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**The Whole-transcriptome Landscape of Diabetes-related Sarcopenia Reveals the Specific
Function of Novel lncRNA Gm20743**

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23 **Abstract**

24 **Background:** While the exact mechanism remains unclear, type 2 diabetes mellitus (T2DM) has been
25 shown to increase the risk of sarcopenia that is characterized by decreased muscle mass, strength, and
26 function. This study aimed to identify the pathophysiological role of lncRNAs in diabetic sarcopenia.
27 **Methods:** Whole-transcriptome RNA sequencing was performed on the gastrocnemius from the overt
28 diabetes-induced sarcopenia model of *db/db* mice. Informatics and co-expression networks were
29 performed to predict key lncRNAs and their potential regulation circuits in diabetes-induced sarcopenia.
30 To determine the specific function of lncRNA Gm20743, the detection of Mito-Sox, reactive oxygen
31 species, Ethynyl-2'-deoxyuridine, and myosin heavy chain was performed in overexpressed-Gm20743
32 C2C12 cells. **Results:** RNA-seq data and informatics revealed the key lncRNA-mRNA interactions
33 and indicated a potential regulatory role of lncRNAs. Meanwhile, we characterized three core
34 candidate lncRNAs Gm20743, Gm35438, and 1700047G03Rik and their potential function.
35 Furthermore, the results suggested lncRNA Gm20743 may be involved in regulating mitochondrial
36 function, oxidative stress, cell proliferation, and myotube differentiation in skeletal muscle cells.
37 **Conclusion:** These findings significantly improve our understanding of lncRNAs that may mediate
38 muscle mass, strength, and function in diabetes and represent potential therapeutic targets for diabetes-
39 induced sarcopenia.

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46 **Keywords:** obese; T2DM; transcriptional regulation; lncRNAs; Gm20743

Introduction

Obesity and diabetes are serious global health problems. Obesity is a global chronic health problem attributed to almost 44% of type 2 diabetes mellitus (T2DM) ^{1,2}. Skeletal muscle is a major organ for glucose metabolism. In addition, skeletal muscle loss is closely related to insulin resistance and metabolic syndromes such as obesity and diabetes ³. Loss of skeletal muscle and intramuscular fat disposition is associated with various pathological mechanisms, including insulin resistance, oxidative stress, mitochondrial dysfunction, and inflammatory cytokines ⁴. As a chronic complication of diabetes, the progress of sarcopenia was further exacerbated in patients with T2DM and is a serious risk factor that may result in mortality ⁵. Sarcopenia is characterized by a loss of skeletal muscle strength, mass, and physical function ⁶. According to the review in the Lancet recently, sarcopenia can occur in mid-life associated with a range of conditions such as obesity and T2DM ⁷. Decreased muscle strength, mass, and physical function were reported in T2DM patients compared to healthy individuals ^{8,9}. Rodent research also found that the accumulation of advanced glycation end-products may impair muscle mass and function, leading to muscle loss in diabetic mice ¹⁰. Moreover, with a higher risk of falls, disability, and mortality, sarcopenia associated with obesity and diabetes impairs the individual's quality of life and the prognosis of T2DM ^{11,12}. Although there are many known risk factors and symptoms of diabetes-associated sarcopenia, the underlying mechanism remains unclear.

Most of the human genome is expressed as primary transcripts. However, only approximately 2% of transcripts (known as messenger RNAs, mRNAs) encode functional proteins, while non-coding transcripts (known as non-coding RNAs, ncRNAs) account for approximately 98% of transcripts ¹³. NcRNAs are mainly divided into two categories: long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) ¹⁴. LncRNAs are a class of longer than 200nt RNAs with weak or no protein-coding potential ¹⁵. LncRNAs have a lower transcript abundance but show stronger tissue specificity ¹⁶.

LncRNAs can bind to miRNA sites as competing endogenous RNAs (ceRNAs), and the co-expression of lncRNA-mRNA is involved in pathological and physiological functions in skeletal muscle cells¹⁷. LncRNAs are closely related to the progression of skeletal muscle and diabetes development¹⁸, therefore serving as therapeutic targets, potential biomarkers, or indicators of prognosis. However, the gene expression profiling and precise regulatory roles of lncRNAs during diabetes-induced sarcopenia are still unknown.

In our study, we utilized an overt obese and diabetes *db/db* model in which muscle loss was evidenced and an acute stimulation *in vitro* model of palmitic acid (PA)-treatment in C2C12 myotubes. Skeletal muscle fibres can be categorized into slow (Type I) and fast-twitch fibres (Type II)¹⁹, the preferred atrophy of type II fast-twitch fibres like gastrocnemius (GAS) seems to occur in patients with diabetes and muscle atrophy²⁰. Therefore whole-transcriptome sequencing was performed on the GAS from *db/db* mice and their littermates *db/m* mice. Subsequently, differential expression and functional prediction analysis were performed to explore the gene-regulatory circuits that are associated with diabetes-related sarcopenia. In addition, the candidate lncRNAs (such as Gm20743 (log2FC = -1.57, *q*-value < 0.001), Gm35438 (log2FC = -1.90, *q*-value < 0.001) and 1700047G03Rik (log2FC = 6.34, *q*-value < 0.001)) and their potential function were identified by co-expression network and informatics. In this study, we will focus on the specific function of lncRNA Gm20743 in the development of diabetes-related sarcopenia. An *in vitro* cultured skeletal muscle cells with overexpressed lncRNA Gm20743 by lentivirus transfection were applied to further determine the role of Gm20743 in regulating mitochondrial function, oxidative stress, cell proliferation, and myotube differentiation. Together, this study provides novel insights into the regulatory molecular mechanism of the progression of diabetes-related sarcopenia and potential targets for early diagnosis and therapeutic implications.

96 **Materials and Methods**

97 **Animals**

98 Eight-week-old male *db/db* mice (C57BLKS- *Lepr*^{db/db} diabetic mice) (n=10) and *db/m* male wild-type
99 littermates (C57BLKS- *Lepr*^{db/m} mice) (n=10) were purchased from SPF Biotechnology Company
100 (Beijing, China). *Db/m* mice were considered as the normal control group. All mice were fed with a
101 standard chow diet (18 kcal% fat, 24 kcal% protein, 58 kcal% carbohydrates; Research Diets, Inc) for
102 seven weeks. Bodyweight and blood glucose were measured. Grip strength was measured by using an
103 electronic grip strength metre (Cat. 47200, Ugo Basil) as described previously ²¹. The grip index is
104 reported as grip strength divided by body weight. Body composition was measured as described
105 previously ²¹ by dual-energy X-ray absorptiometry (DEXA, Hologic Discovery A, Hologic Inc) (n=5).
106 After fasting for 6 hours, mice were anaesthetized and sacrificed with 2% pentobarbital sodium
107 solution at fifteen-week of age. Blood was harvested, and serum biochemical indexes were determined
108 by an automatic biochemistry analyser (n=5). GAS was harvested and stored in liquid nitrogen for
109 RNA-seq and molecular biological examinations. Approval for this research was obtained by the Ethics
110 Committee of Chongqing Medical University (Chongqing, China), and all procedures complied with
111 the guidelines and principles for the care and use of laboratory animals approved by Chongqing
112 Medical University.

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114 **Histological analysis**

115 GAS was fixed in 4% paraformaldehyde for 24 hours, dehydrated in gradient alcohol, and then
116 embedded in paraffin. The samples were cut into 6-μm-thick sections. GAS sections were stained with
117 haematoxylin and eosin (H&E) and Masson trichrome, as described previously ²². Frozen sections were

118 stained with Oil-red O²¹. The average cross-sectional area (CSA), fibrosis, and Oil-red O-positive area
119 of GAS myofibres were measured and analyzed by ImageJ software (NIH, USA).

121 **Transmission electron microscopy (TEM)**

122 GAS was cut into 1 mm³ cube and fixed rapidly. After dehydration, infiltration, embedding, and
123 polymerization, samples were processed for TEM analysis as described previously²¹. The images were
124 taken at ×20 000 and ×50 000 magnification by a transmission electron microscope (HT7700,
125 HITACHI).

127 **RNA isolation and RNA-seq**

128 Total RNA of GAS (medial and lateral portions) was extracted from each mouse (each group n=3)
129 using TRIzol (Invitrogen) according to the instructions. The integrity and purity of total RNA were
130 measured by using a NanoDrop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific). RNA
131 samples with RIN≥8 and 28S/18S≥1.5 were sequenced. mRNA was purified by using a Dynabeads
132 mRNA Purification Kit (Invitrogen). The products were amplified using PCR to construct the final
133 cDNA library. The qualified cDNA library was constructed and sequenced using the paired-end mode
134 on the BGISEQ-500/MGISEQ-2000 System of the BGI-Tech Bioinformatics Institute (Shenzhen,
135 China)

137 **Analysis of RNA-seq data**

138 All profiling analyses for RNA-seq were processed with the assistance of BGI. The raw sequencing
139 data were mapped to the reference genome by using the HISAT2 programme. The raw data were
140 aligned to the database built by BGI, and the expression level of genes was calculated by using the
141 RSEM programme. DESeq2 was used to analyse the differential expression of genes (DEGs) with the
142 following criteria: $|\log_2 FC| > 0.585$, and q-value or false discovery rate (FDR) < 0.05. The heatmap

was generated by using the pheatmap (v1.0.8) programme according to the gene expression in each sample.

Functional enrichment analysis of DEGs

Gene Ontology (GO) enrichment analysis was performed to construct the three main functions of differentially expressed mRNAs (dif-mRNAs) ($q\text{-value} < 0.05$). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted to obtain pathway sets containing dysregulated mRNAs ($q\text{-value} < 0.05$) in the molecular biological mechanism network. An enrichment score with a $-\log_{10}(q\text{-value})$ value denotes significance for the GO terms and KEGG pathways ($q\text{-value} < 0.05$). Gene set enrichment analysis (GSEA) was performed to determine novel and potential biological functions with a predefined set of genes ($p\text{-value} < 0.05$).

Construction of co-expression networks

Co-expression analysis of dif-mRNAs and differentially expressed lncRNAs (dif-lncRNAs) was based on the calculation of the Pearson correlation coefficient (PCC)²³. Dif-lncRNAs and dif-mRNAs ($q\text{-value} < 0.05$) with a $PCC > 0.95$ were selected to construct lncRNA-mRNA co-expression networks. Visual networks were produced with Cytoscape software.

Cell culture and lentiviral transfection

Mouse C2C12 myoblasts (ATCC, USA), skeletal muscle precursor cells with the potential of myotube differentiation and muscle regeneration, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% foetal bovine serum (Bioind, Israel), and 1% penicillin–streptomycin (Gibco, USA). All cells were incubated in 5% CO₂ atmosphere at 37 °C. After the cells reached 70%-95% confluence, the culture medium was switched to a differentiation medium with DMEM containing 2% horse serum (Bioind, Israel) to induce differentiation into myotubes. Myotubes

appeared after 4-5 days of differentiation and were used for subsequent experiments. For stable overexpression, lentiviral vectors were used for gene delivery. The recombinant lentiviral vectors HBLV-m-Gm20743-Null-PURO (Lv-Gm20743), HBLV-ZsGreen-PURO (Lv-Green-vector), and HBLV-PURO (Lv-vector) were purchased from HanBio Technology Co., Ltd. (Shanghai, China). According to the transfection of multiplicity of infection (MOI) via Lv-Green-vector, the lentiviruses Lv-Gm20743 and Lv-vector were each added separately into the culture medium of C2C12 myoblasts at an MOI of 10. Stably transfected cells were selected by the addition of 4 µg/ml puromycin (HanBio, Shanghai, China) until uninfected control cells were completely dead. PA was diluted with a differentiation medium that contained fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich, USA). Consistent with the concentration of PA and intervention time of insulin resistance cell model in our previous study ^{21,24}, C2C12 cells were treated with 0.5 mM PA (Sigma-Aldrich, USA) for 24 hours. The experiments are grouped into NC group, PA-treated group, Lv-vector group, PA+Lv-vector group, PA+Lv-Gm20743 group. All experiments were repeated three times.

Real-Time Quantitative PCR (RT-qPCR) analysis

cDNA was synthesized from total RNA of GAS (n = 5) by reverse transcription reactions with a PrimeScript RT Reagent Kit (Tiangen, China). qRT-PCR was processed with SYBR Premix (Tiangen, China) by using a CFX Real-Time PCR Detection System (Bio-Rad, CA). Primers are presented in Table S1. The equation $2^{-\Delta\Delta C_t}$ was used to calculate the relative fold changes of RNA expression. ACTB was performed as a reference gene. The mean values of the control group were set to 1. All experiments were repeated at least three times.

Fluorescence in situ hybridization (FISH)

After seeding, cells were incubated with 4% paraformaldehyde for 15 min and then permeated with 0.3% Triton for 15 min. Prehybridized with specific solutions in a FISH kit (RiboBio, China), and hybridized with FAM-labeled lncRNA Gm20743 probe (5 µg/mL) overnight at 37°C (Genepharma, China). Subsequently, cells were washed three times with 4× SSC detergent containing 0.01% Tween-20 at 42 °C. After washing by 2× SSC and 1×SSC detergent at 42 °C, cells were stained with DAPI. Cells with FAM-labeled probes were detected by using confocal laser scanning fluorescence microscopy (ZEISS, LSM800, Germany).

Immunofluorescence

Immunofluorescence staining was performed as described previously ²¹. After incubation with anti-myosin heavy chain (MyHC) antibody (1:200, Sigma-Aldrich, USA) overnight at 4 °C, C2C12 myotubes were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:400, ZSGB-BIO, China) for 2 hours. The nuclei were stained with DAPI (Beyotime, China). Myotubes with at least two nuclei were considered MyHC-positive myotubes (>2 nuclei). Images were observed by confocal laser scanning fluorescence microscopy (ZEISS, LSM800, Germany). The diameter of the myotubes was measured by ImageJ software (NIH, USA).

Detection of reactive oxygen species (ROS) and Mito-Sox

The intracellular ROS in C2C12 cells was measured by using a ROS Assay Kit (Beyotime, China). 2', 7'-dichlorofluorescein-diacetate (DCFH-DA), which is the ROS assay kit principal component, is oxidized easily to fluorescent dichlorofluorescein by cellular ROS. Therefore, intracellular ROS levels were detected. The mitochondrial ROS in C2C12 cells were detected by a Mito-Sox Assay Kit (Invitrogen, USA). According to the manufacturer's protocol, C2C12 cells in different groups (Lv-

vector, PA+Lv-vector, and PA+Lv-Gm20743) were washed with serum-free medium 3 times and treated with DCFH-DA and Mito-Sox for 20 mins respectively. After washing, C2C12 cells were stained with Hoechst (Beyotime, China) for 5 mins and then intracellular ROS was observed by fluorescence microscopy (Olympus, Japan) and mitochondrial ROS was observed by confocal laser scanning fluorescence microscopy (ZEISS, LSM800, Germany). The relative fluorescence intensive was measured by ImageJ software (NIH, USA).

Detection of Ethynyl-2'-deoxyuridine (EdU) assay

After the intervention, C2C12 cells seeded in 6-well plates were washed 3 times. According to the manufacturer's protocol, C2C12 cells were fixed and stained with an EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, China). The number of EdU-positive cells was observed by fluorescence microscopy (Olympus, Japan). The EdU-positive cell numbers were measured by ImageJ software (NIH, USA).

Statistical analysis

All data are presented as the mean $\pm$ standard deviation (SD). GraphPad Prism (San Diego, CA) was used to analyze the data. The comparisons of the two groups were detected by using an unpaired two-tailed Student's t-test. Three or more groups were measured by one-way analysis of variance. Pearson's correlation analysis was used to detect the correlation between two groups. The significance was regarded as p -values < 0.05 . All experiments were repeated at least three times.

Results

Model-identification of diabetes-related sarcopenia in *db/db* mouse

Fifteen-week-old *db/db* mice showed a phenotype of severe obesity (Figure 1A-B) with obvious hyperglycaemia (>25 mmol/L) (Figure 1C) compared to normal control *db/m*. Morphological changes in GAS were observed by H&E, Oil-Red O, and Masson's trichrome staining compared to *db/m mice* (Figure 1D-F). The CSA of muscle fibres was markedly reduced (Figure 1G), and lipid droplet infiltration and increased fibrosis were observed in GAS of *db/db* mice, indicating evident pathological changes of diabetes-induced sarcopenia. The forelimb grip strength of *db/db* mice was significantly decreased (Figure 1H), and the mass and size of the quadriceps (QUA), GAS, and tibialis anterior (TA) were significantly decreased (Figure 1I, M). These results indicate that muscle strength and mass were significantly decreased in *db/db* mice. Decreased bone mineral density (BMD), lower lean mass, and increased fat mass were observed by DEXA analysis in the *db/db* group (Figure 1J-L). Serum biochemical parameters were indicative of the systematic metabolic disorder. Triglycerides, glucose, total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were increased significantly in *db/db* mice compared to *db/m* control (Table 1). Morphological abnormalities of mitochondria, swelling of the endoplasmic reticulum, reduced muscular glycogen, and sarcomere damage was observed in GAS of *db/db* compared with *db/m* group (Figure 1N, O). Our data showed that muscular weakness and loss with subcellular structural dysfunction are evident in *db/db* mice.

Identification of dif-mRNAs in GAS of *db/db* mice

To identify new molecular mechanisms during diabetes-accelerated sarcopenia, total RNA from GAS of *db/db* and *db/m* mice was used for whole transcriptome sequencing. Raw sequencing data were filtered, and clean reads were mapped to the reference genome (reference species: *Mus_musculus*).

Reference Genome Version: GCF_000001635.26_GRCm38.p6). Filtered clean reads were spliced into putative transcripts (Table S2). An absolute $\log_2$ FC value of 0.585 was used as the standard to confirm the dif-mRNAs (FDR < 0.05). Based on that standard, 607 upregulated mRNAs and 1332 downregulated mRNAs were identified in *db/db* vs. *db/m* (Table S3-4). The heatmap and a volcano plot (Figure 2A-B) showed differential mRNA expression between *db/db* vs. *db/m* (FDR < 0.05). The top 10 dysregulated mRNAs are shown in Table 2.

Functional prediction of Dif-mRNAs

GO and KEGG pathway enrichment analyses were performed to identify potential biological functions and implications. Dif-mRNAs were exhibited in three modules of the GO pathway enrichment analysis in GAS of the *db/db* group. Upregulated mRNAs were mainly enriched in the extracellular matrix (ECM) organization (GO:0030198), negative regulation of the Wnt pathway (GO:0030178), retinoic acid catabolic process (GO:0034653), inflammatory response (GO:0006954), and collagen fibril organization (GO:0030199) in the biological process category of GO terms (Figure 2C). Downregulated mRNAs were primarily enriched in terms such as immune system process (GO:0002376), and skeletal muscle contraction (GO:0006936) (Figure 2D). The top 10 GO terms associated with dif-mRNAs of *db/db* mice are shown in Table S5-6. For dif-mRNAs, enriched transcription factors (TFs), including Nr4a1, Mafa, Jun (also known as AP-1), ATF5, Trp63, and Tbx1, were differentially expressed in the *db/db* group (Table S7). These data showed that differential TFs might be essential for the progression of diabetes-induced sarcopenia.

Then, the dif-mRNAs were enriched in dysregulated pathways by KEGG analysis. Our data showed that 607 upregulated genes were enriched in 204 signalling pathways, which were mainly enriched in the pathway of neuroactive ligand-receptor interaction (PATH: map04080), cAMP signaling (PATH:

map04024), ECM-receptor interaction (PATH: map04512), regulation lipolysis in adipocytes (PATH: map04923) and protein digestion and absorption (PATH: map04974) (Figure 2E). KEGG enrichment showed that 1332 downregulated genes were enriched in 249 significant pathways, including the pathway of muscle contraction (PATH: map04260), cell adhesion molecules (PATH: map04514), TNF signaling (PATH: map04668), arginine and proline metabolism (PATH: map00330), and glutathione metabolism (PATH: map00480) (Figure 2F). The top 10 KEGG pathways associated with dysregulated mRNAs of diabetes-related sarcopenia are shown in Table S8-9, indicating that these significant changes in pathways may contribute to the development of diabetes-induced sarcopenia.

Based on shared biological function, location, or expression of genes, GSEA has a broad scope of enrichment analysis and discovers potential mechanisms in disease. The dif-mRNAs were associated with significant pathways in GSEA, as shown in Table S10 (p-value < 0.05). The GSEA results showed that the top 2 upregulated mRNAs were closely related to neuroactive ligand-receptor interactions (PATH: map04080) and the renin secretion pathway (PATH: map04924) (Figure S1A, B). In contrast, top 2 downregulated mRNAs were highly correlated with the pathway of oxidative phosphorylation (PATH: map00190), arginine and proline metabolism (PATH: map00330) (Figure S1C, D).

Pathway-act-network

To investigate deeper interactions in significant pathways, 370 dif-mRNAs enriched into 10 mainly significant pathways of KEGG were subjected to a visual pathway-act-network analysis (Figure S2A). A pathway-act network was constructed with 233 dif-mRNAs enriched with 10 significant pathways of GSEA (Figure S2B). These data showed that significant metabolic pathway regulation might be involved in the development of diabetes-induced sarcopenia.

Identification of dif-lncRNAs in GAS of *db/db* vs. *db/m*

According to the screening criteria, 306 upregulated lncRNAs and 554 downregulated lncRNAs were identified in *db/db* vs. *db/m* (Table S11-12). All dif-lncRNAs were widely found on all chromosomes, including the X and Y sex chromosomes (Figure 3A). These dif-lncRNAs were divided into four categories based on different genic loci: exonic sense, intronic sense, antisense, and intergenic (Figure 3B). Heatmap and volcano plot were constructed (Figure 3C-D). We also identified the top 10 dysregulated lncRNAs in *db/db* mice (Table 3).

Co-expression network construction and RT-qPCR validation

Based on the co-expression relationship of lncRNA-mRNA, we generated diabetes-induced sarcopenia-specific lncRNA-mRNA co-expression networks. Through using correlation analysis of the expression, 24 lncRNA ($\log_2 \text{FC} > 1$, $q\text{-value} < 0.05$ and expression > 2) and 860 mRNAs ($\text{PCC} > 0.95$, $p\text{-value} < 0.05$) were used to construct a highly correlated co-expression network. Several core dif-lncRNAs in the co-expression network were validated by RT-qPCR in *db/db* and *db/m* mice. The results showed that 1700047G03Rik and Gm31814 were upregulated, while Gm20743, Gm35438, Gm36131, A330074k22rik were downregulated in GAS of the *db/db* group compared with *db/m* mice (Figure 4A-F). Accumulated evidence showed that the elevated serum PA level in T2DM is involved in the development of insulin resistance and implicated in skeletal muscle inflammation, oxidative stress, and mitochondrial dysfunction by causing lipotoxicity, which leads to skeletal muscle loss^{25,26}. In our results, PA-treated C2C12 myotubes decreased the diameter of and inhibited the differentiation level of myotubes using fusion index analysis (Figure 5A-B). Atrogin-1, MuRF-1, and Mstn, which serve as muscle atrophy markers, were higher relative to NC (Figure 5C-E). These results suggested that PA efficiently induced muscle atrophy in C2C12 myotubes. The expression of these six dif-lncRNAs was further confirmed in a PA-induced muscle atrophy C2C12 cell model by RT-qPCR

(Figure 6A-F). The results found that the expression of most lncRNAs was consistent with animal models and RNA-seq data except for Gm38141 (Figure 6E), which may be due to the differences in tissue and cellular components. Overall, the RT-qPCR validation of dysregulated lncRNAs of co-expression network in diabetes-associated sarcopenia mouse models and PA-treated C2C12 cells provide us with further indications to characterize their functions in the development of diabetes-associated sarcopenia. In addition, we found that 3 core dif-lncRNAs named Gm20743 ($\log_2FC = -1.57$, $q\text{-value} < 0.001$), Gm35438 ($\log_2FC = -1.90$, $q\text{-value} < 0.001$) and 1700047G03Rik ($\log_2FC = 6.34$, $q\text{-value} < 0.001$) had the highest number of interactions in the co-expression network and may contribute to the development of diabetes-related sarcopenia (Figure 7aA and Figure 7bC).

The potential function of candidate lncRNAs

We performed functional prediction of the co-expressed dif-mRNAs highly correlated with the three candidate lncRNAs by KEGG analysis, respectively. Functional analysis revealed that Gm20743 may be involved in mitochondrial functions like oxidative phosphorylation and glutathione pathway (Figure 7aB). Through RT-qPCR verification, the key genes (like ATP1B1, Gclc, Gclm, GPX4) in the co-expression network which contributed to oxidative phosphorylation and glutathione pathway were downregulated *in vivo* (Figure 4G-J) and *in vitro* (Figure 6G-J) consistent with the RNA-seq results. These results suggested Gm20743 may be involved in the regulation of mitochondrial function and redox homeostasis in skeletal muscle. KEGG analysis found that the dif-mRNAs co-expressed with Gm35438 are mainly enriched in muscle contraction and adrenergic signaling pathways (Figure 7aC). The co-expressed contractile proteins Tnncl and TPM3 in these differentially enriched pathways were downregulated in *db/db* mice (Figure 4K-L) and PA-treated C2C12 cells (Figure 6K-L) compared to their control groups, and these results suggested that Gm35438 may be related to skeletal muscle contraction and strength. Through co-expression network and bioinformatic analysis, we found the dif-mRNAs co-expressed with 1700047G03Rik (the top 1 upregulated lncRNA in Table 3) were mainly

enriched in the neuroactive ligand-receptor interaction and glycolipid metabolism pathway (Figure 7bE). The RT-qPCR validation found co-expressed key catalytic enzymes ACSL3 and DGAT2 in the glycolipid metabolism pathway were upregulated in both animal (Figure 4M-N) and cell models of diabetic sarcopenia (Figure 6M-N). It is suggested that 1700047G03Rik may be involved in the intramuscular lipid deposition of diabetes-related sarcopenia.

The role of Gm20743 in the development of diabetic sarcopenia.

In this study, we specifically focused on the role of lncRNA Gm20743 on mitochondrial function and redox homeostasis in skeletal muscle cells. Key reasons are 1) First, through a systematic literature review, many studies found that mitochondrial dysfunction and oxidative stress play an important role in sarcopenia; 2) Secondly, our previous study found that insulin resistance, mitochondrial dysfunction, and oxidative stress were involved in the development of muscle atrophy in PA-treated C2C12 cells^{21,24}; 3) In addition, through functional prediction of dif-mRNAs and lncRNA-mRNA co-expression network. These results showed that the down-regulated mitochondrial oxidative phosphorylation, citrate cycle, and glutathione metabolism were the most significantly enriched pathways based on GSEA (Figure S1C, E, F). The KEGG analysis also revealed that the genes highly correlated with Gm20743 were mainly enriched in down-regulated oxidative phosphorylation and glutathione metabolism pathways (Figure 7aB). The above findings suggested that Gm20743 may play an important role in regulating mitochondrial dysfunction and oxidative stress in diabetic sarcopenia.

To verify the role of Gm20743 in diabetes-related sarcopenia, Through FISH, our results found that the distribution of Gm20743 was found in both cytoplasm and nucleus of skeletal muscle C2C12 cells (Figure 8aA). Gm20743 was overexpressed in C2C12 cells by lentiviral transfection and the expression of Lv-Gm20743 was verified by RT-qPCR (Figure 8aB). Through the detection of DCFH-DA and Mito-Sox, our results showed that overexpression of Gm20743 significantly reduced intracellular ROS

(Figure 8aC-D) and mitochondrial ROS (Figure 8aE-F) in PA-induced C2C12 cells. These data suggest that Gm20743 overexpression alleviates PA-induced mitochondrial dysfunction and oxidative stress in C2C12 cells. Previous studies found that mitochondrial dysfunction and oxidative stress prevent the proliferation and differentiation in skeletal muscle myoblasts²⁷⁻²⁹. To further assess whether Gm20743 is involved in the cell proliferation and myotube differentiation in skeletal muscle myoblasts, the detection of ethynyl-2'-deoxyuridine and myosin heavy chain staining was determined. In our results, we found that overexpressed Gm20743 increased the number of EdU-positive cells in PA-induced C2C12 myoblasts (Figure 8bG-H), suggesting Gm20743 improves cell proliferation. Consistently, we also observed that overexpressed Gm20743 significantly increased the number and diameter of myotube in PA-induced C2C12 myoblasts (Figure 8bI-J), compared to Lv-vector control. Together, these results indicate that overexpression of Gm20743 may promote cell proliferation and myotube differentiation by preventing mitochondrial dysfunction and oxidative stress in skeletal muscle cells.

Discussion

Accumulating evidence illustrated that emerging lncRNAs were involved in skeletal muscle growth and regeneration³⁰⁻³⁶. However, the regulatory mechanism of lncRNAs in sarcopenia during diabetes pathogenesis remains unclear. Our study shows that thousands of differential gene expressions were found in *db/db* mice by whole transcriptome RNA-seq. Our results indicated that down-regulated "oxidative phosphorylation", "metabolic pathways" and "muscle contraction" pathways and up-regulated "neuroactive ligand-receptor interaction" and "glucolipid metabolism" pathways were significantly enriched in *db/db* mice according to functional enrichment analysis. We have revealed three core significant candidate lncRNAs Gm20743, Gm35438, 1700047G03Rik, and their potential function based on co-expression network and functional prediction. The candidate lncRNAs and their closely correlated gene expression were validated by RT-qPCR in *db/db* mice and PA-treated C2C12 cells. In this study, we examined the specific role of lncRNA Gm20743 in skeletal muscle cells, and our results have shown that the level of mitochondrial and intracellular ROS in PA-treated C2C12 cells was reduced by overexpressed Gm20743 and indicated that overexpressed Gm20743 could prevent mitochondrial dysfunction and oxidative stress in C2C12 cells. The results also provided evidence that overexpressed Gm20743 could promote cell proliferation and myotube differentiation in PA-induced C2C12 cells. All these data identify already known interactions and novel changes in the lncRNAs regulation in diabetes-related sarcopenia and an emphasis on the specific function of novel lncRNA Gm20743 in the development of diabetes-related sarcopenia.

According to lncRNA-mRNA co-expression networks, we found three candidate lncRNAs Gm20743, Gm35438, and 1700047G03Rik that may have vital regulatory roles in diabetes-related sarcopenia. Surprisingly, down-regulated lncRNA Gm20743 is a key lncRNAs with 86 co-expressed DEGs in the co-expression network, and these highly correlated DEGs were mainly enriched in down-regulated oxidative phosphorylation and glutathione pathway. Mitochondrial oxidative phosphorylation converts

the energy released by nutrient oxidation into ATP. Studies indicated defective mitochondrial oxidative phosphorylation and reactive oxygen species (ROS) removal systems lead to ROS imbalance and oxidative stress, which in turn damages mitochondrial function in skeletal muscle^{37,38}. Numerous studies also have reported that the complication of obesity and diabetes induces mitochondrial dysfunction with the activation of oxidative stress, ultimately leading to decreased muscle mass and strength.³⁹⁻⁴¹. Our results found that the down-regulated oxidative phosphorylation pathway was the most significantly enriched in db/db mice based on GSEA (Figure S1C). In addition, the altered mitochondrial morphology was found in GAS tissue of db/db mice compared to the control group by TEM (Figure 1N-O). These results were consistent with the performance of mitochondrial dysfunction in diabetic skeletal muscle. A recent study had described that the glutathione pathway plays a positively substantial role in removing ROS and skeletal muscle mass by modulating redox homeostasis⁴²⁻⁴⁵ which is consistent with our findings with the down-regulated glutathione pathway in *db/db* mice. Moreover, the downregulated expression of highly co-expressed genes (such as ATP1b1, Gclc, Gclm, and GPX4) closely related with Gm20743 was consistent with downregulated oxidative phosphorylation and glutathione pathway^{46,47}. Therefore, we more focused on the specific function of lncRNA Gm20743 on mitochondrial function and oxidative stress in this study. Through *in vitro* experiments, Lv-Gm20743 C2C12 cells exhibited a lower mitochondrial and intracellular ROS accumulation in PA-treated atrophic muscle cells. The imbalance in the enzymatic activity of mitochondrial complexes, mitochondrial complexes I and III generate ROS production^{48,49}. Although our study did not provide sufficient direct evidence, it has been proposed that high levels of mitochondrial ROS lead to downregulation of mitochondrial membrane potential and further decreased mitochondrial oxidative phosphorylation activity, ultimately leading to mitochondrial dysfunction³⁸. These findings suggest that overexpressed Gm20743 ameliorate mitochondrial function and oxidative stress via decreasing the production of mitochondrial and intracellular ROS. Thus, these findings strongly suggest that overexpressed Gm20743 have a very important role in the regulation of

mitochondrial function and redox homeostasis via a lower level of mitochondrial and intracellular ROS in PA-treated C2C12 cells. Moreover, the results demonstrate that overexpression of Gm20743 improves cell proliferation and myotube differentiation in PA-treated skeletal muscle cells. Collectively, it is important to highlight the fact that GM20743 may be involved in promoting mitochondrial function and redox homeostasis to regulate cell proliferation and myotube differentiation in skeletal muscle cells. However, the function of Gm20743 has not been observed in skeletal muscle of any disease models in public databases such as GEO profiles and GTEx. To our knowledge, this is the first time we report the specific function of Gm20743 in mouse skeletal muscle cells. In addition, through gene alignment in the public human database, we found that some sequences on human chromosome 1 were highly homologous to the sequences of lncRNA Gm20743 on mouse chromosome 1, and the proportion of homologous genes reached 70-80%. Therefore, this study on the role of the novel lncRNA Gm20743 in diabetes-related sarcopenia makes it very meaningful to further explore the human gene homologous to Gm20743 as a target or biomarker for clinical diagnosis and therapy in the future.

Our results also revealed the potential functions of the other two candidate lncRNAs Gm35438 and 1700047G03Rik according to the co-expression networks. The KEGG enrichment analysis revealed that 86 DEGs co-expressed with Gm35438 were mainly enriched in muscle contraction and adrenergic pathways. Exactly as the muscle contraction pathway, the adrenergic signaling pathway is also considered to regulate skeletal muscle contraction and muscle mass through excitation-contraction coupling⁵⁰. The key contractile proteins troponin C1 (Tnnc1) and tropomyosin3 (TPM3) which are involved in both muscle contraction and adrenergic signaling pathway were down-regulated in the Gm35438-mRNA co-expression network. Tnnc1 is one of the TnC isoforms encoded by homologous genes in slow skeletal and cardiac muscle⁵¹. Tnnc1 has also been recognized as a key biological marker for assessing muscle fiber type and quality⁵². TPM3 is a slow-twitch muscle fiber tropomyosin

involved in skeletal muscle contraction⁵³. Studies have found that maximal contractile force in skeletal muscle is decreased in patients with tpm3 mutations^{54,55}. Compared to their controls, downregulated expression of Tnncl and TPM3 were validated in db/db mice (Figure 4K-L) and PA-treated C2C12 cells (Figure 6K-L) which is consistent with the RNA-seq data. Thus, we speculate that Gm35438 is likely involved in the regulation of skeletal muscle contraction and needs further explorations in the future. Our results showed that 45 co-expressed DEGs were closely correlated to core lncRNA 1700047G03Rik (the top 1 of dif-lncRNAs), which were mainly enriched in the up-regulated neuroactive ligand-receptor interaction and glycolipid metabolism pathways based on KEGG analysis. The results showed that up-regulated neuroactive ligand-receptor interaction pathway is the most differentially enriched pathway in db/db mice based on GSEA (Figure S1A). Although a clear correlation of neuroactive ligand-receptor interactions in skeletal muscle atrophy or sarcopenia has not yet been reported, it may be a new mechanism of diabetes-related sarcopenia. Recent studies have demonstrated that sarcopenia is accompanied by accumulated intramuscular fat (IMAT) infiltration⁵⁶. In skeletal muscle, IMAT is a common feature in several myopathies and is related to muscular dysfunction and insulin resistance⁵⁷⁻⁵⁹. ACSL3 and DGAT2 are localized on the endoplasmic reticulum and act as a key catalytic enzyme to promote the synthesis of lipids and TAGs^{60,61}. A study found that increased lipid metabolism pathways such as the glycolipid pathway may contribute to insulin resistance and muscular dysfunction, leading to skeletal muscle loss and low muscle strength⁶². Consistent with the RNA-seq results, the up-regulated expressions of ACSL3 and DGAT2 which are involved in the co-expression network with core 1700047G03Rik were confirmed by RT-qPCR in db/db mice (Figure 4A, M-N) and PA-treated C2C12 cells (Figure 6 A, M-N) compared with their controls. These results supported that 1700047G03Rik related to the function of ACSL3 and DGAT2 which are involved in lipid synthesis may play vital roles in diabetes-related sarcopenia. Therefore, we speculate that 1700047G03Rik may regulate lipid metabolism to change fatty acid content or

composition in skeletal muscle, ultimately causing skeletal muscular dysfunction, atrophy, and weakness, and further verification will be needed in the future.

The leptin-deficient *db/db* mouse model used in our study has previously been shown to be affected by the metabolic disorders of obesity and T2DM, including elevated blood glucose and abnormal insulin secretion^{63,64}. In our data, the DEXA test has found that the lean mass of 15-week-old *db/db* mice is significantly lower than that of control *db/m*, accompanied by a decrease in forelimb grip strength, although *db/db* mice are severely obese and overweight. These results strongly demonstrated that muscle atrophy is evident in *db/db* mice of the T2DM model. Thus, the *db/db* mice are a suitable and reliable model for obesity and diabetes-induced sarcopenia. Our data showed that reduced skeletal muscle mass and strength, decreased CSA, increased body fat and a high level of triacylglycerols were found in *db/db* mice, and a recently published study by Pandeya confirms our findings in this study⁶⁵. Based on the available literature, several RNA-seq research on skeletal muscle insulin resistance were performed *in vivo* and *in vitro*, and these studies are mainly aimed at detecting the novel changes of lncRNAs and their potential function in insulin-resistant skeletal muscle^{66,67}. Compared with these studies, the strengths of our investigation include 1) we predicted the regulation of three key candidate lncRNAs (Gm20743, Gm35438, 1700047G03Rik) with potential significant pathways in diabetes-related sarcopenia. 2) our study focused on the specific function of novel lncRNA Gm20743 in the development of diabetes-related sarcopenia. Compared to these published RNA-seq studies, we identified partial inconsistencies in the dif-lncRNAs, dif-mRNAs, and significantly enriched pathways in our study, which may be related to differences in muscle tissue composition, PA concentration, time of intervention, and the stage or development of insulin resistance, diabetes, and muscle atrophy.

However, this study has certain limitations. Our study underscores the ability of whole transcriptome RNA-seq analysis to provide specific information in diabetes-induced sarcopenia. Although the sequencing data is derived from *db/db* mice, the mouse pathological phenotype already closely mimics human obese and T2DM induced sarcopenia. The state of T2DM can partially present the pathophysiological process of human diabetes-induced sarcopenia in midlife. Despite the small samples size, given the sequencing depth obtained per sample, we believe that our results could provide complex and comprehensive information and insight into differential gene expression profiles in overt obese and diabetes *db/db* mice. Future studies with the consideration of human skeletal muscle samples and increased sample size would allow further analysis to determine gene signatures that correspond to differential responses to diabetic sarcopenia in different disease stages.

Conclusions

Collectively, the findings of this study improve our understanding of novel lncRNAs that regulate skeletal muscle mass, strength, and physical function in T2DM and identify several candidate lncRNAs such as Gm20743, Gm35438, 1700047G03Rik that may serve as potential molecular targets to regulate sarcopenia associated with T2DM. Particularly, our data identified that lncRNA Gm20743 may play a substantially important role in the development of diabetes-related sarcopenia via regulating mitochondrial function and oxidative stress.

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Data Availability Statement: The original RNA sequencing data of this manuscript was uploaded in Sequence Read Archive (SRA) successfully, and the accession number is SUB10570221. We choose to keep the data private until acceptance. We will provide a confirmation from a screenshot showing data upload completed in revised manuscript and release the deposited data upon acceptance.

576 **Supplementary Materials:**

577 Table S1. RT-qPCR Primers.xlsx

578 Table S2. Mapping statistics of RNA sequencing reads.xlsx

579 Table S3. Up-regulated mRNAs in GAS of *db/db* vs. *db/m* mice (FDR <0.05).

580 Table S4. Down-regulated mRNAs in GAS of *db/db* vs. *db/m* mice (FDR <0.05).

581 Table S5. GO analysis of up-regulated mRNAs in GAS of *db/db* vs. *db/m* mice. Top 10 significantly
582 changed terms are shown.xlsx

583 Table S6. GO analysis of down-regulated mRNAs in GAS of *db/db* vs. *db/m* mice. Top 10 significantly
584 changed terms are shown.xlsx

585 Table S7. Transcription factors were enriched within differentially expressed mRNAs.xlsx

586 Table S8. KEGG pathway enrichment up-regulated mRNAs with top ten Enrichment score.xlsx

587 Table S9. KEGG pathway enrichment down-regulated mRNAs with top ten Enrichment score.xlsx

588 Table S10. Gene set enrichment analysis of differentially expressed mRNAs in GAS of *db/db* vs. *db/m*
589 mice.xlsx

590 Table S11. up-regulated lncRNAs in GAS of *db/db* vs. *db/m* mice (FDR <0.05).xlsx

591 Table S12. Down-regulated lncRNAs in GAS of *db/db* vs. *db/m* mice (FDR <0.05).xlsx

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792 **Figure 1. A metabolic disorder, loss of muscle mass and strength, and structural abnormalities**
793 **are evident in the diabetic muscle atrophy model of *db/db* mice. Compared to *db/m* group, *db/db***
794 **mice extremely developed obesity (A-B), hyperglycaemia (C) (n = 10). H&E (D), Oil-red O staining**

795 (E), and Masson staining(F) in GAS of *db/db* and *db/m* group. Scale bar, 20 μ M. Red arrow indicates
796 adipocytes; orange arrow indicates atrophic myofibres; green arrows indicate lipid droplets; black
797 arrows indicate muscle fibrosis. Quantitative analysis of the average cross-sectional area (CSA) of
798 myofibres (G). The forelimb grasping strength (H) and muscle mass of GAS, quadriceps (QUA) and
799 tibialis anterior (TA) were decreased in *db/db* mice compared with *db/m* mice (I, M). The whole-body
800 composition analysis of dual-energy X-ray absorptiometry (DEXA) (J) displayed more fat mass, less
801 lean mass (K) and decreased bone mineral density (BMD) (L) in *db/db* vs. *db/m*. Transmission electron
802 microscopy (TEM) of GAS from *db/db* and *db/m* mice (N-O). The long red arrow indicates
803 mitochondrial abnormalities and the short one indicates normal mitochondria; the blue arrow indicates
804 myelinbody; the yellow arrows indicate muscular glycogen; the long green arrow indicates swelling
805 endoplasmic reticulum and the short one indicates normal endoplasmic reticulum. Data were expressed
806 as mean $\pm$ SD. * $P < 0.05$ ** $P < 0.01$. *Note:* H&E: haematoxylin and eosin; GAS: gastrocnemius;
807 CSA: average cross-sectional area; QUA: quadriceps; TA: tibialis anterior; DEXA: dual-energy X-ray
808 absorptiometry; BMD: decreased bone mineral density; TEM: Transmission electron microscopy.

Figure 2. RNA sequencing profile and functional enrichment analysis of DEGs. Hierarchical clustering of mRNA sequencing showed mRNA differential expression profiles ($|\log_2 FC| < 0.585$, $FDR < 0.05$) of GAS between *db/db* and *db/m* (NC) group (A). Each row indicates a single gene, and each column indicates the GAS tissue of a mouse. The scatter plot was performed for the alteration of dif-mRNAs in GAS of *db/db* vs. *db/m* (B). Red plots represent up-regulated mRNAs ($\log_2 FC > 0.585$, $FDR < 0.05$) and green plots represent down-regulated genes ($\log_2 FC < -0.585$, $FDR < 0.05$). The significant GO terms ($q\text{-value} < 0.05$) of mRNA expression variation between two compared groups were listed (C-D). The top significant KEGG pathway terms ($q\text{-value} < 0.05$) of dif-mRNAs were listed (E-F). *Note*: NC: normal control group. GAS: gastrocnemius; dif-mRNAs: differentially expressed mRNAs; DEGs: differential expression of genes; FC: fold change; FDR: false discovery rate; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

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Figure 3. Differentially expressed lncRNAs in *db/db* vs. *db/m*. Circos plots showed dif-lncRNAs on mouse chromosomes for *db/db* vs. *db/m* (A). The outer circle is the chromosome, the second circle is all the differential dysregulated lncRNAs, and the inner circle is the significantly dif-lncRNAs ($|\log_2 FC| > 1$ q-value < 0.05). Pie charts showed that the percentage of four types (exonic sense, intronic sense, antisense, intergenic sense) of dysregulated lncRNAs in *db/db* mice and the controls (B). The cluster heatmaps (C) and scatter plots (D) showing the dif-lncRNAs in *db/db* and *db/m* mice, respectively. *Note:* dif-lncRNAs: differentially expressed lncRNAs.

Figure 4. Validation of the expression of dif-lncRNAs and dif-mRNAs in *db/db* and *db/m* mice. The relative expression of dif-lncRNAs ($|\log_2 \text{FC}| > 1$, expression > 2 and $q\text{-value} < 0.01$) was detected by RT-qPCR in GAS of *db/db* and *db/m* mice (A-F). The relative expression of closely related dif-mRNAs involved in significant pathways was detected in GAS of *db/db* and *db/m* mice (G-N). Data were expressed as mean $\pm$ SD. * $P < 0.05$ ** $P < 0.01$. *Note:* RT-qPCR: Real-Time Quantitative PCR.

Figure 5. PA induced muscle atrophy model in C2C12 cells. The decreased CSA and numbers of myotubes were observed in PA-treated C2C12 cells through fluorescently labeled MyHC by confocal microscopy (A) and light microscopy (B). The higher expression of Fbox32, Trim63, Mstn suggested that PA induced muscle atrophy in C2C12 cells (C-E). Data were expressed as mean $\pm$ SD. * $P < 0.05$ ** $P < 0.01$. *Note:* PA: palmitic acid; MyHC: myosin heavy chain.

Figure 6. Validation of the expression of dif-lncRNAs and dif-mRNAs in C2C12 cells exposed to PA. The relative expression of dif-lncRNAs ($|\log_2 \text{FC}| > 1$, expression > 2 and $q\text{-value} < 0.01$) was detected in PA-induced C2C12 cells compared with NC group (A-F). The relative expression of highly correlated dif-mRNAs involved in co-expression network was detected in PA-induced C2C12 cells and control group (G-N). Data were expressed as mean $\pm$ SD. * $P < 0.05$ ** $P < 0.01$.

Figure 7a. Co-expression network analysis and functional prediction of dif-lncRNAs.

Figure 7b. Co-expression network analysis and functional prediction of dif-lncRNAs.

Figure 7. Co-expression network analysis and functional prediction of dif-lncRNAs. The lncRNA Gm35438 and Gm20743 and 137 mRNAs were constructed in a co-expression network with a $PCC > 0.95$ (A). The red color represents upregulated genes, and the green color indicates down-regulation expression. Moreover, the top significant pathway terms of dysregulated genes associated with Gm35438 and Gm20743 were displayed according to KEGG analysis (B and C), respectively. In addition, the lncRNA 1700047G03Rik and 45 mRNAs were constructed in a co-expression network with a $PCC > 0.95$ (D). The top significant pathway terms of dysregulated genes associated with 1700047G03Rik were displayed according to KEGG analysis (E). *Note*: PCC: Pearson correlation coefficient.

Figure 8a. The role of lncRNA Gm20743 in diabetes-related sarcopenia.

Figure 8b. The role of lncRNA Gm20743 in diabetes-related sarcopenia.

Figure 8. The role of lncRNA Gm20743 in diabetes-related sarcopenia. Fluorescence in situ hybridization was used to observe the distribution of Gm20743 in C2C12 cells (A). The expression of Gm20743 in C2C12 cells transfected with Lv-Gm20743 was determined by RT-qPCR (B). DCFH-DA staining was performed to evaluate intracellular ROS level in C2C12 cells (scale bar = 100 μ m) (C-D). Mito-Sox staining was applied to detect mitochondrial ROS level in C2C12 cells (scale bar = 10 μ m) (E-F). EdU staining was measured to test cell proliferation in C2C12 cells (scale bar = 100 μ m) (G-H). Immunofluorescence staining of MyHC was determined to test myotube differentiation (scale bar = 50 μ m) (I-J). Data were expressed as mean $\pm$ SD. * $P < 0.05$ ** $P < 0.01$. Note: DCFH-DA: 2', 7'-dichlorofluorescein-diacetate; ROS: reactive oxygen species; EdU: 5-ethynyl-2'-deoxyuridine; MyHC: myosin heavy chain.

FIGURE S1. Gene set enrichment analysis of DEGs. The top 2 dysregulated pathways of GSEA: neuroactive ligand-receptor interaction(A), renin secretion(B), oxidative phosphorylation (C) and arginine proline metabolism (D) in *db/db* vs. *db/m*. The pathway of citrate cycle (E) and Glutathione metabolism (F) were significantly enriched based on GSEA. The ES was exhibited by green curves for each gene shown by a black line, and are ranked all the differential expressions indicating the observed

fold change in GAS from *db/db* vs. *db/m*. The NES and nominal p-value are displayed. *Note*: GSEA: Gene set enrichment analysis; ES: enrichment score; NES: normalized enrichment scores.

Figure S2. Pathway-act-network in DEGs. The links and overlaps among differentially expressed mRNAs in significant pathway terms of KEGG (A) and a visual pathway-act-network were constructed with DEGs which involved in 10 significant pathways of GSEA (B).

896 **Captions for Tables**

897 **Table 1. Serum biochemical parameters of the *db/db* vs. *db/m* (n=5)**

Variable	<i>db/db</i>	<i>db/m</i>
Total cholesterol (mM)	4.06±0.47*	2.31±0.25
Glucose (mM)	33.73±7.79*	7.55±3.68
Triglycerides (mM)	1.31±0.11*	0.91±0.05
HDL cholesterol (mM)	2.88±0.27*	1.60±0.08
LDL cholesterol (mM)	0.60±0.07*	0.49±0.08

898 *Note:* HDL=high-density lipid; LDL= low-density lipid. Results are presented as mean ± standard
899 deviation; * $P < 0.05$.

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913 **Table 2. Top 10 differential expressed mRNAs in GAS of *db/db* vs. *db/m* (FDR <0.05)**

Gene ID	Gene Symbol	Log ₂ FC	<i>p</i> -value	<i>q</i> -value	style
102639653	LOC102639653	7.241607714	1.24E-18	1.52E-17	Up
63993	Slc5a7	6.845998839	2.88E-107	1.38E-105	Up
240916	Vsig8	6.7491373	4.68E-14	4.62E-13	Up
108167806	Gm46221	6.053698263	1.54E-05	7.34E-05	Up
67717	Lipf	5.994249798	4.21E-09	2.93E-08	Up
195646	Hs3st2	5.638105987	2.04E-07	1.21E-06	Up
216343	Tph2	5.556492222	2.90E-46	7.15E-45	Up
24108	Ubd	5.548838649	1.07E-12	9.77E-12	Up
50765	Tfr2	5.496750138	9.55E-34	1.84E-32	Up
14765	Gpr50	5.38656722	1.80E-11	1.51E-10	Up
14559	Gdf1	-11.96878857	4.56E-158	2.99E-156	Down
100039008	Mup10	-8.794305417	2.17E-44	5.18E-43	Down
105243585	Gm39469	-7.629265944	3.83E-23	5.60E-22	Down
14283	Fosl1	-7.382179513	4.09E-20	5.34E-19	Down
15077	Hist2h3c1	-7.283363073	5.16E-19	6.45E-18	Down
544791	Myh13	-6.953416359	9.54E-16	1.02E-14	Down
105244831	Gm40367	-6.819274892	1.40E-14	1.42E-13	Down
100043914	Zfp968	-6.228013806	2.23E-10	1.71E-09	Down
100861909	Gm21320	-6.089096556	1.28E-129	7.14E-128	Down
100042295	Gm3776	-6.015634791	3.48E-09	2.44E-08	Down

914 *Note:* mRNAs: messenger RNAs; FC: fold change; FDR: false discovery rate.

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916 **Table 3. Top 10 differential expressed lncRNAs in GAS of *db/db* vs. *db/m* (FDR <0.05).**

Gene ID	Gene Symbol	Log ₂ FC	<i>p</i> -value	<i>q</i> -value	style
73339	1700047G03Rik	6.34827874	3.25E-97	1.44E-95	Up
102642392	Gm38610	5.08171264	2.11E-05	9.89E-05	Up
105245123	Gm40617	5.08171264	2.11E-05	9.89E-05	Up
434510	Gm5627	4.81867823	1.23E-40	2.79E-39	Up
105247666	LOC105247666	4.72217025	2.05E-04	8.28E-04	Up
74654	4930455H04Rik	4.69468952	2.39E-04	9.55E-04	Up
403202	A430093F15Rik	4.69468952	2.39E-04	9.55E-04	Up
102640807	Gm36788	4.69468952	2.39E-04	9.55E-04	Up
74341	G630025P09Rik	4.58041404	4.41E-04	0.00168321	Up
73904	4833412C05Rik	4.5792123	4.44E-04	0.00169328	Up
108168736	1700047G03Rik	-5.5504829	4.95E-07	2.83E-06	Down
108169071	Gm46728	-5.0057502	3.69E-05	1.67E-04	Down
329483	Gm43001	-4.9126408	6.76E-05	2.95E-04	Down
102638215	Gm14015	-4.9126408	6.76E-05	2.95E-04	Down
102639612	Gm34828	-4.9126408	6.76E-05	2.95E-04	Down
100043729	Gm35882	-4.8438674	2.90E-05	1.33E-04	Down
102634658	Gm14010	-4.8131051	1.24E-04	5.21E-04	Down
105245193	Gm27198	-4.718346	2.15E-04	8.68E-04	Down
75589	4930563J15Rik	-4.5907127	6.35E-07	3.59E-06	Down
433751	2310043L19Rik	-4.4651818	8.04E-04	0.00292604	Down

917 *Note:* lncRNAs: long non-coding RNAs.

Supplementary Files

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

- [SupplementaryTable1.RTqPCRPrimers.pdf](#)
- [SupplementaryTable2.MappingstatisticsofRNAsequencingreads.pdf](#)
- [SupplementaryTable3.UpregulatedmRNAsinGASofdbdbvs.dbmmice.pdf](#)
- [SupplementaryTable4.DownregulatedmRNAsinGASofdbdbvs.dbmmice.pdf](#)
- [SupplementaryTable5.GOanalysisofupregulatedmRNAsinGASofdbdbvs.dbmmice.Top10significantlychangedtermsareshowen.pdf](#)
- [SupplementaryTable6.GOanalysisofdownregulatedmRNAsinGASofdbdbvs.dbmmice.Top10significantlychangedtermsareshowen.pdf](#)
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- [SupplementaryTable12.DownregulatedlncRNAsinGASofdbdbvs.dbmmice.pdf](#)