study displayed significant emaciation after 24 weeks of continuous feeding from baseline (8 weeks). There was no notable difference in heart size or weight or cardiac decompensation. This may be associated with the severe protrusion of the enlarged bladder into the gastrointestinal tract, which affects feeding and inflammatory responses in the mice. Cardiac tissue staining indicated increased myocardial fibrosis, myocardial hypertrophy, reduced myofibrillar interstitial space and low expression of HK2, which was consistent with the present bioinformatics analysis.

Previous studies have reported decreased HK2 expression in diabetic mice (34-36). HK2-OE has been demonstrated to improve cardiomyocyte contractile function and elevate ATP levels in hypoxic diabetic mice (37). During aging, methyltransferase-like 3 induces HK2 expression, leading to metabolic reprogramming that promotes liquid-liquid phase separation (LLPS) (38). LLPS is a biophysical phenomenon characterized by the condensation of biomolecules, such as protein or nucleic acids, into membraneless, liquid-like droplets within cells. This process is primarily driven by multivalent interactions and the presence of intrinsically disordered regions within proteins (39). These dynamic condensates serve a key role in cellular function by spatially organizing molecules and promoting specific biochemical reactions (40). Notably, LLPS was more prominent in HK2-OE compared with HK2-WT cells. This suggests HK2-OE may facilitate the formation of biomolecular condensates, potentially via enhanced interactions with protein or nucleic acids that drive phase separation. Previous studies have demonstrated that metabolic enzymes, including HK2, engage in LLPS, thereby influencing cellular metabolism and stress responses (41,42). The increased LLPS in HK2-OE cells may indicate a role for HK2 in the assembly of metabolic compartments or stress granules, which may be vital for maintaining cardiomyocyte function under both physiological and pathological conditions (43). The detection of LLPS in cardiomyocytes underscores its potential role in cardiac biology. For example, LLPS is associated with the regulation of sarcomere organization, calcium signaling and cellular stress responses in the heart (44). Further exploration of the molecular mechanisms governing LLPS in cardiomyocytes, particularly in the context of HK2-OE, may yield valuable insights into the pathogenesis of cardiac disorders, including hypertrophy, heart failure and ischemia-reperfusion injury. Purines serve a key role in cellular metabolism, serving as an energy source and essential component in ATP synthesis. HK2 is a key enzyme that regulates purine metabolism, and is involved in the metabolic processes of AMP, ADP, ATP, inosine monophosphate, adenosine and inosine (38). The present energy analysis revealed that HK2 modulated

the purine metabolites adenine, dAMP, guanosine and IMP through 14 downstream genes. In a normal resting state, 70% of total cardiac energy production from ATP is derived from fatty acid oxidation. However, in hearts affected by DCM, glucose use is notably altered, and the energy source of the heart predominantly relies on fatty acids (45).

T2D is a chronic inflammatory disease characterized by inflammation and immune senescence (46). Metabolic stress-driven immune senescence is a key factor contributing to cardiac dysfunction in DCM (47). AGEs are formed when protein, lipid and nucleic acids undergo non-enzymatic modification through glycosylation (involving glucose, fructose and pentose) via the Maillard reaction (48). The interaction of AGEs with RAGE triggers oxidative stress, inflammatory response and increased extracellular matrix accumulation (49). Blocking RAGE prevents cardiac dysfunction in db/db mice (50). The present study identified that HK2 regulated 30 lipid submetabolites (including glycerolipids and ceramides) through 14 downstream genes (I16, I11A, THBD, AGT, COL1A1, COL3A1, EDN1, PLCB1, COL4A2, CCL2, TGFB2, SERPINE1, PIK3CD, PRKCB). The accumulation of toxic lipid metabolites is associated with mitochondrial function, bioenergetics and metabolic regulation, leading to metabolic remodeling of the DCM heart (51) and supporting alterations in the cardiomyocyte microenvironment (52).

THBD is implicated in coagulation, innate immunity, inflammation and cell proliferation, and is primarily located in the vascular endothelium (53). THBD-dependent activated protein C formation protects glomerular cells and podocytes from glucose-induced injury (54). Through its lectin-like domain, THBD mitigates ischemia-reperfusion injury in cardiomyocytes (55).

EDN1 may mediate cardiomyocyte hypertrophy in DCM via the MAPK pathway, while EDN1 antagonists prevent glucose-induced cardiomyocyte hypertrophy and AGT expression (56). AGT, the precursor of all angiotensin peptides, not only serves as a renin-angiotensin system substrate but also promotes macrophage infiltration, enhances adipocyte metabolic activity (57) and accelerates cancer progression (58). Hepatocytes are the primary source of AGT, with adipose tissue contributing ~25% of plasma AGT levels. Elevated AGT expression is associated with cardiovascular disease and obesity (59). The upregulation of COL1A1 and COL3A1 promotes myocardial fibrosis in DCM, facilitated by an increase in circular RNA homeodomain-Interacting Protein Kinase 3 (circHIPK3) in DCM mice (60). COL4A2 encodes type IV collagen fibers, which are key for the integrity and function of the basement membrane (61) and are poorly expressed in DCM (62).

The PLC enzyme hydrolyzes phosphatidylinositol diphosphate into DG and inositol triphosphate, which are key lipid mediators involved in cardiomyocyte regulatory signaling as second messengers (63). Elevated PLCB1 expression is associated with atrial dilation in both humans and mice (64). High-glucose induces an increase in CCL2 and IL-6 expression, with glycemic control decreasing IL-6 but not CCL2 levels (65). This may explain why partial glycemic control is ineffective in alleviating DCM progression. High-glucose-induced expression of TGFB and receptor activation promotes myocardial fibrosis in DCM (66).

SERPINE1 is a key gene in DCM and may be associated with impaired mitochondrial function, although the exact mechanism remains unclear (67). Hepatic PIK3CD levels are significantly decreased in mouse models of diabetes, and PIK3CD is associated with insulin resistance (68). High-glucose triggers the activation of PRKCB and increases DG levels. Overexpression of PRKCB induces cardiac hypertrophy and heart failure in mice. HG-induced oxidative stress may promote diabetic complications via the DG/PRKCB pathway (69). Consequently, downstream HK2-regulated genes involved in the AGE/RAGE pathway are associated with DCM.

Although the present study provided insight into the role of HK2 in DCM and its potential as a therapeutic target, there are limitations. First, the findings are primarily derived from preclinical models, including HL-1 cardiomyocytes and db/db mice, which may not fully replicate the complexity of the human DCM pathophysiology. Second, the present study lacked transcriptomic and metabolomic analyses of myocardial tissue, as well as investigation of the functional effects of altered metabolite levels on DCM progression. Third, the lack of validation using clinical samples limits the direct translation of the present results to human patients. Future studies should incorporate patient-derived samples or clinical data to validate the therapeutic potential of HK2 and explore the mechanisms of HK2 by manipulating key genes or metabolites within the metabolic pathway to facilitate clinical application.

In conclusion, HK2 is a key enzyme in glycolysis and associated with the pathogenesis of DCM. HK2-OE in DCM cardiomyocytes markedly protects against apoptosis and decreased ROS levels. Additionally, HK2 modulates glucose and lipid metabolism in cardiomyocytes through numerous metabolic pathways. Metabolic intermediates and end products may inversely regulate gene and protein expression. The present study suggested that the AGE/RAGE signaling pathway is a notable mechanism by which HK2 regulates lipid metabolism and is associated with diabetic complications.

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Availability of data and materials

The data generated in the present study may be found in the National Center for Biotechnology Information BioProject and European Molecular Biology Laboratory's European Bioinformatics Institute MetaboLights under accession number PRJNA1234119 and MTBLS12315 and MTBLS12314, respectively, or at the following URL: (<https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1234119>; <https://www.ebi.ac.uk/metabolights/MTBLS12315>; <https://www.ebi.ac.uk/metabolights/MTBLS12314>).

Authors' contributions

YD and YZ conceived the study. BL and XZ designed experiments, analyzed data and constructed figures. BL, XZ, LM, XW, YD and YZ performed experiments. YD supervised the study. BL, XZ, LM and XW wrote the manuscript. BL, XZ, YD and YZ edited the manuscript. YD and YZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Center's Experimental Animal Ethics Committee of Hubei University of Medicine (approval no 2024S071; Shiyan, China) and were conducted in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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