

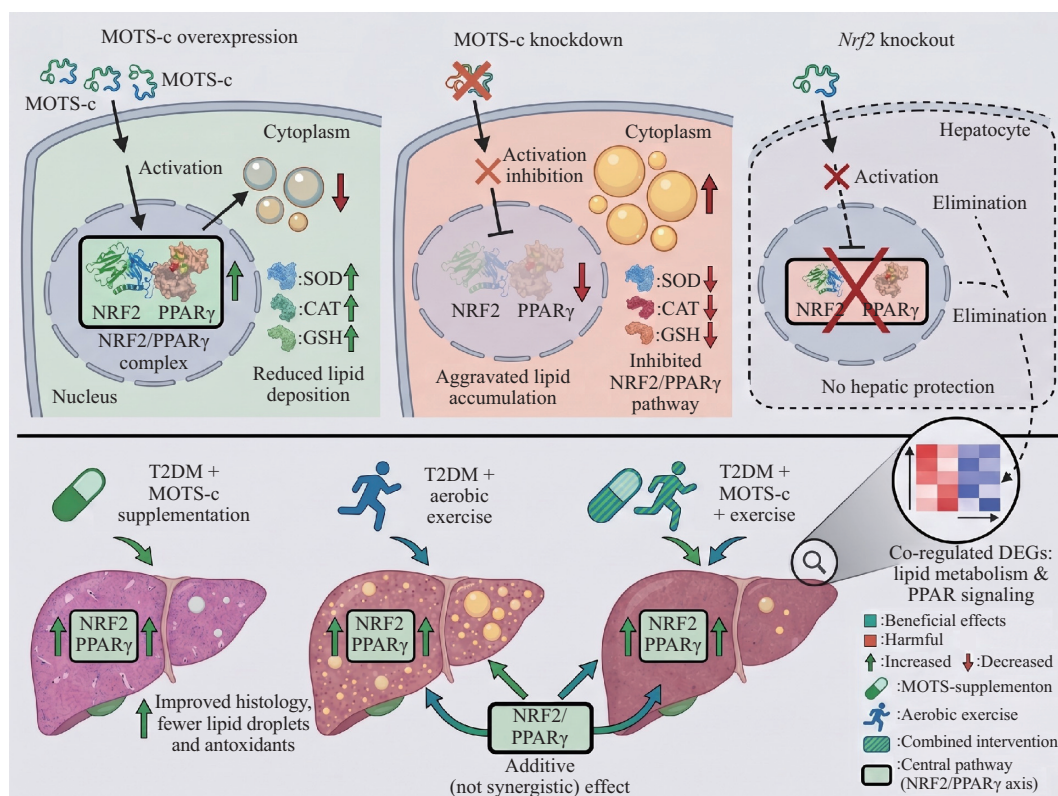


Aerobic Exercise and MOTS-c Ameliorate Hepatic Oxidative Stress and Metabolic Disorder in Type 2 Diabetes *via* The NRF2/PPAR γ Axis*

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Graphical abstract



Abstract Objective Type 2 diabetes mellitus (T2DM) often causes severe hepatic metabolic complications, dominated by metabolic associated fatty liver disease (MAFLD). Persistent hepatic steatosis and oxidative stress further trigger steatohepatitis and progressive liver damage, increasing the mortality risk of diabetic patients. Aerobic exercise effectively improves hepatic lipid metabolism and antioxidant capacity, but poor patient adherence restricts its long-term clinical application. Mitochondrial-derived

* This work was supported by grants from Sichuan Provincial Natural Science Foundation (2025NSFSC2021), the China Postdoctoral Science Foundation (2023M740381, GZB20240083), and Sports Medicine Key Laboratory of Sichuan Province (2026-A001).

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Received: March 23, 2026 Accepted: June 15, 2026

mitochondrial open reading frame of the 12S rRNA type-c (MOTS-c) is a key peptide regulating insulin sensitivity and hepatic redox homeostasis. This study aimed to explore the protective mechanism of MOTS-c against T2DM-related liver injury and its combined beneficial effect with aerobic exercise *via* the NRF2/PPAR γ signaling axis. This study aimed to investigate whether MOTS-c cooperates with aerobic exercise to alleviate T2DM-associated hepatic oxidative stress and metabolic dysfunction by activating the NRF2/PPAR γ axis, and to clarify the molecular and transcriptomic characteristics of their combined intervention. **Methods** Stable MOTS-c overexpression and knockdown HepG2 cell lines were constructed using lentiviral transfection. An oleic acid-induced cellular lipid accumulation model and NRF2-knockout cell model were applied to verify the NRF2-dependent mechanism of MOTS-c. Intracellular lipid deposition, triglyceride levels, antioxidant enzyme activities, and the expression of NRF2/PPAR γ pathway-related genes and proteins were detected. *In vivo*, a T2DM rat model with obvious hepatic steatosis was established *via* a high-fat and high-sucrose diet combined with streptozotocin injection. Model rats received aerobic exercise, MOTS-c intraperitoneal injection, or combined intervention. We detected systemic glycolipid metabolic indicators, hepatic histopathological changes, and the expression of core proteins in the hepatic NRF2/PPAR γ axis. Hepatic transcriptomic sequencing was performed to screen differentially expressed genes (DEGs) and enrich key pathways co-regulated by MOTS-c and aerobic exercise. **Results** Cellular results showed that MOTS-c overexpression significantly reduced oleic acid-induced lipid deposition, enhanced antioxidant enzyme activity, and upregulated NRF2 and PPAR γ expression. Conversely, MOTS-c knockdown aggravated hepatic lipid accumulation and oxidative damage and inhibited NRF2/PPAR γ pathway activation. NRF2 knockout completely eliminated the protective effects of MOTS-c on lipid metabolism and redox balance, confirming its NRF2-dependent regulatory mechanism. In T2DM rats, both MOTS-c supplementation and aerobic exercise effectively improved insulin resistance, corrected glycolipid metabolic disorders, and alleviated hepatic steatosis, while consistently activating the hepatic NRF2/PPAR γ axis. Compared with single intervention, the combined treatment showed a better improvement trend in hepatic metabolic and oxidative injury, without definitive synergistic effects. Transcriptomic analysis revealed that the co-regulated DEGs of MOTS-c and aerobic exercise were primarily enriched in lipid metabolism and PPAR signaling pathways, with multiple antioxidant and lipid-regulating genes significantly modulated by combined intervention. **Conclusion** MOTS-c exhibits obvious exercise-mimetic hepatoprotective effects in T2DM. It activates the hepatic NRF2/PPAR γ axis to strengthen antioxidant defense, stabilize lipid metabolism, and relieve T2DM-associated hepatic steatosis and oxidative damage. Furthermore, MOTS-c produces additive beneficial effects with aerobic exercise, showing a superior intervention trend on diabetic liver dysfunction. This study identifies the NRF2/PPAR γ axis as the core mechanism of MOTS-c-regulated hepatic protection, elucidates the transcriptomic basis of combined intervention, and provides a reliable theoretical basis and potential therapeutic target for clinical intervention in T2DM-complicated MAFLD.

Key words MOTS-c, T2DM, aerobic exercise, liver metabolism, antioxidative stress response, NRF2/PPAR γ axis

DOI: 10.3724/j.pibb.2026.0149

CSTR: 32369.14.pibb.20260149

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by impaired blood glucose regulation resulting from insulin resistance and/or insufficient insulin secretion^[1]. It is projected that the number of individuals with diabetes in China will reach 174 million by 2045, accounting for 12.5% of the total population^[2]. Diabetic complications are the primary cause of mortality in T2DM patients. While previous research on T2DM complications has predominantly focused on conditions such as hypertension, obesity, and heart failure, less attention has been paid to T2DM-induced liver injury^[3]. However, the liver is also an important target organ for diabetic injury. This oversight is largely attributable to the liver's strong compensatory capacity and the often asymptomatic nature of early-stage liver pathology. Among various forms of T2DM-related liver damage, metabolic associated fatty liver disease (MAFLD) is one of the most frequent complications. As one of the most frequent complications of T2DM, MAFLD exhibits a rising incidence and has become a leading cause of chronic liver disease globally, with an estimated prevalence of approximately 5% in China^[4]. Hepatic steatosis

constitutes the pathological foundation of MAFLD. Clinical studies indicate that patients with concurrent T2DM and hepatic steatosis experience greater metabolic dysregulation and higher intrahepatic lipid levels compared to those with either condition alone^[5]. Therefore, investigating the pathogenesis of T2DM complicated by hepatic steatosis is crucial for early diagnosis and preventing disease progression.

The initial hepatic pathological change in T2DM patients is steatosis. Without intervention, this can progress through a spectrum of conditions—from hepatic steatosis to non-alcoholic steatohepatitis (NASH) and liver fibrosis, potentially culminating in cirrhosis and hepatocellular carcinoma, thereby increasing patient mortality^[6-7]. The clinical hallmark of hepatic steatosis is the presence of triglyceride-containing lipid droplets in more than 5% of hepatocytes, as assessed by light microscopy of thin sections^[8]. Since the pathological alteration at this stage is confined to simple steatosis, timely removal or control of causative factors can slow or even reverse fat accumulation. Thus, appropriate interventions (such as exercise or medication) at this stage can mitigate or reverse hepatic steatosis.

Current pharmacological treatments for T2DM with hepatic steatosis primarily include biguanides (*e. g.*, metformin), glucagon-like peptide-1 (GLP-1) receptor agonists, and statins (*e. g.*, atorvastatin, rosuvastatin, simvastatin). However, their widespread application is limited by suboptimal efficacy and notable side effects^[9-10]. Metabolic dysregulation is a key driver in the onset and worsening of T2DM-related hepatic steatosis. Existing evidence demonstrates that aerobic exercise effectively ameliorates MAFLD symptoms. Studies in MAFLD model mice show that a 3%–5% reduction in body weight improves hepatic steatosis, a 7%–9% reduction alleviates liver inflammation, and a 10% reduction can mitigate NASH and liver fibrosis^[11]. In T2DM rat models, moderate aerobic exercise (150 min/week) has been shown to reduce early liver damage, indicating that aerobic exercise enhances hepatic metabolism and improves pathological changes^[12].

Although aerobic exercise serves as a beneficial “self-care prescription” for preventing and treating T2DM with hepatic steatosis, patient adherence remains low^[13]. Consequently, for patients newly diagnosed with T2DM and hepatic steatosis, intervention with agents that mimic the effects of exercise could potentially reduce the risk of progressing to NASH, liver fibrosis, cirrhosis, and hepatocellular carcinoma. “Exercise mimetics” represent an emerging therapeutic strategy aimed at enhancing or replicating the beneficial effects of physical exercise, such as those observed in the context of diabetes^[14]. Exercise exerts its therapeutic outcomes by activating or inhibiting specific molecular and cellular pathways. Similarly, exercise mimetics partially emulate or augment these exercise-induced benefits by modulating the same processes^[15]. Previously, we sorted exercise mimetics into three categories: pharmacological agonists (*e. g.*, AICAR, GW1516, SR9009), hormones (*e. g.*, irisin, mitochondrial open reading frame of the 12S rRNA type-c (MOTS-c)), and plant extracts (*e. g.*, resveratrol, epicatechol)^[16]. Mitochondria-derived peptides (MDPs) are small bioactive peptides produced from short open reading frames (sORFs) in mitochondrial DNA. They don’t have the typical features of protein-coding genes and mainly help regulate metabolic balance, playing a wide range of physiological roles. MOTS-c, a 16-amino-acid mitochondrial DNA-encoded signaling peptide discovered in 2015, is implicated in metabolic regulation related to insulin. It is widely expressed across various tissues—including the brain, heart,

liver, muscle, testes, kidneys, intestine, and plasma—in both rodents and humans^[17-19].

MOTS-c is a mitochondria-derived peptide. It could serve as a new regulator of liver metabolism and a therapeutic target^[20]. Research indicates that MOTS-c levels correlate with metabolic markers in patients with MAFLD^[21]. Also, MOTS-c can ameliorate mitochondrial dysfunction induced by NASH and alleviate hepatic steatosis^[22-23]. Notably, HEK293 cells stably overexpressing MOTS-c exhibit a significant reduction in long-chain fatty acids and increased fatty acid utilization compared to normal HEK293 cells^[24]. Under resting conditions, MOTS-c is predominantly localized in the mitochondria. Upon oxidative stress, it translocates to the nucleus, where it interacts with Nrf2 and participates in the antioxidant response^[23, 25-27]. The Kelch-like ECH-associated protein 1 (Keap1)-NRF2 pathway is recognized as a critical pharmacological target for liver diseases, as its activation confers protective effects against hepatic injury^[28]. Previous studies show that MOTS-c is a mitochondria-derived peptide. It mainly localizes to mitochondria and plays a key role in metabolism, anti-inflammation, and antioxidative stress response^[29]. However, under oxidative stress, it moves to the nucleus, where it interacts with NRF2 and helps drive the antioxidant response^[30]. In T2DM rat models, both aerobic exercise and intraperitoneal injection of MOTS-c have been shown to activate the Keap1-NRF2 pathway^[31]. As a classic cellular defense pathway, the Keap1-NRF2 pathway is activated under conditions of elevated reactive oxygen species (ROS) or by MOTS-c in the context of T2DM^[22, 31]. Furthermore, studies have shown that NRF2 can induce the expression of PPAR γ by binding to the antioxidant response element (ARE) within the PPAR γ gene promoter^[14, 32]. The activation of both NRF2 and PPAR γ contributes to the amelioration of liver injury, as they play pivotal roles in modulating the expression of genes involved in key processes such as hepatic metabolism, energy homeostasis, and oxidative stress during the progression of liver diseases^[15, 19, 33].

Previous studies from our group have demonstrated that MOTS-c can treat oxidative stress-induced cardiac dysfunction by activating the Keap1-NRF2 signaling pathway^[34]. Moreover, MOTS-c mediates hepatic antioxidant defense systems during aerobic exercise, thereby attenuating diabetic myocardial injury in rat cardiac tissue^[31, 35]. We focused on the NRF2/PPAR γ axis rather than other exercise-related pathways (*e. g.*, AMPK, PGC-1 α) because NRF2 directly transactivates PPAR γ *via* ARE binding, linking oxidative stress to lipid metabolism,

and our preliminary data showed that MOTS-c activates NRF2 but not AMPK in hepatocytes, with NRF2 knockout abolishing its lipid-lowering effects. Our group previously reported that MOTS-c also ameliorates hepatic fibrosis progression in T2DM *via* the KEAP1-NRF2 pathway^[34]. Hepatic metabolic dysfunction accelerates hepatic fibrogenesis; emerging evidence confirms that MOTS-c expression is closely associated with hepatic lipid deposition, and effectively mitigates hepatic injury^[21, 36]. However, despite the established cardioprotective effects of MOTS-c, its role in hepatic metabolism—especially its regulatory potential on the NRF2/PPAR γ signaling axis—remains largely unknown. Three core scientific questions remain unaddressed: does MOTS-c activate the hepatic NRF2/PPAR γ pathway? If so, does this activation improve T2DM-associated hepatic steatosis? Additionally, whether MOTS-c and aerobic exercise produce mutually enhanced therapeutic benefits *via* this axis has not been explored. Current literature has not verified MOTS-c as an exercise mimetic against diabetic hepatic injury, nor clarified the crosstalk between NRF2 and PPAR γ signaling in mediating hepatic antioxidant and lipid-lowering effects.

Treating T2DM patients with MAFLD is a clinical challenge. Current drugs like metformin, GLP-1 agonists, and statins have clear downsides: metformin can cause gut issues and lactic acidosis in liver disease; GLP-1 agonists often bring nausea and pancreatic risks; statins may raise liver enzymes or worsen insulin resistance. More importantly, none of them directly target liver oxidative stress or fat buildup. MOTS-c, on the other hand, is a natural peptide. It mimics exercise, lowers toxicity, and works through the NRF2/PPAR γ pathway to tackle both oxidative stress and lipid problems. That makes it a good candidate for these hard-to-treat patients.

Based on the aforementioned evidence, we hypothesize that MOTS-c, an exercise-responsive mitochondrial peptide, may produce mutually enhanced therapeutic effects alongside aerobic exercise to alleviate hepatic oxidative stress and metabolic dysregulation in T2DM. In this study, “synergy” is defined as the combined treatment (MOTS-c plus aerobic exercise) producing an effect that is significantly greater than the sum of the effects of either treatment alone (*i.e.*, a supra-additive effect), which will be assessed by comparing the combined group with each single-intervention group. However, this study does not carry out formal interaction-effect statistical analyses to verify real synergistic effects, so

we only observe the efficacy trend of combined administration in this experiment. This synergistic benefit is likely mediated through the coordinated activation of the NRF2/PPAR γ signaling axis, enhancing antioxidant defenses and improving lipid metabolism in the liver. This study helps to further clarify that MOTS-c exhibits exercise mimetic effects and shares similarities with aerobically-exerted amelioration of hepatic steatosis in T2DM. It is expected to serve as a potential target for the early prevention and treatment of T2DM-associated hepatic steatosis in clinical practice, while also providing a theoretical basis.

1 Materials and methods

1.1 Cells

The HepG2 cell line used in this study was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The NRF2-knockout cell line (Nrf2^{-/-} cell line) was previously generated in our laboratory by disrupting the NRF2 gene in HepG2 cells^[37]. The lentiviral plasmid vector used was pLVX-mcmv-ZsGreen-puro, which carries a puromycin resistance gene. Lentiviruses for MOTS-c empty vector, overexpression, or knockdown were generated and used to infect HepG2 cells at a multiplicity of infection (MOI) of 10⁻². After infection, cells were selected with puromycin (2 mg/L) for 3 d to enrich successfully transduced cells, and fluorescence microscopy was performed to confirm infection efficiency. Thus, stable cell lines with MOTS-c overexpression or knockdown were established in HepG2 cells. Following lentiviral plasmid infection, all cell lines were selected and maintained in culture medium containing puromycin. All these cell lines were cultured in DMEM (25 mmol/L glucose, 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin) at 37°C in 5% CO₂, prior to subsequent experiments.

1.2 Construction of lentiviral overexpression and knockdown plasmids

Lentiviral plasmids were constructed by Tsingke Biotechnology Co., Ltd. (Beijing, China). The sequence used for MOTS-c overexpression was: ATGAGGTGGCAAGAAATGGGCTACATTTTCTACCCCAGAAACTACGA.

The shRNA targeting MOTS-c contained the following sequences: forward 5'-CAAGAAUUGGCUACAUUU-3' and reverse 5'-AAAUGUAGCCC-AUUUCUUG-3'.

1.3 Measurement of TG, GSH, SOD, and CAT

Cells (4×10^5) were seeded in 6-well plates and cultured for 48 h. After digestion, cells were collected into 1.5-ml centrifuge tubes. NRF2^{+/+}, NRF2^{-/-}, OE-MOTS-c, and sh-MOTS-c cells were treated with oleic acid (OA) (100 μ mol/L) for 24 h, and then TG levels were measured. The OA concentration and treatment time were based on previously optimized conditions established in our laboratory^[38]. Triglyceride (TG) content, total superoxide dismutase (SOD) activity, intracellular reduced glutathione (GSH) level, and catalase (CAT) activity were determined using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; product numbers A110-1-1, A005-1-2, A001-3-2, and A006-2-1, respectively). All experiments were performed independently in triplicate.

1.4 Experimental animals

The experimental protocol was approved by the ethics committee of Chengdu Sport University (approval No: Chengti Ethics [2024] No. 30), and all procedures were conducted in strict accordance with relevant guidelines and regulations. The study followed the animal research: reporting of *in vivo* experiments (ARRIVE) guidelines. Seventy 6-week-old male Sprague-Dawley rats (body weight 170–185 g) were purchased from Chengdu Dossy Experimental Animal Co., Ltd. All animals were housed in the experimental animal facility of the Institute of Sports Medicine and Health at Chengdu Sport University under controlled conditions: temperature 22–25°C, humidity 40%–60%, a 12 h/12 h light/dark cycle, and adequate ventilation. After random grouping, rats were fed either a standard chow diet or a high-fat/high-sucrose diet (consisting of 67% standard chow, 20% sucrose, 10% lard, 2% cholesterol, and 1% sodium cholate; provided by Chengdu Dossy Experimental Animal Co., Ltd.). The production license number and animal quality certificate number of Chengdu Dashuo Experimental Animal Co., Ltd. are SCXK (Chuan) 2025-0030 and SYXK (Chuan) 2023-0211, respectively. A rat model of T2DM with concurrent hepatic steatosis was induced by a modified high-fat/high-sucrose diet and injection of a low dose of streptozotocin (STZ, 30 mg/kg) dissolved in citrate buffer (pH 4.5). After 7 weeks of feeding with the high-fat/high-sucrose diet, fasting blood glucose (FBG) and fasting serum insulin (FINS) were measured in the model group *via* tail vein blood sampling, the insulin resistance index was calculated, and the same STZ solution was injected.

The control group received an equal volume of citrate buffer (0.25 ml/kg). Prior to liver tissue collection at the experimental endpoint, all animals were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg).

1.5 Animal model establishment

After a one-week acclimatization period with no significant changes in body weight or mortality observed, the rats were assigned to experimental groups. Ten rats were randomly selected as the normal control group (Group C) and maintained on a standard diet. The remaining 60 rats were fed a high-fat/high-sucrose diet to establish a T2DM model, following methods previously published by our team^[18, 35]. Upon successful model induction, the 60 diabetic rats were randomly divided into four groups ($n=10$ per group): (1) T2DM group (Group D): no exercise or drug treatment; (2) T2DM+exercise group (Group DE): regular aerobic exercise intervention; (3) T2DM+MOTS-c group (Group DM): MOTS-c pharmacological intervention; (4) T2DM+exercise+MOTS-c group (Group DME): combined aerobic exercise and MOTS-c intervention.

1.6 Aerobic exercise protocol

Aerobic exercise training was performed using a motorized treadmill following the Bedford running model^[39]. Aerobic exercise training was performed on a small animal treadmill. Before formal training, rats underwent a 3-d acclimation period on the treadmill set at 0° grade: first running at 8 m/min for 5 min, then accelerating to 10 m/min for 10 min. For the formal training, the treadmill grade remained at 0°, and the rats ran at 20 m/min for 1 h, which corresponds to approximately 50%–60% of their maximal oxygen consumption. During the training, manual prodding and tail stimulation were used to encourage continuous running. Training was conducted 5 d/week for 8 weeks.

Rats underwent a 3-d adaptation period at 15 m/min, followed by a formal 8-week training regimen at 20 m/min for 60 min per session, 5 d per week.

1.7 MOTS-c administration

Rats in the DM and DME groups received a daily intraperitoneal injection of MOTS-c (0.5 mg/kg, GL Biochem Shanghai Ltd., China) for 8 weeks^[19]. Control rats in Groups C and D were injected with an equal volume of normal saline following the same schedule. MOTS-c was stored as a solid at –20°C, dissolved in saline before injection, and administered intraperitoneally. No significant degradation occurred during the experimental period under these conditions.

1.8 Analysis of body weight, blood glucose, insulin, and glycated hemoglobin (HbA1c)

Body weight was monitored regularly using an electronic animal scale (precision 0.01 g, Shanghai Yuyan Scientific Instrument Co., Ltd.). To assess glycemic status, approximately 0.05 ml of blood was collected from the tail vein at specified time points for FBG measurement with a portable glucometer. At the experimental endpoint, rats were anesthetized and whole blood was collected from the abdominal aorta. Serum was separated by centrifugation at 2 500 r/min for 10 min at 4°C and stored at -80°C for subsequent analysis. FINS levels were measured using a commercially available ELISA kit (ImmunoWay Biotechnology, USA) according to the manufacturer's instructions, with absorbance read at 450 nm on a SpectraMax M5 microplate reader (Thermo Scientific, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as: $\text{HOMA-IR} = [\text{FBG}/(\text{mmol} \cdot \text{L}^{-1})] \times [\text{FINS}/(\text{mIU} \cdot \text{L}^{-1})] / 22.5$. HbA1c was measured with a commercial kit (ABclonal, RK03710) following a published protocol. The procedure includes preparing a hemoglobin solution, performing acid hydrolysis, allowing color development, and using spectrophotometric quantification to calculate the HbA1c percentage.

1.9 Analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Serum levels of ALT and AST in the five experimental groups (C, D, DE, DM, and DME) were measured using commercial ELISA kits (ALT, Cat# ab234579; AST, Cat# ab263883; both from Abcam, Waltham, MA, USA) according to the manufacturers' instructions.

1.10 Analysis of total cholesterol (TC), TG, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)

Serum samples from the five groups were collected for lipid profile analysis. The concentrations of TC, TG, HDL-C, and LDL-C were determined using corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; Cat# A111-1-1, A110-1-1, A112-1-1, and A113-1-1, respectively). All measurements were performed in triplicate.

1.11 Hematoxylin and eosin (HE) staining and immunohistochemical (IHC) analysis

Liver tissues from each group were immediately fixed in 4% paraformaldehyde (Servicebio) for 24 h, followed by standard dehydration, clearing, paraffin embedding, and sectioning. To evaluate the expression and localization of key proteins involved in hepatic oxidative stress signaling, IHC staining was performed on liver sections from all five groups using

specific antibodies against NRF2, Keap1, and PPAR γ (Servicebio, Inc.). Staining results were semi-quantitatively analyzed using the histochemical score (H-score). HE staining was also conducted to assess overall liver morphology and steatosis for systematic pathological evaluation. All staining reagents and antibodies used were obtained from Servicebio, Inc.

1.12 Protein extraction and Western blot analysis

Total protein was extracted from harvested cells or liver tissues after washing three times with ice-cold phosphate buffered saline (PBS). Samples were then lysed in radio-immunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors on ice. The resulting lysates were denatured at 100 °C for 10 min and stored at -20°C until use. For immunoblotting, equal amounts of protein were separated by SDS-PAGE on 8%–15% polyacrylamide gels and transferred onto PVDF membranes. After blocking, membranes were incubated with specific primary and secondary antibodies. Protein bands were visualized using a chemiluminescence imaging system. Notably, due to the small molecular mass (approximately 2 ku) of the target protein MOTS-c, a 15% SDS-PAGE gel (10 wells) and a 3–40 ku protein ladder (Beijing Tsingke Biotechnology Co., Ltd.) was employed. Electrophoresis was conducted at a constant voltage of 60 V. The separated proteins were transferred onto a 0.22 μm PVDF membrane under a constant current of 200 mA for 60 min. Detailed antibody information is listed in Table 1.

Table 1 Main antibody information in the text

Antibodies	Identifier	Source
MOTS-c	101AP	FabGennix
NRF2	A0674	ABclonal
KEAP1	ab227828	Abcam
HO-1	ab189491	Abcam
PPAR γ	ab209350	Abcam
PGC-1 α	ab191838	Abcam
GAPDH	A19056	ABclonal

1.13 RNA isolation and quantitative real-time PCR (q-PCR)

Total RNA was extracted from cultured cells or liver tissues using an RNA extraction kit (DP419, Tiangen Biotech, Beijing, China). Subsequently, equal amounts of RNA were reverse-transcribed into cDNA with the revert aid first strand cDNA synthesis kit (Thermo Fisher Scientific). q-PCR was performed using gene-specific primers listed in Table 2, with GoTaq® qPCR master mix on a CFX 96 Real-Time PCR system (Bio-Rad). Data were collected and

analyzed with Bio-Rad CFX Manager 3.1 software. The relative expression levels of target genes were calculated using the $\Delta\Delta C_t$ method and are presented graphically.

Table 2 Primer pairs used for q-PCR analysis

Gene ID	Name	Description	Forward primer (5'-3')	Reverse primer (5'-3')
4549	MOTS-c	Human	CAAGAAATGGGCTACATTTTC	GAAAATGTAGCCCATTCTTG
9817	KEAP1	Human	CGTGGCTGTCTCAATCGTCT	CATTGCTGTGATCATTCGCCACT
117519	Keap1	Rat	CTCGCAGGATGGTAACCGAACCTT	CCGAAGCTCGTTCATGATGCCAA
4780	NRF2	Human	GAGAGCCCAGTCTTCATTGCTA	CCGTCTAAATCAACAGGGGGCTA
83619	Nrf2	Rat	AGTCTTCCATTTACGGAGACCC	ACTGTATCCCCAGAAGAATGTGT
2730	GCLM	Human	TGCTGTGTGATGCCACCAGA	CGCTTGAATGTCAGGAATGCTT
2729	GCLC	Human	CACCCTCGTTCAGTACCTT	AATTGCCCCATTCCAAATCCCA
25283	Gclc	Rat	CCAGACGAAGCCATAACAAGC	ACAACCTTTTCTCCTCTCCGAT
3162	HO-1	Human	CAGGCTCCGCTTCTCCGAT	AAGCCAGCCAAGAGAATTCCC
24451	Ho-1	Rat	TTCAAACAGCTCTATCGTGCTC	TTAGCCTCTTCTGTACCCCT
5468	PPAR γ	Human	ACTTCTCCAGCATTTCTACTCC	GGCTCCACTTTGATTGCACT
25664	Ppar γ	Rat	TGATCTTAAGTGTGGATCCAC	CCAAACCTGATGGCATTGTGA
10891	PGC-1 α	Human	CCTGCTATTACTATGAGTCAAGCC	ATTCCTCGTAGCTGTCATACCTG
83516	Pgc-1 α	Rat	TACACAACCGCAGTCGCAAC	TTCCACACTTAAGGTTTCGCTCA
2597	GAPDH	Human	CAACAGCCTCAAGATCATCAGC	TGAGTCCTTCCACGATACCAA
24383	Gapdh	Rat	TCAACGGGAAACCCATCACC	TAGACTCCACGACATACTCAGC

1.14 Transcriptomic sequencing analysis

Transcriptome sequencing of rat liver tissues was performed by BGI (Shenzhen, China) on the BGISEQ-500 platform. A total of 15 samples were sequenced. Total RNA was extracted, and RNA integrity was assessed using an Agilent 2100 Bioanalyzer; the RNA integrity number (RIN) was ≥ 7.0 for all samples. Libraries were constructed following standard protocols: mRNA enrichment, fragmentation, cDNA synthesis, adapter ligation, and PCR amplification. Paired-end (PE100) sequencing was carried out. Each sample produced an average of 6.69 Gb of raw data (approximately 22 Gb raw reads per sample). Raw reads were filtered to remove low-quality reads, adapters, and reads with high N content, yielding high-quality clean reads. The clean reads were aligned to the rat reference genome, with an average alignment rate of 91.23%. Differentially expressed genes (DEGs) were identified based on a Poisson distribution model (PoissonDis) with the criteria: fold change ≥ 2 or ≤ 0.5 , and false discovery rate (FDR) ≤ 0.001 . The raw sequencing data have been deposited in the NCBI public database (BioProject accession number: PRJNA1189138; to be released). Genes meeting these thresholds were defined as DEGs and used for subsequent bioinformatic analyses.

1.15 Statistical analysis

All data were analyzed using IBM SPSS

Statistics 22 software. The data are presented as the mean $\pm$ standard deviation (SD) of three independent experiments. A one-way analysis of variance (ANOVA) was first performed to assess overall differences among multiple groups. If the ANOVA result was statistically significant ($P<0.05$), Student's t -test with Bonferroni correction was used for pairwise comparisons. A P -value <0.05 was considered statistically significant, and $P<0.01$ was considered highly significant, which are indicated in the figures by * $P<0.05$, \$ $P<0.05$ and ** $P<0.01$, respectively.

2 Results

2.1 MOTS-c modulates lipid metabolism and activates the NRF2/PPAR γ pathway in HepG2 cells

To investigate the impact of endogenous MOTS-c on hepatic oxidative stress and lipid accumulation, we conducted experiments using the HepG2 cell line, a well-established model for studying liver metabolism^[35]. First, we generated three stable cell lines *via* lentiviral infection: a control group infected with empty vector (Control), a group overexpressing MOTS-c (OE-MOTS-c), and a group with MOTS-c knockdown (sh-MOTS-c). Fluorescence microscopy confirmed successful infection across all groups (Figure 1a). Furthermore, q-PCR and Western blot analyses validated that MOTS-c expression was

significantly increased in the OE-MOTS-c group and decreased in the sh-MOTS-c group compared with the Control group (Figure 1b).

Secondly, lipid droplets (LDs)—mainly composed of neutral lipids such as triglycerides and cholesteryl esters—serve not only as storage sites for lipids and energy but also as dynamic, signaling-competent organelles implicated in various metabolic diseases. Conditions such as overnutrition-induced fatty liver and diabetes are often accompanied by abnormal LD accumulation. After treatment with 100 $\mu\text{mol/L}$ OA for 24 h, Oil Red O staining and the fluorescent lipid droplet assay were quantified using Image J. Relative to the Control group (set as 1), the OE-MOTS-c group showed significantly lower lipid droplet area and mean fluorescence intensity (both <1 , $P<0.05$), whereas the sh-MOTS-c group showed significantly higher values (both >1 , $P<0.05$) (Figure 1c, d). Measurement of intracellular TG content using an enzymatic assay further demonstrated that, under basal conditions, relative to the Control group (set as 1), TG levels were significantly lower in the OE-MOTS-c group (approximately 0.76-fold, $P<0.05$) and significantly higher in the sh-MOTS-c group (approximately 5.2-fold, $P<0.05$) (Figure 1e). Following OA treatment, relative to the Control group (set as 1), the OE-MOTS-c group still showed significantly lower TG levels (approximately 0.72-fold, $P<0.05$), while the sh-MOTS-c group exhibited significantly higher levels (approximately 4.4-fold, $P<0.05$). All comparisons were adjusted using Bonferroni correction for multiple comparisons. OA treatment increased TG content in all groups, but the rise was most pronounced in the sh-MOTS-c cells. These results indicate that suppressing MOTS-c promotes lipid deposition, whereas MOTS-c overexpression attenuates lipid synthesis and/or uptake.

Next, to examine whether MOTS-c is involved in OA-induced oxidative stress, we measured typical antioxidant biomarkers including reduced GSH, SOD, and CAT^[40]. As shown in Figure 1f–h, MOTS-c overexpression elevated the levels of GSH, SOD, and CAT, enhancing antioxidant enzyme activity, whereas MOTS-c knockdown reduced their expression. Furthermore, using previously established NRF2-knockout HepG2 cells (NRF2^{-/-}), we observed that NRF2 deletion led to decreased mRNA and protein levels of heme oxygenase-1 (HO-1), glutamate-cysteine ligase catalytic subunit (GCLC), and glutamate-cysteine ligase modulatory subunit (GCLM), accompanied by diminished antioxidant capacity^[37]. In Figure S1a, treatment with 100 $\mu\text{mol/L}$

OA significantly elevated TG levels in both NRF2^{+/+} (NRF2 wild-type) and NRF2^{-/-} (knockout) cells, indicating that OA-induced lipid accumulation is independent of NRF2. Figure S1b–d showed that compared with NRF2^{+/+} cells, NRF2^{-/-} cells exhibited markedly lower levels of GSH, SOD, and CAT, establishing NRF2 as a key factor in maintaining the activity of these antioxidant enzymes. These results are consistent with those in Figure 1e–h, which showed that MOTS-c overexpression reduced TG levels and increased GSH/SOD/CAT activities, whereas MOTS-c interference produced opposite effects. NRF2 deletion in Figure S1 led to decreased antioxidant enzymes, and MOTS-c overexpression in Figure 1e–h upregulated NRF2 and reversed these changes.

NRF2 is a key transcription factor in the cellular antioxidant defense system. Previous studies indicate that MOTS-c can directly bind NRF2 and affect its activity^[41]. Moreover, NRF2 binding to the ARE in the PPAR γ promoter induces PPAR γ expression, and the PPAR γ /NRF2 axis can modulate lipid metabolism by regulating fatty acid oxidation^[42]. The expression of antioxidant and lipid metabolism-related genes was detected by q-PCR and Western blot, and the Western blot results were subjected to quantitative analysis. q-PCR analysis showed that, compared with the Control, the OE-MOTS-c group exhibited up-regulated mRNA levels of *NRF2*, *HO-1*, *GCLC*, *GCLM*, *PPAR γ* , and *PGC-1 α* , along with down-regulated *KEAP1* expression, while the opposite pattern was observed in the sh-MOTS-c group (Figure 1i). Western blot results confirmed these trends at the protein level for the examined genes, with the exception of GCLC and GCLM, whose protein expression was not determined (Figure 1j, k).

Collectively, these findings suggest that MOTS-c activates the NRF2/PPAR γ signaling axis in HepG2 cells, thereby enhancing antioxidant defense, regulating lipid metabolism, and alleviating liver injury associated with metabolic disturbance.

2.2 MOTS-c ameliorates systemic metabolic parameters and hepatic steatosis in T2DM rats

To investigate whether the exercise-mimetic peptide MOTS-c improves disordered hepatic lipid metabolism in a manner comparable to aerobic exercise, we established a rat model of T2DM with concurrent hepatic steatosis. The modeling scheme is outlined in Figure 2a. After 7 weeks on either a normal diet (PC group) or a high-fat/high-sucrose diet (PD group), PD rats exhibited significantly elevated FBG, FINS, and HOMA-IR index compared with PC rats (Figure 2b–d).

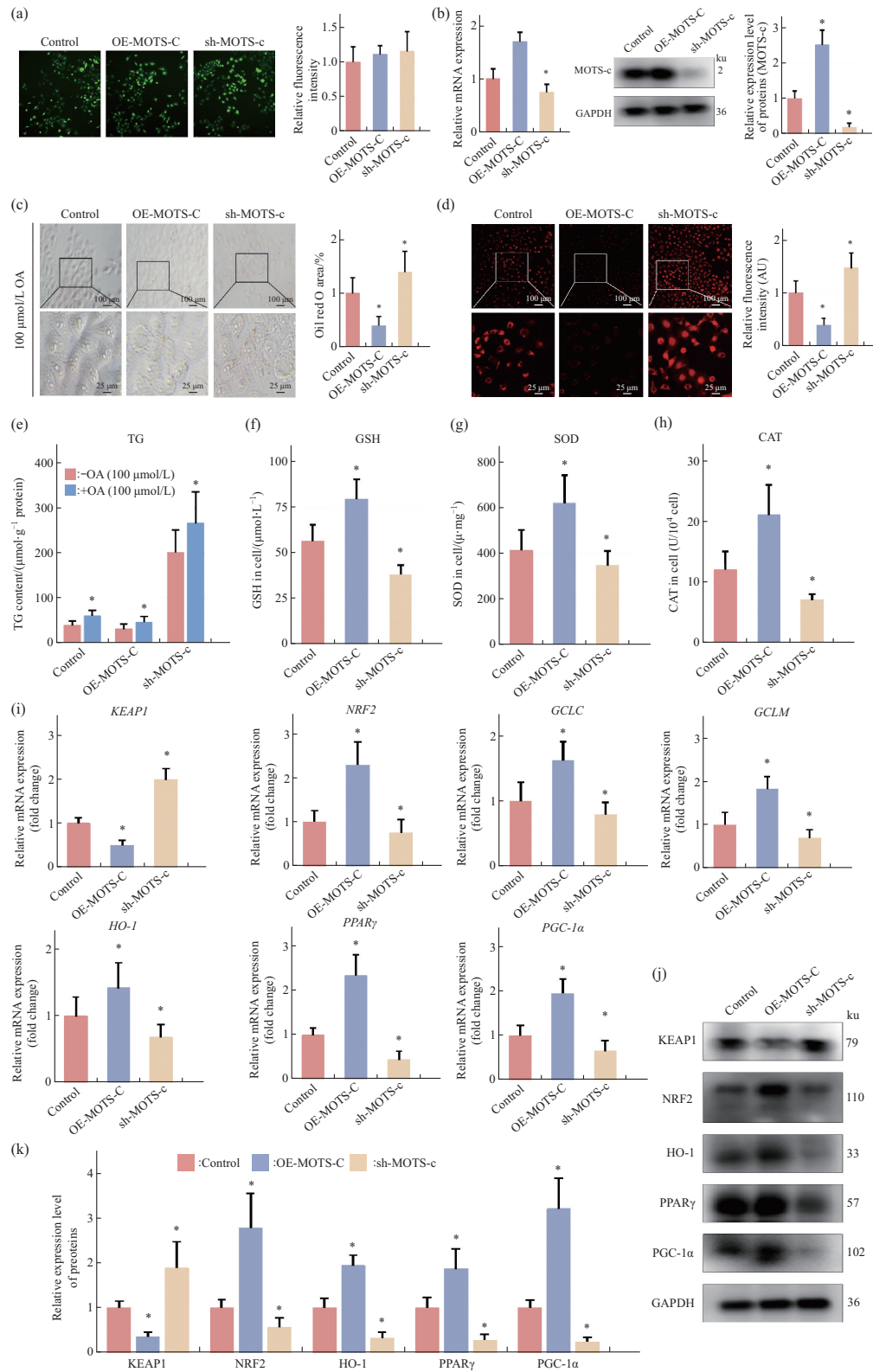


Fig. 1 MOTS-c modulates lipid metabolism and activates the NRF2/PPAR γ pathway in HepG2 cells

(a) Establishment of stable cell lines via lentiviral transduction, observed under a fluorescence microscope. Control: cells transduced with the empty lentiviral vector; OE-MOTS-c: MOTS-c-overexpressing cells; sh-MOTS-c: MOTS-c-knockdown cells. (b) Expression levels of MOTS-c in the established cell lines were verified by q-PCR and Western blot analysis. (c, d) After treating the three cell lines with OA (100 μ mol/L) for 24 h, lipid droplets were assessed by Oil Red O staining (c) and a fluorescent lipid droplet assay kit (d). (e-h) Levels of triglycerides (TG), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) were measured in the three cell lines. (i-k) q-PCR (i) and Western blot (j, k) were used to measure the mRNA and protein expression levels of relevant targets in the cell lines (i, j). The Western blot results were then quantified and presented as bar graphs (k). All results are given as the mean $\pm$ SD. $n=3$, * $P<0.05$.

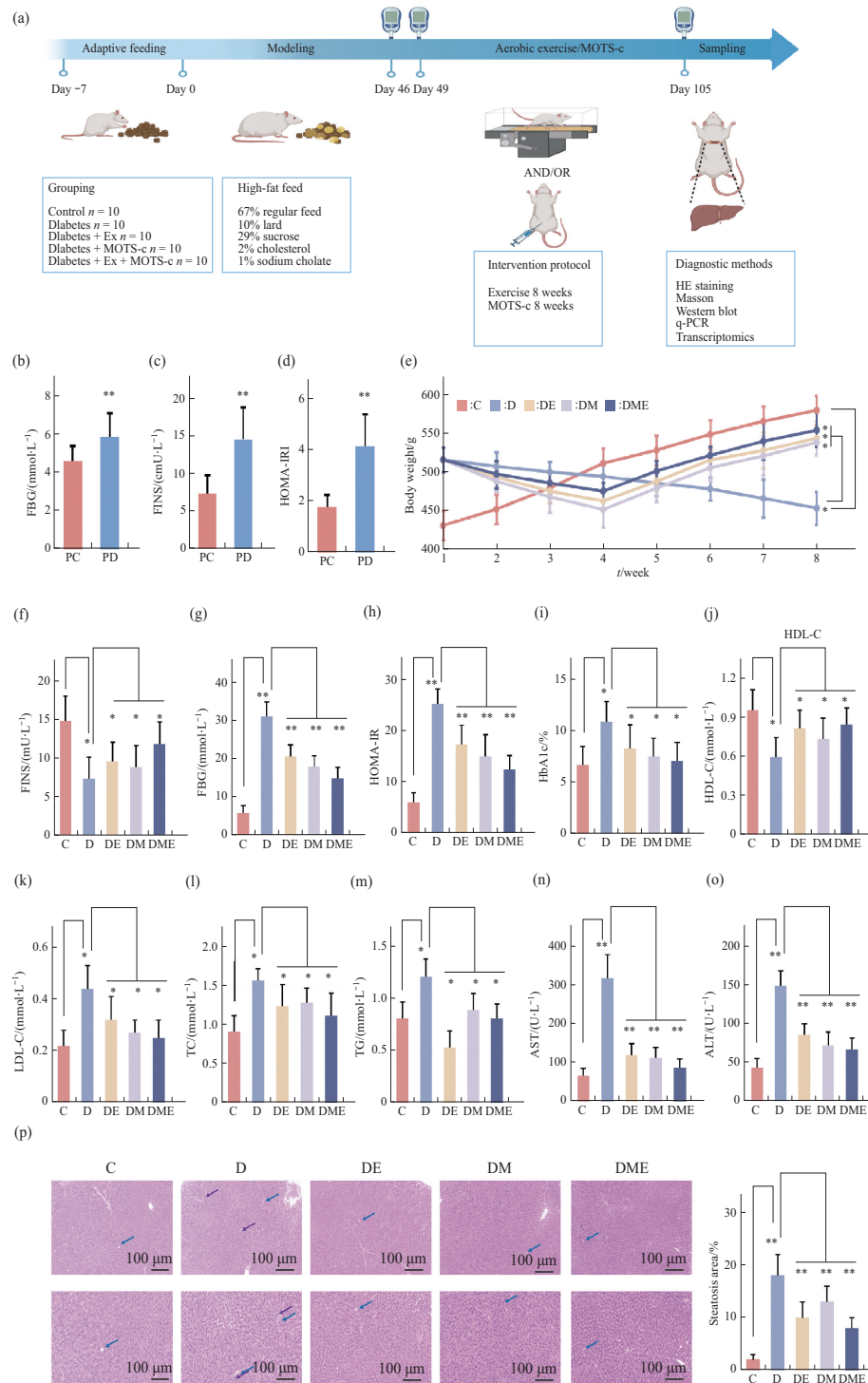


Fig. 2 MOTS-c ameliorates systemic metabolic parameters and hepatic steatosis in T2DM rats

(a) Schematic diagram of the animal model establishment process. (b-d) After 7 weeks of feeding with either a normal diet (PC group) or a high-fat/high-sucrose diet (PD group), fasting blood glucose (FBG), (b), fasting serum insulin (FINS), (c), and the homeostasis model assessment of insulin resistance (HOMA-IR) index (d) were measured in the two groups. (e) PD rats were divided into groups receiving 8-week aerobic exercise training (DE group), MOTS-c injection (DM group), combined treatment (DME group), or no intervention (D group), with healthy rats serving as the control (C group). Body weight changes during the experimental period were recorded and presented as a line graph. (f-i) Metabolic parameters including fasting insulin (FINS), (f), fasting blood glucose (FBG), (g), HOMA-IR (h), and glycated hemoglobin (HbA1c), (i) were measured in each group. (j-m) Serum lipid profiles were determined: high-density lipoprotein cholesterol (HDL-C), (j), low-density lipoprotein cholesterol (LDL-C), (k), triglycerides (TC), (l), and total cholesterol (TG), (m). (n, o) Liver function markers were assessed: alanine aminotransferase (AST), (n) and aspartate aminotransferase (ALT), (o). (p) Representative hematoxylin and eosin (HE)-stained liver sections from each group were examined. Statistical analysis of the HE staining results was performed, and the data are presented as a bar graph, with the percentage of liver steatosis area per group on the y-axis and the experimental groups on the x-axis. All results are given as mean±SD. $n=3$, * $P<0.05$, ** $P<0.01$.

The PD rats were then divided into groups receiving 8 weeks of aerobic exercise training, MOTS-c injection, combined treatment, or no intervention (D group). Compared with the control group (C), diabetic rats (D) showed sustained weight loss over 8 weeks. In contrast, rats subjected to exercise (DE), MOTS-c treatment (DM), or their combination (DME) lost weight only during the first 4 weeks, after which their body weight gradually increased (Figure 2e). By week 8, body weight in the D group was significantly lower than in the C group, whereas all treatment groups showed marked weight recovery compared with the D group. FINS levels were significantly higher in the DE, DM, and DME groups than in the D group (Figure 2f). Moreover, FBG, HOMA-IR, and HbA1c levels were notably reduced in the treatment groups relative to the D group (Figure 2g-i).

We next assessed whether aerobic exercise, MOTS-c, or their combination alleviated T2DM-induced liver injury and metabolic disturbances by examining liver function markers (ALT, AST) and serum lipid profiles (LDL-C, HDL-C, TG, TC). Compared with the D group, HDL-C levels increased while LDL-C, TC, and TG levels decreased in all intervention groups, indicating improved dyslipidemia (Figure 2j-m). ALT and AST levels, which were markedly elevated in the D group compared with the C group, were significantly lowered by each treatment (Figure 2n, o). Histopathological evaluation of liver sections *via* HE staining further revealed the beneficial effects of MOTS-c and exercise on hepatic tissue (Figure 2p). Compared with the C group, the D group exhibited pronounced hepatic steatosis, characterized by cytoplasmic vacuoles of varying sizes (blue arrows), along with noticeable perivascular inflammatory infiltration marked by sparse lymphocyte clusters around vessels (purple arrows). In contrast, these pathological features were substantially attenuated in all treatment groups, with reduced steatosis and no detectable perivascular lymphocyte infiltration.

Collectively, these findings demonstrate that MOTS-c, like aerobic exercise, ameliorates T2DM-induced liver injury and improves abnormal hepatic lipid metabolism in rats.

2.3 MOTS-c activates hepatic NRF2 and PPAR γ signaling pathways in T2DM rats

Previous *in vitro* experiments elucidated that MOTS-c overexpression activates the NRF2/PPAR γ signaling axis in HepG2 cells, enhancing antioxidant defenses and improving lipid metabolism, thereby attenuating hepatic injury induced by metabolic disruption. To determine whether aerobic exercise,

intraperitoneal MOTS-c injection, or their combination similarly activates this pathway *in vivo*, we examined the NRF2/PPAR γ axis in the T2DM rat model with hepatic steatosis. Immunohistochemical staining was performed to assess the expression of NRF2, KEAP1, and PPAR γ in liver tissues across the five experimental groups, with quantitative analysis conducted using the histochemical score (H-score). As shown in Figure 3a, compared with the control group (C), the diabetic group (D) exhibited decreased expression of Nrf2 and PPAR γ , while Keap1 expression was increased. In contrast, the exercise (DE), MOTS-c (DM), and combined intervention (DME) groups showed markedly elevated levels of NRF2 and PPAR γ , along with reduced Keap1 expression relative to the D group.

We further verified these findings by measuring mRNA and protein levels of key antioxidant and lipid metabolism-related genes. q-PCR analysis revealed that, compared with the D group, the DE, DM, and DME groups displayed significantly higher mRNA expression of *Nrf2*, *HO-1*, *GCLC*, *PPAR γ* , and *PGC-1 α* , whereas *Keap1* expression was downregulated (Figure 3b). These trends were consistently confirmed at the protein level by Western blot and densitometric analysis (with the exception of GCLC, for which protein expression was not determined) (Figure 3c, d). Taken together, these data indicate that MOTS-c, like aerobic exercise, activates the hepatic NRF2/PPAR γ signaling pathway in T2DM rats, promoting antioxidant responses and contributing to the improvement of lipid metabolism.

2.4 MOTS-c reprograms the hepatic transcriptome and activates cytoprotective pathways in diabetic liver

Transcriptome sequencing is an effective approach for screening DEGs. We performed RNA sequencing on liver tissues from the five experimental groups (C, D, DE, DM, and DME). A bar chart was used to visualize DEGs between groups, with red and blue bars representing up-regulated and down-regulated genes, respectively (Figure 4a). Compared with the C group, the D, DE, DM, and DME groups exhibited 160, 125, 321, and 73 up-regulated genes, and 99, 122, 573, and 54 down-regulated genes, respectively. When each treatment group (DE, DM, DME) was compared with the D group, 85, 87, and 67 genes were up-regulated, while 112, 227, and 124 genes were down-regulated. Further comparisons between DE *vs* DM, DE *vs* DME, and DM *vs* DME revealed distinct DEG profiles, with the highest number of DEGs observed between DM and C (894 genes) and the lowest between DME and DE

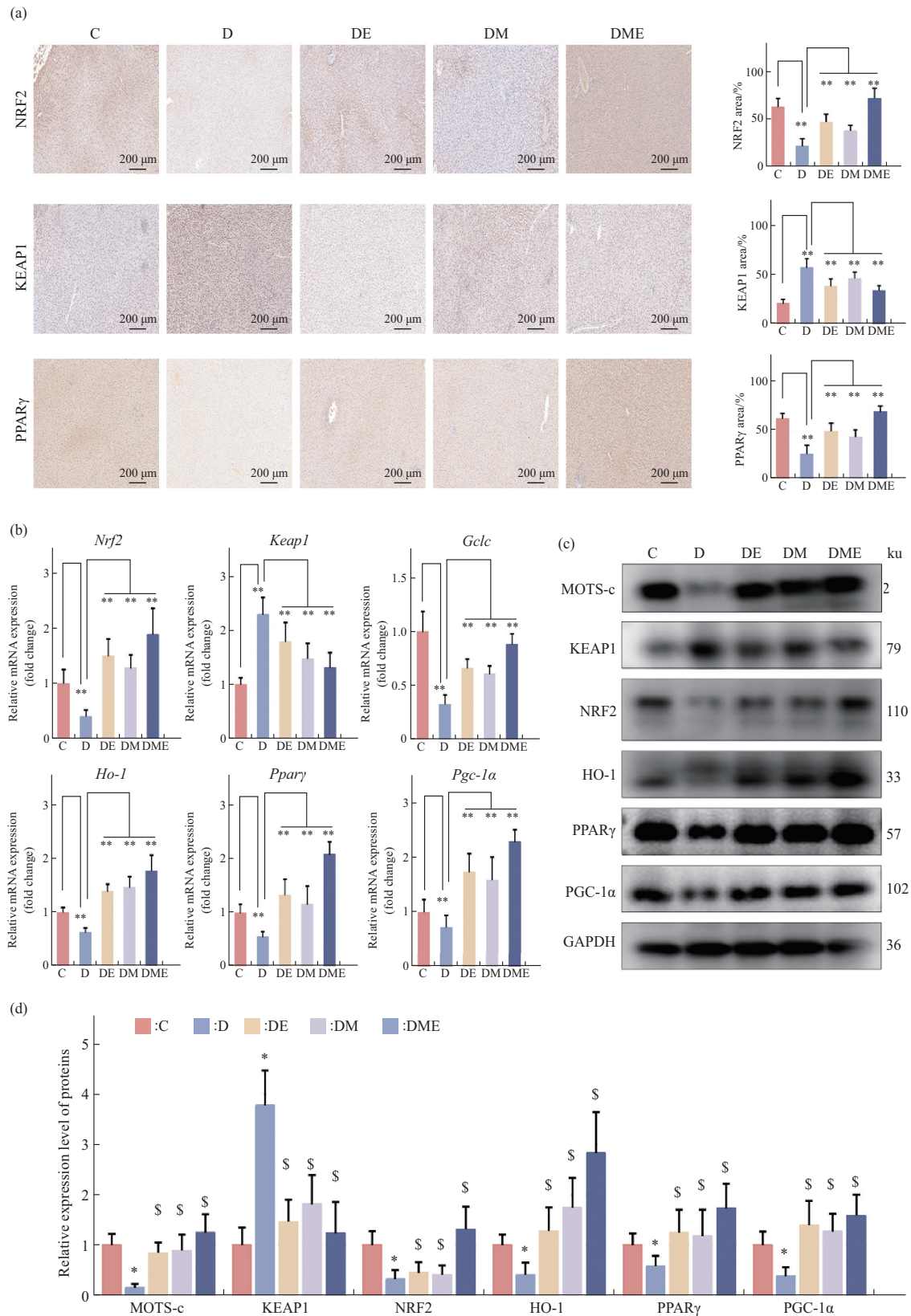


Fig. 3 MOTS-c activates hepatic NRF2 and PPAR γ signaling pathways in T2DM rats

(a) Expression of NRF2, KEAP1, and PPAR γ in liver tissues from the five experimental groups was assessed by immunohistochemistry and analyzed using the histochemistry score (H-score). (b–d) mRNA and protein expression levels of related targets in the five groups were detected by q-PCR (b) and Western blot analysis (c), respectively. Western blot results were then quantified and presented as bar graphs (d). The asterisk (*) indicates a comparison between group D and group C, while the symbol (\$) indicates comparisons between group DE, group DM, or group DME and group D, respectively. $n=3$, \$ $P<0.05$, * $P<0.05$, ** $P<0.01$.

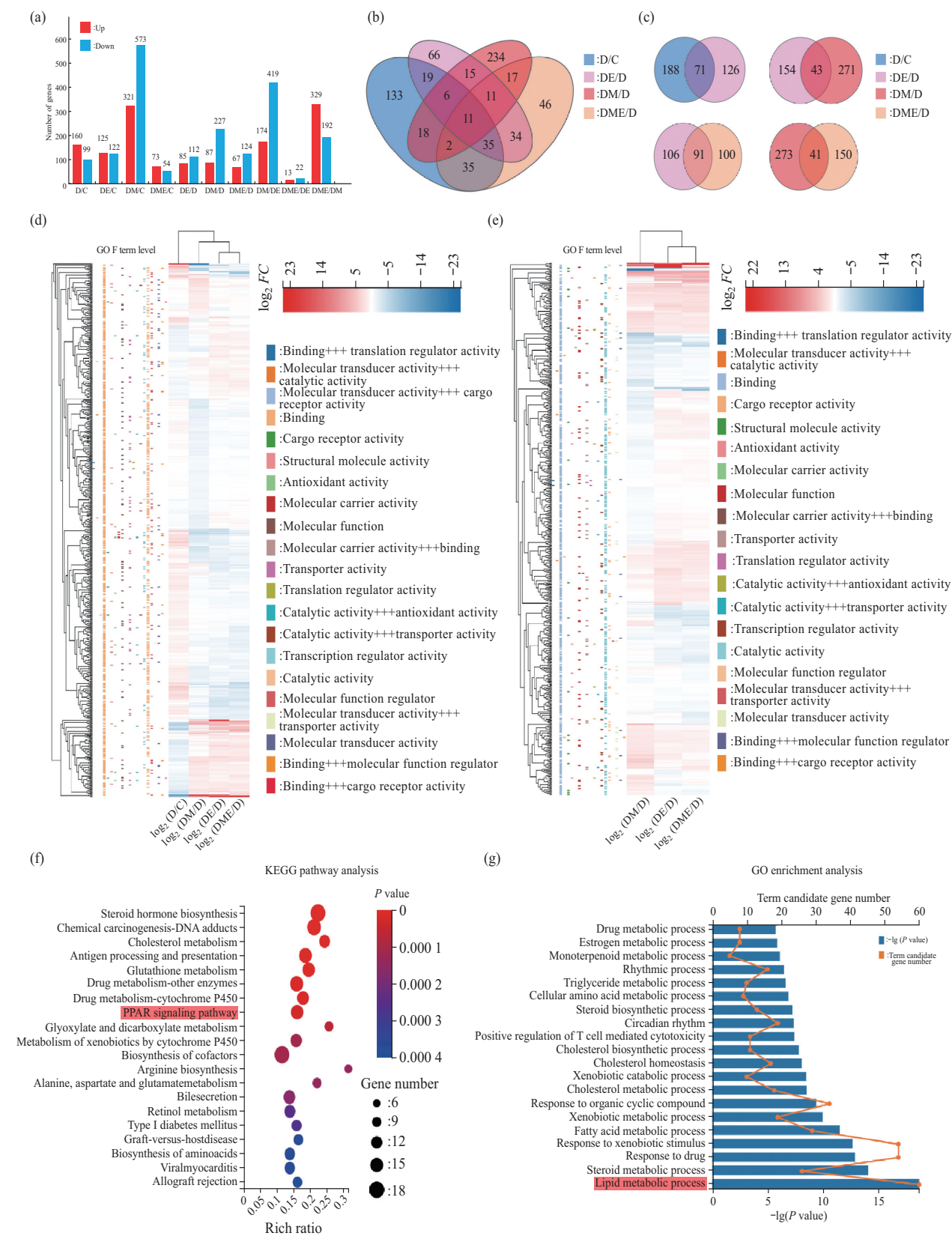


Fig. 4 MOTS-c reprograms the hepatic transcriptome and activates cytoprotective pathways in the diabetic liver
(a) Bar graph illustrating the number of upregulated (red) and downregulated (blue) differentially expressed genes (DEGs) identified by transcriptome sequencing across the five groups (C, D, DE, DM, and DME groups). (b) Venn diagram showing the overlap of DEGs among the five experimental groups. (c) Pairwise comparisons between treatment groups presented as Venn diagrams. (d) Hierarchical clustering heatmap of all 682 DEGs identified in the analysis shown in panel b. (e) Hierarchical clustering heatmap of 549 DEGs obtained from the comparison between the D group and other groups. (f, g) KEGG pathway (f) and GO term enrichment analyses (g) performed on the 682 DEGs identified in panel b.

(35 genes).

A Venn diagram analysis (Figure 4b) comparing DEGs from D vs C and from each treatment vs D identified 11 common DEGs. The eleven common differentially expressed genes are as follows: *Rasgef1b*, *LOC100910829*, *LOC102553715*, *Hspb1*, *Rell1*, *Thnsl2*, *Chd1l*, *Wfdc21*, *ste2*, *Cml2*, and *Prodh1*. Among them, *Hspb1*, *Wfdc21*, and *Prodh1* are associated with antioxidant stress and lipid metabolism. Detailed information is provided in Table S1. The highest number of unique DEGs was found in the DM vs D comparison (234 genes). To better understand the functional similarities and differences among the five groups, we analyzed shared and distinct DEG sets using Venn diagrams (Figure 4c). For instance, D/C and DE/D shared 71 DEGs, with 188 and 126 unique to each comparison, respectively. Comparisons between DE/D and DM/D, DE/D and DME/D, and DM/D and DME/D further revealed group-specific transcriptional changes.

Next, hierarchical clustering based on all 682 DEGs identified in Figure 4b showed clear separation among groups (Figure 4d). Gene ontology (GO) analysis indicated that these DEGs were mainly associated with biological processes such as binding, catalytic activity, molecular function, transporter activity, and transcription regulator activity (Figure 4d). Similarly, clustering of 549 DEGs from the treatment-vs-D comparisons also displayed distinct expression patterns across groups (Figure 4e), with enrichment in comparable functional categories. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the 682 DEGs highlighted the top 20 enriched pathways (Figure 4f), including steroid hormone biosynthesis, chemical carcinogenesis, and the PPAR signaling pathway—a key pathway involved in lipid metabolism. Correspondingly, GO enrichment analysis of the same gene set revealed a strong association with lipid metabolic processes among the top 20 terms (Figure 4g). D comparisons also displayed distinct expression patterns across groups (Figure 4e), with enrichment in comparable functional categories.

2.5 Comparative transcriptomics reveals pathway-specific regulation by MOTS-c in T2DM liver

While Venn diagram analysis revealed both unique and shared DEGs between experimental groups (Figure 4c), their associated pathways and biological functions remained unclear. To address this, we performed a series of functional enrichment analyses. First, KEGG pathway analysis was conducted for each paired comparison (D vs C, DE vs

D, DM vs D, DME vs D). The top 20 enriched pathways from each comparison are presented in Figure 5. Notably, many of these pathways are related to lipid metabolism. Importantly, all comparisons indicated a significant impact on the PPAR signaling pathway, a canonical pathway regulating lipid metabolism (Figure 5). Further categorization of these KEGG results into major classes—cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems—demonstrated that the most affected categories included transport and catabolism, signal transduction, folding/sorting/degradation, cancer: overview, lipid metabolism, and the endocrine system (Figure S2). The consistent enrichment in lipid metabolism across comparisons is particularly noteworthy. Subsequently, GO enrichment analysis was performed for the categories: biological process, cellular component, and molecular function. The results showed that the dominant terms were “metabolic process” (biological process), “cell” (cellular component), and “binding” (molecular function) (Figure S3). Finally, a detailed GO enrichment analysis focusing specifically on biological processes was carried out. The top 20 enriched terms were predominantly associated with metabolic activities, especially those influencing lipid metabolism. In summary, integrated KEGG pathway and GO enrichment analyses of DEGs from paired group comparisons suggest that MOTS-c treatment, aerobic exercise, and their combination likely modulate lipid metabolism, potentially through the PPAR signaling pathway.

3 Discussion

While it is known that T2DM accelerates liver disease progression and increases mortality risk^[43], the molecular mechanisms linking exercise-mimetic peptides to hepatoprotection remain poorly defined. Here we identify MOTS-c as an activator of the hepatic NRF2/PPAR γ axis, a role not previously reported for MOTS-c. Unlike prior studies that simply described MOTS-c as an exercise-mimetic peptide^[44], the present work shows that MOTS-c improves antioxidant defense and lipid metabolism specifically through this axis, thereby providing a distinct molecular pathway for intervening in T2DM-associated MAFLD. By combining evidence from cell, animal, and omics studies, this work shows that the mitochondrial peptide MOTS-c acts like an exercise mimetic. MOTS-c may cooperate with aerobic exercise to jointly activate the NRF2/PPAR γ

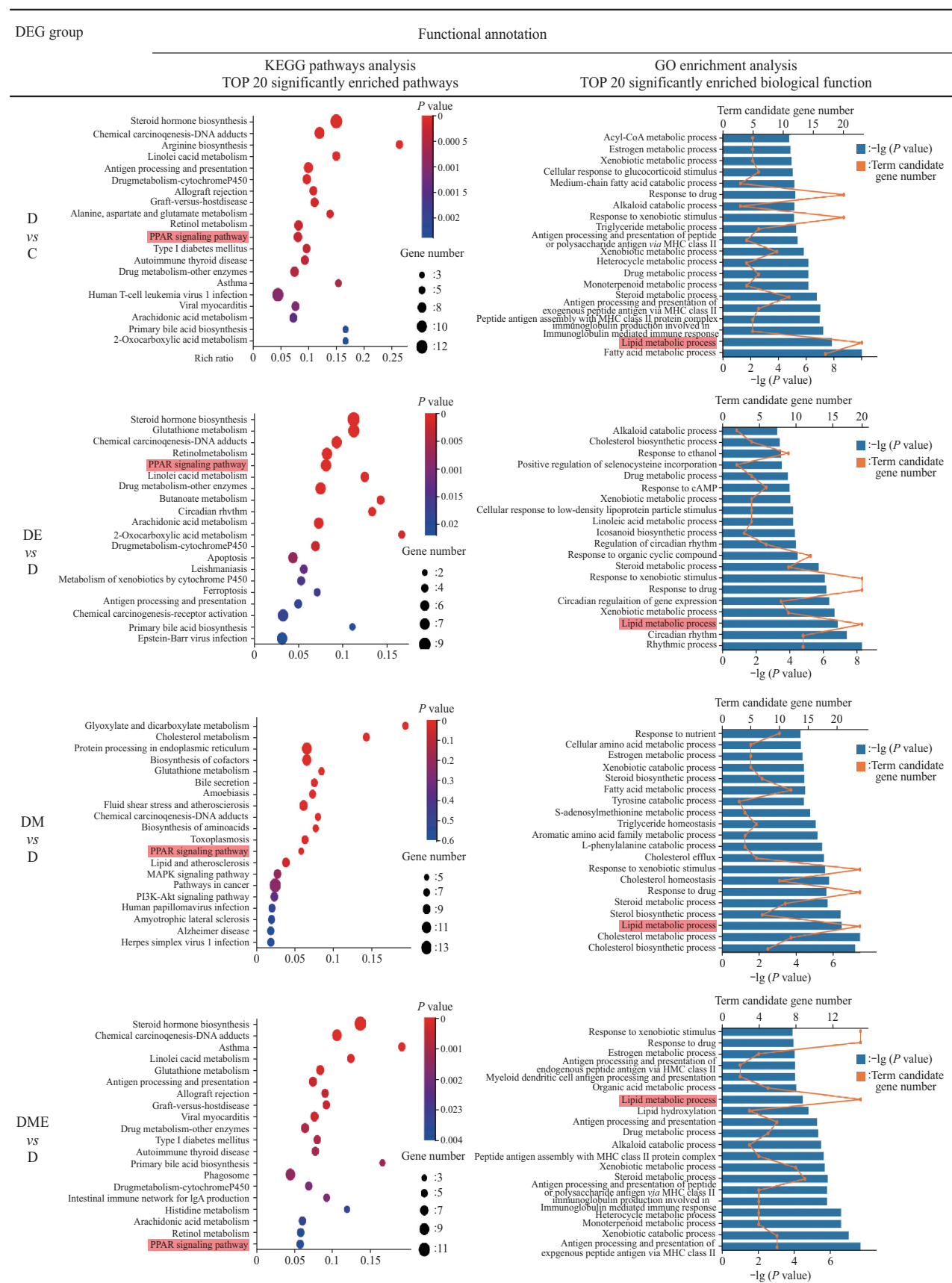


Fig. 5 Comparative transcriptomics reveals pathway-specific regulation by MOTS-c in T2DM liver

KEGG pathway and GO term enrichment analyses were conducted for pairwise comparisons between treatment groups. The top 20 significantly enriched pathways and biological functions are presented.

pathway without definitive synergistic validation, thereby reducing liver oxidative stress and lipid metabolism problems in T2DM. This finding diverges from previous research by establishing a direct, NRF2/PPAR γ -dependent mechanism downstream of MOTS-c, rather than a general exercise-mimetic effect.

The logical starting point of this study was to clarify the direct effect of MOTS-c on hepatocytes. Our experiments in HepG2 cells provided robust mechanistic evidence (Figure 1, S1): overexpression of MOTS-c significantly suppressed oleic acid-induced lipid droplet formation and triglyceride accumulation, whereas its knockdown exacerbated lipid deposition. This directly establishes MOTS-c as an intrinsic regulator of lipid metabolism in hepatocytes. Moreover, MOTS-c overexpression markedly increased levels of endogenous antioxidants, including reduced GSH and SOD, indicating enhanced cellular antioxidant capacity. Crucially, in NRF2-knockout cells, these regulatory effects of MOTS-c were abolished, with elevated TG and suppressed antioxidant enzyme activity—demonstrating for the first time in hepatocytes that NRF2 is essential for MOTS-c function. Further molecular analyses revealed that MOTS-c upregulates NRF2 and its downstream targets (HO-1, GCLC) while concurrently activating PPAR γ , which is closely linked to lipid metabolism. Together, these results construct a clear cellular model: MOTS-c activates NRF2, initiating an antioxidant program and, potentially through NRF2-mediated transcriptional regulation of PPAR γ , establishes a coordinated “NRF2/PPAR γ ” defensive axis that directly counteracts lipotoxicity and oxidative stress at the cellular level. This provides a direct molecular target and mechanistic hypothesis for subsequent animal experiments.

Evidence has indicated that NRF2 is a central regulator of cellular oxidative stress and can transcriptionally activate PPAR γ to modulate lipid metabolism. Based on our cellular findings, we validated the therapeutic potential of MOTS-c and its possible combined beneficial relationship with aerobic exercise in a T2DM rat model^[45–46]. As shown in Figure 2, 3, exogenous MOTS-c supplementation recapitulated key benefits of aerobic exercise: improving insulin resistance, glucose and lipid metabolism, and significantly alleviating hepatic steatosis and injury (reflected by reduced ALT/AST and improved liver histopathology)^[47]. This aligns with previous reports and confirms the *in vivo* efficacy of MOTS-c as an exercise mimetic^[19, 48]. More importantly, liver tissue analysis showed that

both MOTS-c and exercise consistently activated the NRF2/PPAR γ axis identified in cellular experiments—upregulating hepatic NRF2, PPAR γ , and PGC-1 α protein expression, while downregulating KEAP1. We examined endogenous MOTS-c expression in rat livers. As shown in Figure 3c, D group had lower MOTS-c protein levels than C group. Endogenous MOTS-c deficiency may contribute to diabetic liver disease. Cellular overexpression and intraperitoneal injection both restore or supplement MOTS-c levels. Overexpression in HepG2 cells provides sustained intracellular production. Intraperitoneal injection delivers exogenous peptide, which is likely taken up by hepatocytes *via* peptide transporters or endocytosis. Both approaches increase intracellular MOTS-c and activate the NRF2/PPAR γ axis. This striking consistency from cells to animals and from molecules to phenotype establishes the MOTS-c-NRF2-PPAR γ pathway as a core mechanism in ameliorating diabetic liver disease. The unique advantages observed in the combined intervention group suggest that exercise may complement and produce additive benefits alongside the molecular action of MOTS-c through broader physiological networks, such as systemic energy expenditure and endocrine regulation. Mechanistically, aerobic exercise increases whole-body energy expenditure and improves insulin sensitivity, thereby reducing the systemic metabolic burden on the liver. This systemic effect may act in concert with the direct hepatoprotective action of MOTS-c *via* the NRF2/PPAR γ axis, which independently counteracts lipotoxicity and oxidative stress in hepatocytes. The convergence of systemic metabolic benefits (from exercise) and direct hepatic protection (from MOTS-c) likely underlies the enhanced effects observed in the combined intervention group.

Transcriptomic sequencing provided an unbiased global perspective that corroborated and extended the above findings (Figure 4, 5, S2, S3). Differential gene expression profiles and enrichment analyses revealed that MOTS-c and exercise significantly reprogram the gene expression landscape in diabetic liver, with shared targets markedly enriched in processes such as “lipid metabolic process” and the “PPAR signaling pathway”. Among the 11 common differentially expressed genes (Figure 4b, Table S1), *Hspb1*, *Wfdc21*, and *Prodh1* are associated with antioxidant stress and lipid metabolism. *Hspb1* activates the NRF2/HO-1 pathway, *Wfdc21* participates in adipocyte differentiation, and *Prodh1* regulates proline catabolism and cellular redox balance—all of which align with the NRF2/PPAR γ axis. The

remaining eight common DEGs showed no direct association with this axis. This systems-level evidence reinforces the central role of the NRF2/PPAR γ axis and suggests it governs a broad network of metabolically protective genes. The study holds clear translational promise: MOTS-c and its associated pathway may serve as targets for novel hepatoprotective agents. For patients unable to engage in adequate physical activity, drugs targeting this pathway or MOTS-c analogs could offer effective alternative or adjuvant treatment strategies. Compared with established NAFLD targets such as PPAR α agonists (fenofibrate), PPAR γ agonists (pioglitazone), and FXR agonists (obeticholic acid), MOTS-c activates both NRF2 and PPAR γ *via* an endogenous exercise-mimetic mechanism. Unlike small-molecule drugs, MOTS-c as a peptide has different pharmacokinetic properties (see below). Head-to-head efficacy comparisons and combination studies are needed to position MOTS-c in the current therapeutic landscape. Before clinical translation, several pharmacokinetic limitations must be considered. As a peptide, MOTS-c has a short plasma half-life and low oral bioavailability due to gastrointestinal degradation and rapid renal clearance. Our animal study used injection, which is less convenient for chronic use. Peptide modification (cyclization, Fc conjugation, or nanoparticle encapsulation) may improve stability and delivery.

This study has several limitations. First, the animal model primarily represents the early stage of T2DM with concurrent hepatic steatosis and does not encompass later pathological stages such as NASH and fibrosis. Therefore, the therapeutic effects of MOTS-c and exercise *via* this axis must be extended to and validated in more severe liver injury models. Second, although NRF2 was demonstrated to be essential for MOTS-c action at the cellular level, the necessity of the NRF2/PPAR γ axis *in vivo* has yet to be directly demonstrated using liver-specific knockout models. Third, we observed that combining exercise with MOTS-c may bring superior therapeutic outcomes compared to monotherapy, and the exact molecular basis deserves further research; formal interaction-effect analyses will be supplemented in follow-up research to judge whether true synergy exists. Fourth, hepatic mitochondrial function was not evaluated. Whether MOTS-c improves mitochondrial respiration or reduces mitochondrial ROS production remains unknown. Fifth, markers of hepatocyte apoptosis (Caspase-3, Bax/Bcl-2) and liver fibrosis (alpha-smooth muscle actin (α -SMA) and collagen deposition) were not assessed. Our model mainly

reflects early steatosis, not advanced NASH or fibrosis.

In summary, this study builds a coherent chain of evidence from molecules to the whole organism: the mitochondrial peptide MOTS-c autonomously regulates lipid metabolism and antioxidant balance in hepatocytes *via* activation of the NRF2/PPAR γ axis. In a T2DM model with hepatic steatosis, it mimics aerobic exercise, improving whole-body metabolism and liver fat buildup through the same pathways, and shows a trend toward enhanced efficacy when combined with aerobic exercise, without verified synergistic effects. These findings not only deepen the understanding of the mechanisms underlying exercise-induced hepatoprotection but also provide an innovative theoretical foundation and potential intervention strategies for T2DM-MAFLD. Future studies should address: (1) validate the NRF2/PPAR γ axis in more advanced disease stages (NASH, fibrosis) and in liver-specific knockout models; (2) evaluate hepatic mitochondrial function and apoptosis/fibrosis markers; (3) examine endogenous MOTS-c expression in diabetic liver; (4) optimize peptide pharmacokinetics for oral delivery; (5) compare MOTS-c head-to-head with existing NAFLD drugs.

4 Conclusion

This study demonstrates the protective effect of MOTS-c against type 2 diabetes-related hepatic injury and its underlying molecular mechanism. MOTS-c functions as an exercise-mimetic peptide and protects the liver by activating the hepatic NRF2/PPAR γ signaling axis. This activation enhances antioxidant capacity, reduces excessive lipid accumulation, and stabilizes hepatic metabolic homeostasis, thereby alleviating hepatic steatosis and oxidative stress under diabetic conditions. Both aerobic exercise and MOTS-c intervention improve diabetic hepatic metabolic dysfunction through NRF2/PPAR γ pathway activation. The combined intervention produces additive protective effects and presents better ameliorative outcomes than single treatments, although no definitive synergistic effect is statistically verified in the present study. Transcriptomic analysis further confirms that MOTS-c and aerobic exercise co-regulate genes primarily involved in lipid metabolism and PPAR signaling, which explains their shared regulatory mechanism in improving diabetic liver damage. In conclusion, this study identifies a novel NRF2/PPAR γ -dependent mechanism by which MOTS-c improves T2DM-associated liver metabolic

disorders. It also validates the protective value of MOTS-c as an endogenous exercise-mimetic factor, providing experimental evidence and a theoretical basis for the mechanism research and comprehensive intervention of type 2 diabetes complicated with MAFLD.

Supplementary Available online (<http://www.pibb.ac.cn>, <http://www.cnki.net>):

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PIBB_20260149_Figure_S2.pdf

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有氧运动与MOTS-c通过NRF2/PPAR γ 轴改善2型糖尿病肝脏氧化应激及代谢紊乱*

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摘要 目的 2型糖尿病 (type 2 diabetes mellitus, T2DM) 并发代谢相关脂肪性肝病 (metabolic associated fatty liver disease, MALD) 等肝脏代谢并发症, 会导致肝脏脂质异常蓄积与氧化应激失衡, 逐步引发脂肪性肝炎、肝纤维化等器质性病变, 增加糖尿病患者的不良预后风险。有氧运动能够有效改善肝脏脂代谢功能、提升机体抗氧化水平, 但患者存在长期依从性不足的情况, 临床应用存在明显局限。线粒体开放阅读框12S rRNA-c (mitochondrial open reading frame of the 12S rRNA type-c, MOTS-c) 参与调控机体胰岛素敏感性 & 肝脏氧化还原平衡, 但其改善 T2DM 引起的肝损伤, 以及与有氧运动联合干预的作用机制研究较少。基于此, 本研究以 NRF2/PPAR γ 信号轴为切入点, 探究 MOTS-c 联合有氧运动改善 T2DM 肝脏氧化应激损伤与代谢紊乱的作用及分子机制。方法 细胞实验方面, 采用慢病毒转染技术构建 MOTS-c 过表达和干扰细胞系, 并通过油酸诱导的方式, 构建肝细胞脂质堆积模型, 同时结合 NRF2 基因敲除细胞模型, 检测细胞脂质沉积、甘油三酯含量、抗氧化酶活性及通路关键分子表达, 验证 MOTS-c 的作用靶点与调控机制。动物实验方面, 采用高糖高脂饮食联合链脲佐菌素构建 T2DM 肝脂肪变性大鼠模型, 设置空白对照、有氧运动、MOTS-c 干预及联合干预组, 检测大鼠糖脂代谢相关指标、肝脏组织病理形态, 检测肝脏 NRF2/PPAR γ 通路核心蛋白表达, 并通过肝脏转录组测序分析两组干预方式共同调控的差异基因及富集通路。结果 细胞实验结果显示, MOTS-c 过表达可有效减轻肝细胞脂质沉积, 提高抗氧化酶活性, 激活 NRF2/PPAR γ 信号通路; 敲除 NRF2 基因后, MOTS-c 对肝细胞的保护效应完全消失, 证实 MOTS-c 的保肝作用依赖 NRF2/PPAR γ 通路。动物实验证实, 有氧运动与 MOTS-c 单独干预均可改善模型大鼠胰岛素抵抗, 改善糖脂代谢紊乱, 缓解肝脏脂肪变性, 显著激活肝脏 NRF2/PPAR γ 通路。相较于单一干预, 联合干预对大鼠肝代谢紊乱与氧化损伤的改善效果更优, 但本研究未证实二者存在明确协同作用。转录组测序结果表明, MOTS-c 与有氧运动共同调控的差异基因主要富集在脂质代谢、PPAR 信号通路。结论 MOTS-c 具备运动模拟效应, 可通过激活肝脏 NRF2/PPAR γ 信号轴, 增强肝脏抗氧化能力、维持脂质代谢稳态, 改善 T2DM 所致肝损伤。MOTS-c 与有氧运动联合干预可产生累加保护效果, 干预优势优于单一方式。本研究明确了 MOTS-c 调控 T2DM 肝损伤的核心分子机制, 为 T2DM 并发非酒精性脂肪肝的临床干预提供了实验依据与新思路。

关键词 MOTS-c, T2DM, 有氧运动, 肝脏代谢, 抗氧化应激反应, NRF2/PPAR γ 轴

中图分类号 Q4, R3

DOI: 10.3724/j.pibb.2026.0149

CSTR: 32369.14.pibb.20260149

* 四川省自然科学基金 (2025NSFSC2021), 中国博士后科学基金 (2023M740381, GZB20240083) 和运动医学四川省重点实验室 (2026-A001) 资助。

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收稿日期: 2026-03-23, 接受日期: 2026-06-15