

SEMA3B Loss Disrupts AMPK-Associated Mitochondrial Homeostasis and Aggravates Cardiac Injury

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Abstract

Background

Cardiac injury is closely linked to defective mitochondrial energy production, yet the factors that preserve mitochondrial function in cardiomyocytes remain incompletely understood. Semaphorin 3B (SEMA3B) participates in metabolic and stress responses, but its contribution to cardiac mitochondrial homeostasis is unclear. We examined whether SEMA3B is linked to AMP-activated protein kinase (AMPK) signaling and myocardial injury.

Methods

Lentiviral short-hairpin RNA was used to reduce SEMA3B expression in human AC16 cardiomyocytes and high-fat-diet-fed mice. Selected groups received the AMPK activator AICAR. We assessed AMPK signaling, mitochondrial-dynamics proteins, membrane potential, ATP, reactive oxygen species, cellular metabolic activity, inflammatory mediators, serum lipids, and cardiac histopathology.

Results

SEMA3B knockdown in AC16 cells reduced phosphorylated AMPK and ACC, together with Mfn1, Mfn2, and Drp1. Mitochondrial membrane potential, ATP content, and MTT reduction declined, whereas oxidant generation increased and mitophagy-related markers changed. AICAR attenuated most of these effects. In mice, SEMA3B knockdown was associated with myocardial necrosis, collagen accumulation, apoptosis, and an adverse serum-lipid profile. AICAR restored AMPK-associated signaling, improved mitochondrial measures, and reduced structural injury.

Conclusion

SEMA3B appears to support mitochondrial and metabolic homeostasis in cardiomyocytes through an AMPK-associated pathway. Loss of SEMA3B worsened biochemical and structural indices of cardiac injury, whereas AICAR produced substantial rescue. Further work is required to identify the receptors involved and establish AMPK-specific causality.

1 Introduction

Coronary artery disease (CAD) remains a major cause of death worldwide and a persistent burden on health-care systems (1). Mitochondrial dysfunction is common to both ischemic and atherosclerotic heart disease, despite their heterogeneous causes (2). The myocardium relies on oxidative phosphorylation for continuous ATP production. Cardiac mitochondria also regulate calcium handling, redox signaling, and cell survival. When this coordination fails, respiratory efficiency, membrane

potential, and mitochondrial quality control deteriorate, as reported in clinical CAD and experimental ischemia-reperfusion injury (3, 4). Changes in mitochondrial DNA copy number, deletions, and oxidative damage have likewise been associated with early atherosclerotic and myocardial injury (5, 6). Current lipid-lowering, antiplatelet, and revascularization strategies improve clinical outcomes but do not address the full range of mitochondrial abnormalities (7). Endogenous pathways that protect cardiac mitochondria may therefore offer complementary therapeutic targets.

SEMA3B is a secreted class 3 semaphorin originally identified as an axonal guidance signal in the developing nervous system (8). It was later shown to limit tumor-cell growth and migration and to regulate apoptosis through neuropilin-plexin receptor complexes (9, 10). SEMA3B binding to neuropilin-1 also restrains endothelial migration and angiogenesis, extending its activity to vascular cells (11). Single-nucleus analysis of human pericoronary epicardial adipose tissue recently identified SEMA3B as a candidate adipokine associated with CAD (12). Other class 3 semaphorins contribute to melanocortin-circuit development, systemic energy balance, and metabolic adaptation (13, 14). These findings prompted us to ask whether SEMA3B also regulates energy homeostasis in cardiac cells.

AMPK is a heterotrimeric kinase that senses cellular energy status and adjusts metabolism during energetic stress. In the heart, AMPK promotes ATP-generating pathways, mitochondrial renewal, and fatty-acid utilization while limiting oxidative stress (15–17). In cancer cells, SEMA3B can inhibit PI3K/Akt signaling by disrupting interaction between the p85 regulatory and p110 α catalytic PI3K subunits, thereby reducing Akt phosphorylation (18). Akt and AMPK participate in intersecting responses to nutrient and energy availability (19). We therefore tested whether SEMA3B influences cardiomyocyte mitochondrial homeostasis through AMPK-associated signaling. SEMA3B was silenced in AC16 cells and in mice to examine mitochondrial dynamics, bioenergetics, oxidative and inflammatory indices, and myocardial structure. AICAR served as a pharmacological rescue to determine whether AMPK activation could counter the effects of SEMA3B deficiency.

2 Materials and methods

2.1 Reagents and antibodies

Cell-culture reagents comprised high-glucose Dulbecco's modified Eagle medium (DMEM; Cat. No. 11965092, Thermo Fisher Scientific, Pittsburgh, PA, USA), fetal bovine serum (FBS; SH30070.03, Hyclone, Logan, UT, USA), penicillin-streptomycin (15140148, Thermo Fisher Scientific), and 0.25% trypsin/0.02% EDTA (15090046, Gibco, Grand Island, NY, USA). Protein-analysis materials included 0.45- μ m PVDF membranes (Millipore, Schwalbach, Germany), a 10-180-kDa prestained protein marker (BL712A, Biosharp, Beijing, China), Ponceau S, Tween 20, acrylamide, SDS, PMSF (Amresco, Solon, OH, USA), RIPA buffer, Tris-HCl buffers (1.5 mol/L; pH 6.8 and 8.8; Beyotime, Shanghai, China), a membrane-regeneration kit (ZN1923, Biolab, Beijing, China), and ECL substrate (Thermo Fisher Scientific). Histology and cell-assay reagents included hematoxylin (G1080, Solarbio, Beijing, China), DAPI (KGA215, Beyotime), one-step TUNEL reagent (C1086, Beyotime), crystal violet (KGA229, Beyotime), Masson's trichrome kit

(Solarbio), ATP assay reagent (S0026, Beyotime), DCFH-DA (S0033, Beyotime), MTT reagent (ab211091, Abcam), the CDK1/CCNB1 assay kit (79597, BPS Bioscience, San Diego, CA, USA), and ELISA kits (R&D Systems, Minneapolis, MN, USA). Molecular-cloning supplies included XhoI (1094A), BamHI (1010A), T4 DNA ligase (2011A), and a plasmid miniprep kit (9783; all Takara, Japan), pHelper 1.0 and 2.0, Genechem transfection reagent (GRC102, Genechem, Shanghai, China), 1B medium (Solarbio), ampicillin (A5354, Sigma, St. Louis, MO, USA), and pLVX-Puro (V010426, NovoPro, Shanghai, China). HRP-linked secondary antibodies were goat anti-rabbit IgG (ab6721) and rabbit anti-mouse IgG (ab6728; Abcam, Cambridge, UK). Primary antibodies recognized SEMA3B (ab48197), phospho-AMPK Thr172 (ab133448), phospho-ACC Ser79 (ab68191), Mfn1/2 (ab57602), Drp1 (ab184247), Parkin (ab77924), PINK1 (ab216144), and LC3-II (ab192890; all Abcam). Serum total cholesterol (C010-1), triglyceride (C010-2), HDL-C (C010-3), and LDL-C (C010-4) kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2 Instruments

Experiments used an XSP-36 optical microscope (Shanghai Optical Instrument No. 1 Factory, Shanghai, China), CFX96 Touch real-time PCR system (1855195, Bio-Rad, Hercules, CA, USA), Criterion electrophoresis and Trans-Blot transfer units (Bio-Rad), a BIOER conventional PCR instrument (Hangzhou, China), RT-6000 microplate reader (Rayto, Shenzhen, China), BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), TS-100 orbital shaker (Kylin-Bell, Haimen, China), and a 4-20R refrigerated centrifuge (Hengnuo, Changsha, China). Supporting equipment included a -80°C freezer (Haier, Qingdao, China), CO₂ incubator, laminar-flow cabinet and thermostatic water bath (Shanghai Boxun, Shanghai, China), XUEKE-IMS-150 ice maker (Tuoheng, Shanghai, China), YDS-175-276 liquid-nitrogen vessel (Huatae Gas, China), and a CENTRA purified-water system (ELGA Veolia, Shanghai, China).

2.3 AC16 cell culture and treatment

AC16 human cardiomyocytes were obtained from MilliporeSigma (Cat. No. SCC109, Burlington, MA, USA) and cultured in high-glucose DMEM containing 10% FBS and 1% penicillin-streptomycin. Cultures were maintained at 37°C in humidified 5% CO₂ and passaged at 80–90% confluence after PBS washing and trypsin-EDTA detachment. Cells from passages 4–12 were included. To silence SEMA3B, cells were exposed for 48 h to lentivirus carrying SEMA3B-targeted shRNA (sh-SEMA3B) or a non-targeting sequence (sh-NC). AICAR was added to the indicated cultures for another 24 h. The five conditions were untreated control, sh-SEMA3B, sh-SEMA3B plus AICAR, sh-NC, and sh-NC plus AICAR.

2.4 Animal model and treatment allocation

Male C57BL/6J mice aged 8–10 weeks and weighing 25–30 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and fed a diet containing 60% energy from fat for 12 weeks. This regimen was used to create a model of metabolic cardiovascular stress. From week 10, mice received weekly tail-vein injections of lentivirus expressing SEMA3B shRNA (shSEMA3B) or non-targeting shRNA (shNC) at 1×10^8 TU/mouse. A polyethylene glycol-based enhancer was administered with the virus to improve cardiac transduction. Experimental groups comprised control, SEMA3B-silenced (shown as SEMA3B-KO in figures), SEMA3B-KO plus AICAR, vector control (KO-NC), and KO-NC

plus AICAR. After viral delivery, AICAR was administered intraperitoneally at 1 mg/kg/day for 2 weeks. SEMA3B-KO and KO-NC are retained only as the original figure labels; both refer to shRNA-mediated knockdown conditions rather than germline knockout genotypes. At the endpoint, mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). Terminal blood and cardiac tissue were collected after deep anesthesia was confirmed, and cervical dislocation was then performed. Cardiac SEMA3B reduction was verified by qRT-PCR and immunoblotting. As the mice were obtained from a licensed commercial laboratory-animal supplier, informed consent from an animal owner was not applicable. All animal procedures were conducted under the ethical oversight of the Ethics Committee of Guangdong Laidi Biomedical Research Institute Co., Ltd. (approval no. 2024078-1) and in accordance with applicable animal-welfare requirements.

2.5 Lentiviral preparation and SEMA3B silencing

Three shRNA sequences targeting SEMA3B were cloned individually into pLKO.1. Supplementary Table S1 lists the corresponding oligonucleotides. A sequence without an intended target was used as the negative control. Each insert was confirmed by Sanger sequencing before lentivirus was produced.

For virus production, 2.25 µg of each expression plasmid was combined with ViraPower packaging components and transfected into 293T cells using Lipofectamine 3000. The plasmid and 0.75 µg P3000 were diluted in 250 µL Opti-MEM. In parallel, 7 µL Lipofectamine 3000 was diluted in a separate 250-µL volume of Opti-MEM. The solutions were combined for 15 min at room temperature and applied to cultures at approximately 80% confluence. Medium was replaced after 8 h. Viral supernatants were harvested at 48 h, filtered through a 0.45-µm membrane, and stored at -80°C. Target cells in six-well plates were incubated with virus and polybrene for 8 h before replacement of the inoculum. Puromycin selection continued for 7–10 days. Silencing efficiency was assessed by qRT-PCR and immunoblotting.

2.6 Immunoblot analysis

RIPA buffer containing 1 mM PMSF and protease inhibitors was used to lyse cultured cells and cardiac tissue. Total protein was measured by BCA assay. Immunoblotting was performed as reported previously (9), and chemiluminescent bands were quantified in Image-Pro Plus 6.0. GAPDH served as the loading control.

Primary antibodies were diluted as follows: SEMA3B, 1:1,000; phospho-AMPK, 1:1,000; phospho-ACC, 1:5,000; Mfn1/2, 1:1,000; Drp1, 1:1,000; Parkin, 1:1,000; PINK1, 1:1,000; and LC3-II, 1:2,000. All other incubation and detection steps followed the manufacturers' protocols.

2.7 Quantitative reverse-transcription PCR

Log-phase cells were plated in six-well dishes at 1×10^6 cells/well and harvested 24 h after treatment. After removal of the medium, 500 µL RL lysis buffer was added to each well. Lysates were centrifuged through a genomic-DNA removal column at $12,000 \times g$ for 30 s. The flow-through was combined with 0.5 volumes of absolute ethanol, transferred to an RNA-binding column, and centrifuged under the same conditions. Columns were washed with 700 µL RW1 and then ethanol-supplemented RW2 buffer. After

centrifugation at 12,000 × g for 2 min and a further 1 min, RNA was eluted in 30 µL RNase-free water following a 3-min incubation at room temperature. The elution step was repeated once. RNA quantity and purity were measured spectrophotometrically.

Reverse-transcription reactions initially contained 500 ng RNA, 2 µL 5× gDNA Wiper Mix, and nuclease-free water to 10 µL. After 2 min at 42°C, 2 µL 10× RT Mix, 2 µL reverse transcriptase, 1 µL oligo(dT)20VN, 1 µL random hexamers, and 4 µL nuclease-free water were added. The final 20-µL reaction was incubated at 37°C for 15 min and then at 85°C for 5 s. cDNA was stored at -20°C.

Each 20-µL qRT-PCR reaction contained 5 µL diluted cDNA, 10 µL SYBR Green master mix, 0.4 µL of each primer, and 4.2 µL nuclease-free water. Cycling began with 30 s at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. Product specificity was examined by melting-curve analysis. GAPDH was the reference transcript, and relative expression was calculated by the 2^{^(-ΔΔCt)} method. Primer sequences are provided in Table 1.

Table 1
Oligonucleotide primers used for qRT-PCR.

Gene	Species	Forward (5'–3')	Reverse (5'–3')
SEMA3B	Human	CAAGTGGACGACGTTCTCTGA	AGAAGACGGCATAGAGCAGC
PGC-1α	Human	TGAAGGGTACTTTTCTGCCCC	GCACAAACTGGATTTCGCCAG
NRF1	Human	ACACAGAAAAGGTGCTCAAAGG	TGTTTCGGTTTGGGTCACTCC
TFAM	Human	GGTGCAGACAGGACAGTGTT	CCCTACTCCCAGACTGCTCT
AMPKα	Human	ACAGCCGAGAAGCAGAAACA	CTTCACTTTGCCGAAGGTGC
ACC	Human	GCCTGACTTTTGATCCGACC	GTTATCCCCAAACCCAGGCA
GAPDH	Human	TGCAACCGGGAAGGAAATGA	GCATCACCCGGAGGAGAAAT
SEMA3B	Mouse	CTGAAGGGAAGGGCAAGAGG	ATTCCAGAGGGCACCAAGTG
AMPKα	Mouse	GAAAGTGAAGGTGGGCAAGC	GATGTGAGGGTGCCTGAACA
ACC	Mouse	GGAGGCGGATATCTGCTGAG	ATCGGGAGTGCTGGTTTAGC
GAPDH	Mouse	CCCTTAAGAGGGATGCTGCC	TACGGCCAAATCCGTTTACA

2.8 Intracellular ATP assay

Intracellular ATP was measured with a luciferase-based luminescence assay. Cell lysates were centrifuged at 12,000 × g for 5 min, and the resulting supernatants were combined with firefly-luciferase reagent. Luminescence was recorded on a microplate reader and normalized to total protein.

2.9 Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_m$) was assessed by loading cells with 10 $\mu\text{g/mL}$ JC-1 for 20 min at 37°C. Fluorescence was acquired by flow cytometry after washing. The ratio of red J-aggregate fluorescence to green J-monomer fluorescence was taken as the membrane-potential index.

2.10 Measurement of intracellular reactive oxygen species

Intracellular oxidant generation was estimated using the cell-permeable probe DCFH-DA. Cells were incubated with 10 μM DCFH-DA for 20 min at 37°C. Flow-cytometric fluorescence was then measured and expressed relative to the untreated control.

2.11 MTT metabolic-activity assay

Exponentially growing cells were seeded at 1×10^4 cells/well and left overnight to attach. Ten microliters of MTT solution (0.5 mg/mL) was added for 4 h at 37°C. The formazan product was dissolved in DMSO, and absorbance was read at 570 nm. MTT reduction was used as an index of viable-cell metabolic activity.

2.12 Cytokine ELISA

Supernatants were collected after 12 h, centrifuged at $1,000 \times g$ for 20 min, and stored at -80°C. TNF- α , IL-6, and IL-1 β were measured with commercial ELISAs (R&D Systems). Standards and 50- μL samples were placed in antibody-coated wells for 30 min at 37°C. HRP-conjugated detection antibody, TMB substrate, and stop solution were added sequentially after washing. Absorbance was measured at 450 nm, and concentrations were derived from assay-specific calibration curves.

2.13 Cardiac histology

2.13.1 Hematoxylin and eosin staining

Paraffin-embedded cardiac sections were cleared twice in xylene for 10 min. Rehydration was performed with two 5-min changes of 100% ethanol, followed by 95%, 90%, 80%, and 70% ethanol for 5 min each. Sections were rinsed in distilled water and stained with Harris hematoxylin for 3 min. They were briefly differentiated in 1% acid alcohol, washed, blued in 0.6% ammonia, and counterstained with eosin for 3 min. Slides were dehydrated through two 5-min changes each of 95% ethanol, absolute ethanol, and xylene. After brief air drying, sections were mounted in neutral balsam and imaged by bright-field microscopy.

2.13.2 Masson's trichrome procedure

Masson's trichrome staining was used to visualize collagen in paraffin sections. Slides were cleared in xylene twice for 10 min, rehydrated in two 5-min changes of 100% ethanol and one 5-min change of 75% ethanol, and washed in distilled water. Masson A solution was applied overnight at room temperature. After rinsing, sections were treated for 1 min with equal volumes of Masson B and C, washed, and differentiated in 1% acid alcohol to minimize nonspecific staining.

2.13.3 TUNEL labeling

DNA fragmentation was measured by TUNEL staining of cardiac sections. After deparaffinization and rehydration, sections were permeabilized with proteinase K and exposed to the labeling mixture at an enzyme-to-fluorophore ratio of 1:9. Incubation proceeded for 1 h at 37°C in the dark, followed by DAPI counterstaining. The apoptotic index was calculated as the proportion of TUNEL-positive nuclei among all nuclei.

2.14 Serum lipid measurements

Serum total cholesterol (TC), triglycerides (TG), HDL-C, and LDL-C were quantified using commercial colorimetric kits according to the supplier instructions. Absorbance was measured at 500 nm for TC, HDL-C, and LDL-C. TG absorbance was recorded at 420 nm after extraction and enzymatic reaction. Each concentration was calculated from the appropriate standard curve.

2.15 Statistical methods

Data are presented as mean $\pm$ SD from at least three independent biological replicates unless stated otherwise in a figure legend. Analyses were performed in GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). An unpaired t test was used for two-group comparisons. Experiments with more than two groups were analyzed by one-way ANOVA followed by Tukey's honestly significant difference test. All tests were two-sided, and $p < 0.05$ was considered statistically significant. Figures were assembled in Adobe Illustrator 2020 (Adobe Systems, San Diego, CA, USA).

3 Results

3.1 SEMA3B silencing suppresses AMPK-associated signaling and alters mitochondrial-dynamics proteins

Among the three SEMA3B-targeted constructs, sh-RNA-1 produced the greatest reduction in SEMA3B mRNA relative to sh-NC (Fig. 1A). Immunoblotting showed a parallel decrease in SEMA3B protein (Fig. 1B). We therefore selected sh-RNA-1 for all subsequent cell experiments.

We then measured AMPK-pathway activity and proteins involved in mitochondrial dynamics (Fig. 1C). Phospho-AMPK and phospho-ACC were both lower in sh-SEMA3B-1 cells than in control-transduced cells ($p < 0.001$). Mfn1, Mfn2, and Drp1 abundance also decreased ($p < 0.001$), showing that SEMA3B loss affected several components of mitochondrial network remodeling. AICAR raised all five markers toward their respective control levels.

3.2 AMPK activation counteracts the bioenergetic defects caused by SEMA3B silencing

To characterize transcriptional changes after SEMA3B loss, we measured AMPK α , ACC, and three genes related to mitochondrial biogenesis. SEMA3B knockdown reduced AMPK α , ACC, PGC-1 α , NRF1, and

TFAM mRNA relative to sh-NC (Fig. 2A). The measured AMPK-associated and mitochondrial-biogenesis transcripts therefore changed in the same direction.

JC-1 measurements showed a lower mitochondrial membrane-potential index after SEMA3B knockdown (Fig. 2B). AICAR increased the red-to-green fluorescence ratio in both sh-NC and sh-SEMA3B cultures. Thus, AMPK activation opposed the membrane-potential defect associated with reduced SEMA3B expression.

ATP content also fell after SEMA3B knockdown and increased after AICAR treatment (Fig. 2C). The MTT signal followed the same pattern (Fig. 2D), consistent with recovery of viable-cell metabolic activity.

Across the transcriptional, membrane-potential, ATP, and MTT measurements, reduced SEMA3B expression was accompanied by impaired cellular bioenergetics. Improvement after AICAR indicates that AMPK activation can offset a substantial part of this phenotype.

3.3 Loss of SEMA3B is associated with lipid dysregulation, inflammation, and oxidative stress

Compared with control cells, SEMA3B-silenced cultures had higher TC, TG, and LDL-C indices and lower HDL-C (Fig. 3A). TNF- α , IL-6, and IL-1 β concentrations also increased, and this response was attenuated by AICAR (Fig. 3B). Ox-LDL abundance was higher after SEMA3B knockdown (Fig. 3C). PINK1 and Parkin expression and the LC3-II/LC3-I ratio were lower (Fig. 3D). These static measurements show altered mitophagy-associated signaling but do not establish mitophagic flux. DCFH-DA fluorescence increased, whereas SOD, catalase, and GPx activities declined (Fig. 3E, F; $p < 0.01$). AICAR moved the lipid indices toward control values, reduced cytokines, ox-LDL, and ROS, and increased antioxidant activity and the measured mitophagy-related proteins. AMPK activation therefore moderated the metabolic, inflammatory, and oxidative changes induced by SEMA3B knockdown.

3.4 AICAR limits myocardial injury and fibrosis in mice with reduced SEMA3B expression

We next asked whether the cellular phenotype was also present in cardiac tissue. Mice in the SEMA3B-KO-labeled knockdown group showed more extensive cardiomyocyte injury and necrotic morphology than controls on H&E staining (Fig. 4A). These lesions were less evident after AICAR treatment. Masson's trichrome staining showed increased interstitial and perivascular collagen after SEMA3B knockdown (Fig. 4B). Collagen deposition was markedly lower in AICAR-treated animals.

The proportion of TUNEL-positive nuclei was higher in SEMA3B-deficient myocardium. AICAR significantly reduced this proportion, indicating less cardiac cell death after pharmacological stimulation of AMPK (Fig. 4C).

Cardiac phospho-AMPK and phospho-ACC were reduced by SEMA3B knockdown and increased by AICAR (Fig. 4D). SEMA3B, AMPK α , and ACC transcript levels were also lower in knockdown hearts. AICAR increased AMPK α and ACC expression (Fig. 4F). The protein and transcript measurements therefore showed concordant disruption of the assayed AMPK-pathway markers and partial or complete recovery after AICAR.

SEMA3B knockdown also reduced cardiac Mfn1/2 and Drp1, whereas AICAR increased their abundance (Fig. 4E). In serum, AICAR lowered TC, TG, LDL-C, and ox-LDL and raised HDL-C relative to untreated SEMA3B-deficient mice (Fig. 4G, H).

The mouse experiments reproduced the main cell-based findings. Reduced SEMA3B expression was associated with cardiac structural damage, apoptosis, altered mitochondrial markers, and an unfavorable lipid profile. Each measured phenotype was less severe after AICAR treatment.

4 Discussion

Our results link SEMA3B to the maintenance of cardiac metabolic homeostasis. In AC16 cells, SEMA3B knockdown reduced AMPK α expression, phospho-AMPK, phospho-ACC, mitochondrial-biogenesis transcripts, and proteins involved in mitochondrial remodeling. Membrane potential, ATP content, and MTT reduction also declined, while redox balance and PINK1/Parkin-LC3 indices were altered. In high-fat-diet-fed mice, cardiac SEMA3B knockdown produced similar signaling changes together with necrotic morphology, collagen deposition, and apoptosis. AICAR improved most outcomes in both models. These data support AMPK pathway involvement, but the rescue experiment does not demonstrate a direct interaction between SEMA3B and AMPK.

The cardiac actions of SEMA3B are less well defined than those of other class 3 semaphorins, and previous studies do not assign SEMA3B a uniformly protective or harmful role (12). SEMA3A, SEMA3C, and SEMA3G regulate cardiovascular development, angiogenesis, and responses to ischemic injury (20). By contrast, most mechanistic work on SEMA3B has been conducted in cancer models. In malignant cells, SEMA3B can suppress PI3K/Akt and MAPK signaling and function as a tumor suppressor (19, 21). Class 3 semaphorins also influence endothelial permeability, inflammation, and vascular organization through neuropilin and VEGF-related signaling (22). The present data extend this context to cardiomyocyte metabolism. SEMA3B knockdown worsened mitochondrial and cardiac injury measures, whereas AICAR reduced these abnormalities. A homeostatic role is therefore plausible in the models tested here, although receptor dependence has not been shown.

AMPK couples cellular energy availability to mitochondrial maintenance by favoring ATP production and regulating biogenesis, dynamics, and autophagy (23, 24). PGC-1 α , NRF1, and TFAM constitute an important transcriptional program for mitochondrial renewal. All three transcripts declined together with AMPK α after SEMA3B knockdown. This pattern raises the possibility that SEMA3B affects transcription or mRNA stability in addition to Thr172 phosphorylation (25, 26). CREB, SIRT1, and other potential intermediates were not examined. The mitochondrial-dynamics findings also require caution. AMPK can

phosphorylate MFF and link energetic stress to DRP1-dependent fission, which may help separate damaged mitochondrial segments (27, 28). Other AMPK-dependent processes support fusion and biogenesis. Here, Mfn1, Mfn2, and Drp1 all decreased after SEMA3B knockdown. Their coordinated loss is more consistent with impaired remodeling capacity than with a simple shift toward fusion or fission. This differs from models in which AMPK inactivation favors a unidirectional fission-dominant state (29). AICAR increased PINK1, Parkin, and LC3-II-associated measures. Without lysosomal inhibition or a flux reporter, however, these measurements cannot establish increased mitophagic flux (30).

Cardiomyocytes contain a high mitochondrial volume because contraction requires continuous ATP production (3). The heart is consequently sensitive to defects in mitochondrial turnover and respiratory efficiency (4). Concurrent reductions in PGC-1 α , TFAM, and mitofusins may impair both organelle renewal and network integrity. In our models, ATP and $\Delta\psi_m$ fell as ROS increased and antioxidant-enzyme activity declined. These changes are consistent with poorer mitochondrial fitness. AICAR restored membrane potential, ATP, and several mitochondrial quality-control markers, suggesting greater resilience of the remaining network. SEMA3B and AMPK-associated signaling may therefore connect energetic stress with cardiomyocyte injury, although the intervening molecular steps remain unknown.

The tissue findings paralleled the cellular phenotype. SEMA3B knockdown was associated with necrotic damage, interstitial and perivascular fibrosis, and more TUNEL-positive nuclei. Each endpoint improved after AICAR treatment. This response is broadly consistent with reports that AMPK modifies remodeling in heart failure, diabetic cardiomyopathy, and pressure-overload models (31–36). AICAR also lowered TC, TG, LDL-C, and ox-LDL while increasing HDL-C. These systemic changes are compatible with reduced SREBP-1c-dependent lipogenesis and increased fatty-acid oxidation after AMPK activation (37, 38). The present design, however, cannot attribute the serum-lipid effects specifically to cardiomyocyte AMPK.

Several limitations restrict interpretation of these findings. First, AICAR affects adenosine metabolism and other AMP-sensitive processes besides AMPK (39). Its rescue effect supports pathway involvement but does not prove AMPK specificity. A stronger test would combine cardiomyocyte-selective AMPK α 1/ α 2 deletion with genetic epistasis or a more selective activator. Second, the mouse model used lentiviral shRNA and should be described as SEMA3B knockdown, not as germline or cardiomyocyte-specific knockout. Variation in tissue transduction and effects outside the heart may have influenced the phenotype. Third, the receptors and proximal signals connecting SEMA3B to AMPK α expression were not identified. Neuropilins, plexins, MAP-kinase or small-GTPase pathways, transcriptional regulation, and RNA stability remain possible mechanisms. Fourth, PINK1, Parkin, and LC3 were measured at single time points and do not quantify autophagic flux. Lysosomal inhibitors or tandem fluorescent reporters will be needed to resolve flux. Fifth, neither the high-fat-diet model nor the short treatment period reproduces ischemia-reperfusion injury or chronic pressure overload. Finally, confirmation in patient myocardium or human induced-pluripotent-stem-cell-derived cardiomyocytes is required before the pathway can be considered clinically relevant.

5 Conclusion

SEMA3B knockdown weakened AMPK-associated signaling and was accompanied by impaired mitochondrial and metabolic measures, oxidative imbalance, and structural cardiac injury. AICAR attenuated these changes in cultured cardiomyocytes and in mice. SEMA3B may therefore contribute to cardiac mitochondrial homeostasis through an AMPK-associated pathway. Receptor-level signaling and AMPK-specific causality must be established genetically before therapeutic translation.

Abbreviations

ACC
acetyl-CoA carboxylase
AICAR
5-aminoimidazole-4-carboxamide ribonucleoside
AMPK
AMP-activated protein kinase
ATP
adenosine triphosphate
DCFH-DA
2',7'-dichlorodihydrofluorescein diacetate
GAPDH
glyceraldehyde-3-phosphate dehydrogenase
HDL-C
high-density lipoprotein cholesterol
LDL-C
low-density lipoprotein cholesterol
MTT
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ROS
reactive oxygen species
SEMA3B
semaphorin 3B
TC
total cholesterol
TG
triglycerides
TUNEL
terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Declarations

Clinical trial number

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Li Ye, Jiayi Guo, and Feiou Zhou conceived the study, analyzed the data, and drafted and revised the manuscript. Li Ye and Haiying He performed data collection, analysis, and investigation and contributed to manuscript preparation. All authors reviewed and approved the final manuscript.

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Not applicable.

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Figures

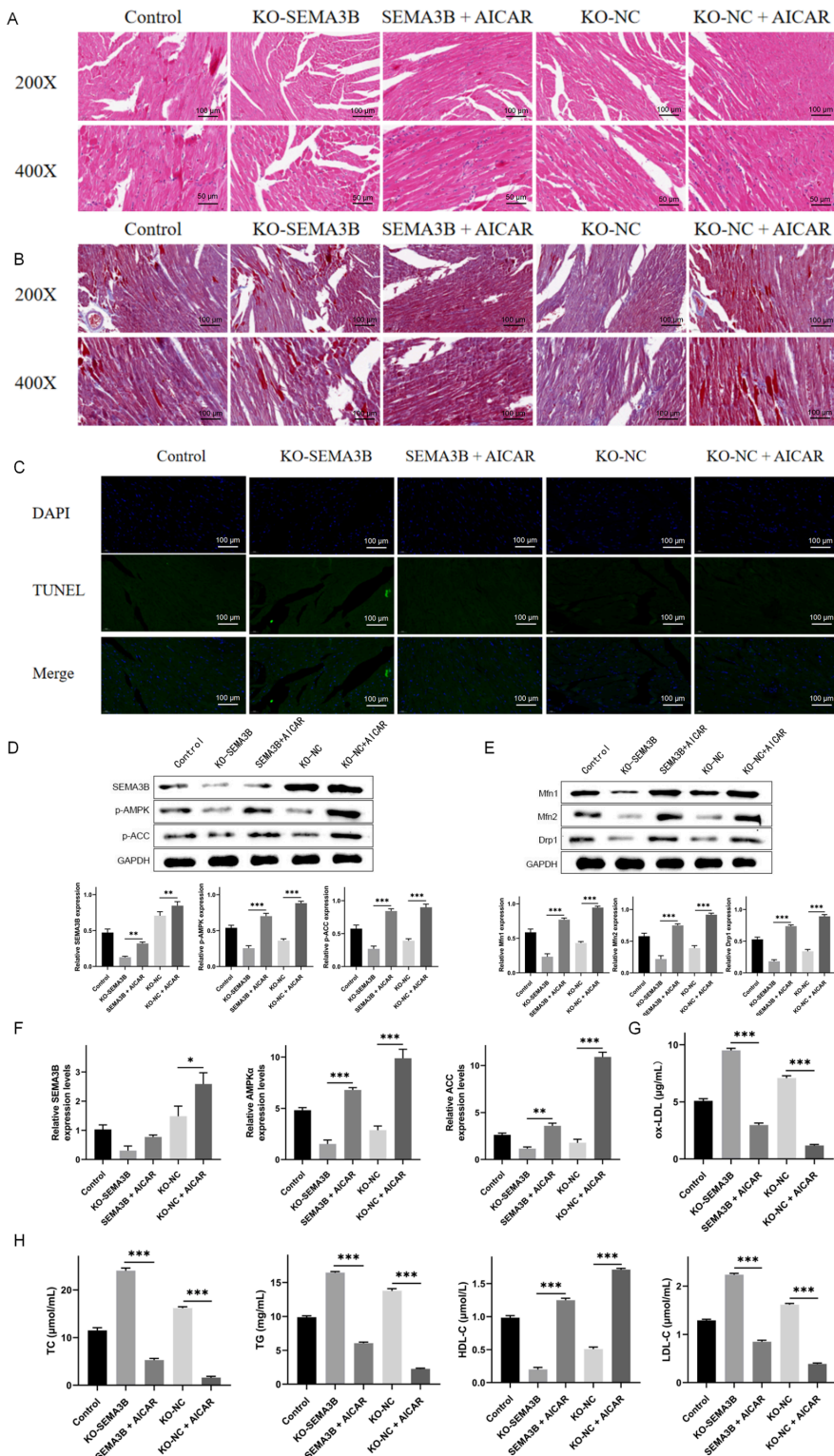


Figure 1

Effects of SEMA3B knockdown on AMPK signaling and mitochondrial-dynamics proteins in AC16 cardiomyocytes. (A) SEMA3B mRNA after transduction with three SEMA3B-targeted shRNAs or non-targeting sh-NC (n = 3 independent experiments). (B) Representative immunoblots and densitometry showing reduced SEMA3B protein for each construct. (C) Phospho-AMPK, phospho-ACC, Mfn1, Mfn2,

and Drp1 after SEMA3B knockdown and AICAR treatment. Data are mean $\pm$ SEM. ** $p < 0.01$; *** $p < 0.001$.

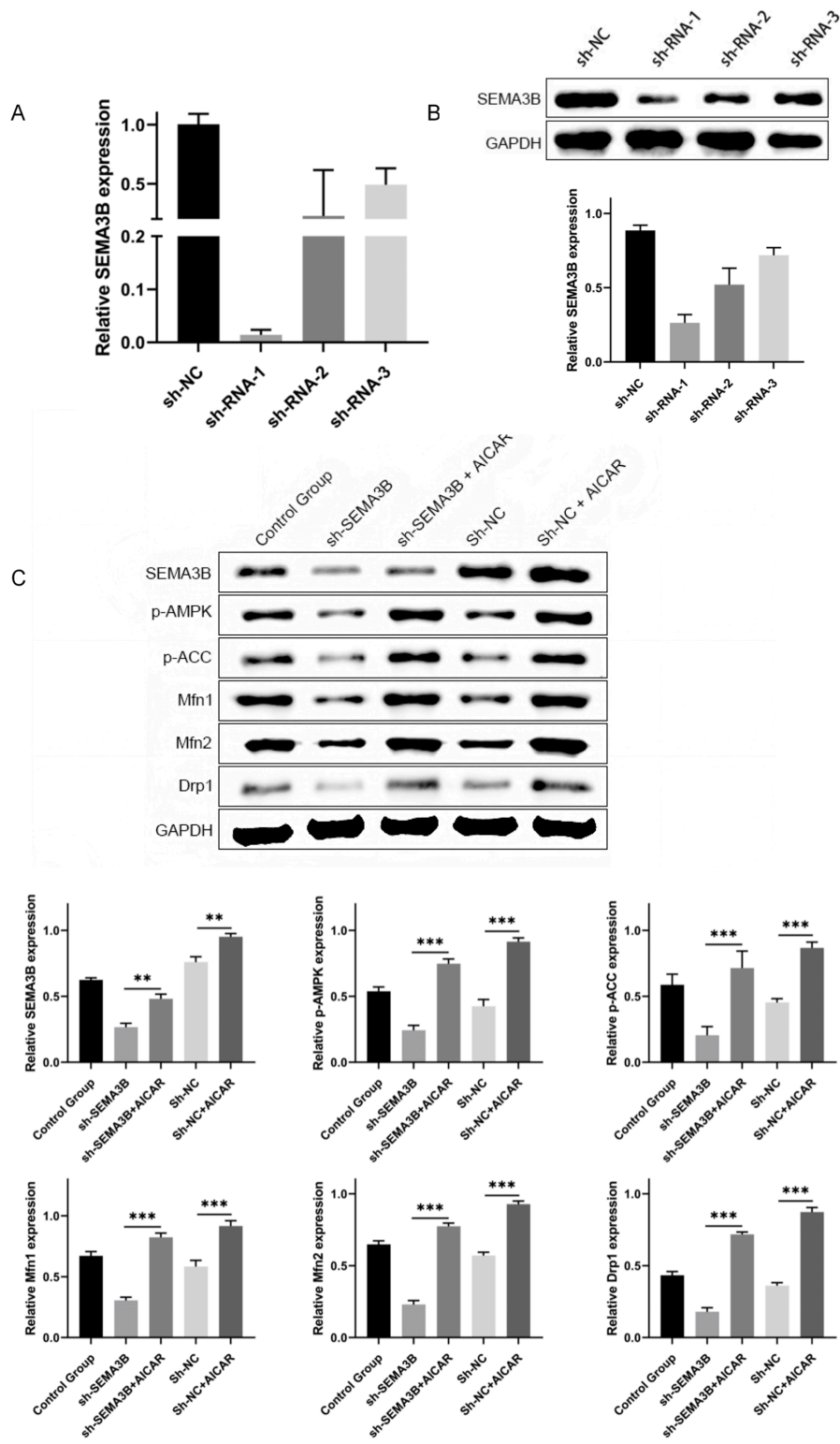


Figure 2

Bioenergetic changes after SEMA3B knockdown and AICAR treatment. (A) Relative AMPK α , ACC, PGC-1 α , NRF1, and TFAM transcript abundance (n = 3). (B) JC-1 measurement of mitochondrial membrane

potential. (C) Intracellular ATP content. (D) MTT reduction as an index of viable-cell metabolic activity. SEMA3B knockdown reduced each outcome, while AICAR produced partial or complete recovery. Data are mean $\pm$ SEM. ** $p < 0.01$; *** $p < 0.001$.

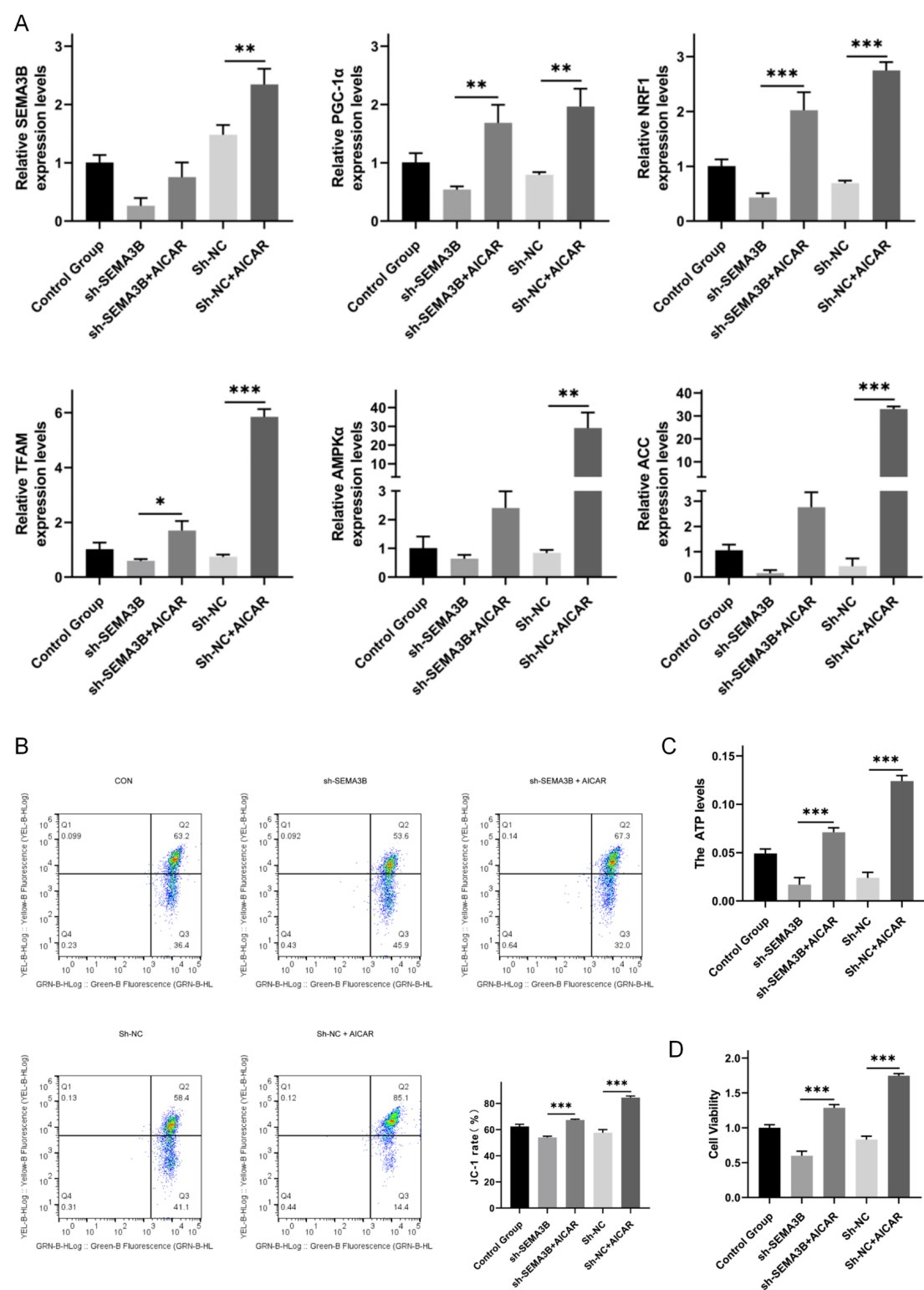


Figure 3

Lipid, inflammatory, oxidative, and mitophagy-associated measurements after SEMA3B knockdown. (A) TC, TG, LDL-C, and HDL-C. (B) TNF- α , IL-6, and IL-1 β . (C) Ox-LDL. (D) PINK1, Parkin, and LC3-II/LC3-I. (E) DCFH-DA fluorescence as an index of intracellular oxidant generation. (F) SOD, catalase, and GPx activity. AICAR shifted the measured outcomes toward their corresponding controls. Data are mean $\pm$ SEM. ***p < 0.001.

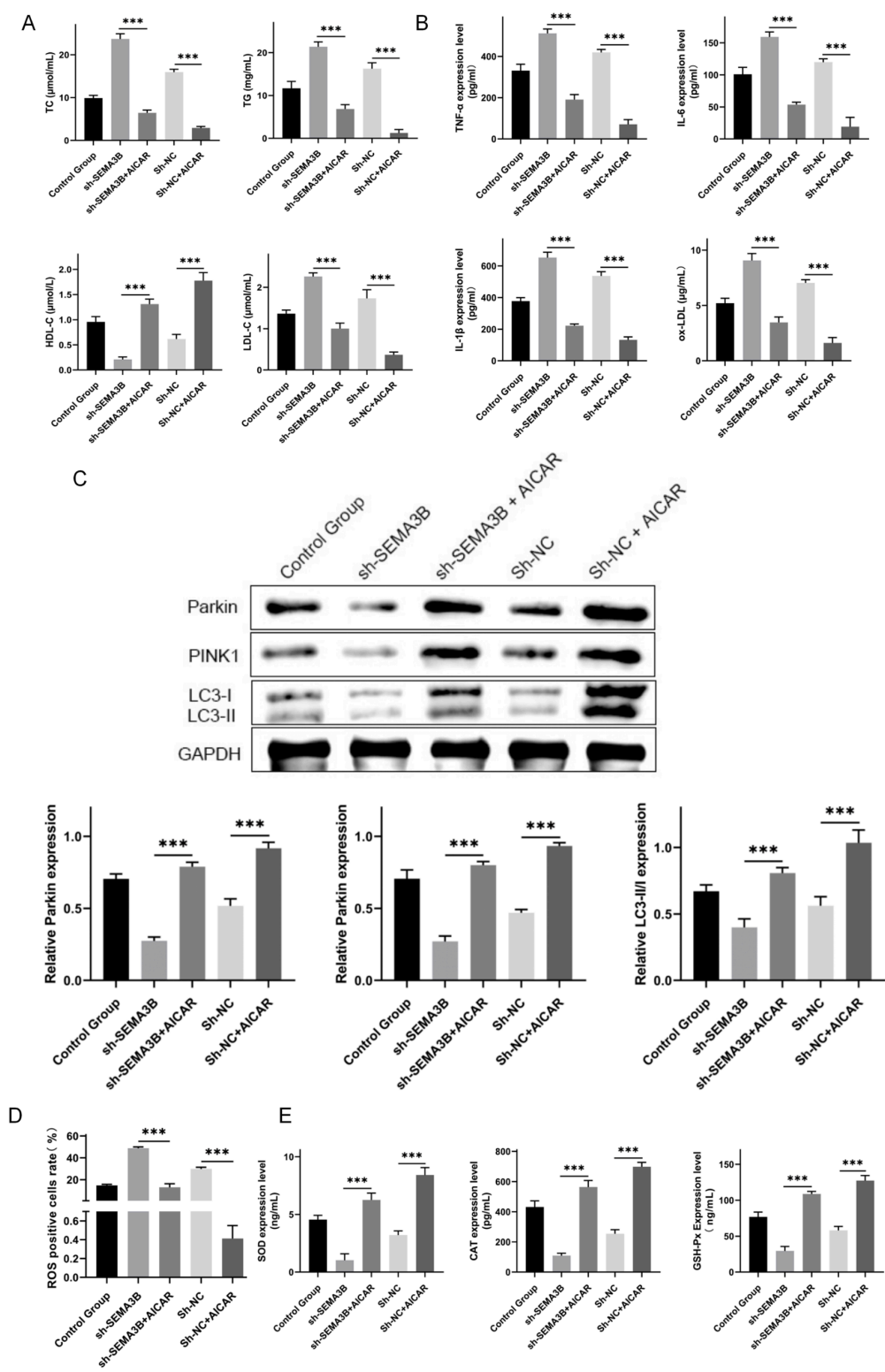


Figure 4

Effect of AICAR on myocardial injury after SEMA3B knockdown. SEMA3B-KO and KO-NC are the original figure labels for the shRNA-mediated knockdown and negative-control groups. (A) H&E assessment of myocardial injury. (B) Masson's trichrome staining of interstitial and perivascular collagen. (C) TUNEL labeling of apoptotic nuclei. (D, E) Cardiac phospho-AMPK, phospho-ACC, Mfn1, Mfn2, and Drp1 immunoblots and quantification. (F) Cardiac SEMA3B, AMPK α , and ACC transcripts. (G, H) Serum ox-LDL and lipid profile. AICAR improved the histological, molecular, and metabolic measurements. Data are mean $\pm$ SEM. **p < 0.01; ***p < 0.001.

Supplementary Files

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