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Pioneering PGC-1α–boosted secretome: a novel approach to combating liver fibrosis

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Purpose: Liver fibrosis is a critical health issue with limited treatment options. This study investigates the potential of PGC-Sec, a secretome derived from peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)-overexpressing adipose-derived stem cells (ASCs), as a novel therapeutic strategy for liver fibrosis.
Methods: Upon achieving a cellular confluence of 70%–80%, ASCs were transfected with pcDNA-PGC-1α. PGC-Sec, obtained through concentration of conditioned media using ultrafiltration units with a 3-kDa cutoff, was assessed through *in vitro* assays and *in vitro* mouse models.
Results: *In vitro*, PGC-Sec significantly reduced LX2 human hepatic stellate cell proliferation and mitigated mitochondrial oxidative stress compared to the control-secretome. In an *in vivo* mouse model, PGC-Sec treatment led to notable reductions in hepatic enzyme activity, serum proinflammatory cytokine concentrations, and fibrosis-related marker expression. Histological analysis demonstrated improved liver histology and reduced fibrosis severity in PGC-Sec–treated mice. Immunohistochemical staining confirmed enhanced expression of PGC-1α, optic atrophy 1 (a mitochondrial function marker), and peroxisome proliferator-activated receptor alpha (an antifibrogenic marker) in the PGC-Sec–treated group, along with reduced collagen type 1A expression (a profibrogenic marker).
Conclusion: These findings highlight the therapeutic potential of PGC-Sec in combating liver fibrosis by enhancing mitochondrial biogenesis and function, and promoting antifibrotic processes. PGC-Sec holds promise as a novel treatment strategy for liver fibrosis.
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Key Words: Adipose-derived stem cells, Liver fibrosis, Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha, Secretome

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INTRODUCTION

Liver fibrosis is a chronic and progressive liver condition marked by an abnormal buildup of proteins in the extracellular matrix, which leads to impaired liver function and ultimately liver failure. This condition is a growing global health concern since it is linked to a range of liver diseases, including alcoholic liver disease, viral hepatitis, and nonalcoholic steatohepatitis [1]. Despite extensive research, effective treatment options for liver fibrosis remain limited, highlighting the urgent need for novel therapeutic strategies.

Mesenchymal stem cells (MSCs), especially adipose-derived stem cells (ASCs), have shown promising capability for liver regeneration attributed to their capacity for differentiation into diverse cell types, secreting bioactive factors, and modulating immune responses. However, the optimization of their regenerative potential is crucial for successful therapeutic applications, as their efficacy can be influenced by factors such as donor age, tissue source, and culture conditions. The proper balance between intracellular reactive oxygen species (ROS) production and their elimination by antioxidant enzymes is a crucial factor influencing the characteristics of MSCs [2,3]. Elevated ROS levels are associated with cellular damage and impaired regenerative potential, while controlled ROS levels play a critical role in maintaining cellular balance and promoting tissue repair [4,5]. Thus, modulating the intracellular redox state of MSCs could potentially enhance their therapeutic efficacy.

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a potent controller of mitochondrial biogenesis and function. When it is overexpressed, it enhances the expression of mitochondrial antioxidant enzymes, effectively reducing oxidative stress and cell death [6,7]. Additionally, PGC-1α has been linked to the regulation of diverse cellular processes, including energy metabolism, inflammation, and cell survival, which are relevant to tissue repair and regeneration. In this study, we aimed to enhance the antifibrogenic potential of ASCs by transfecting them with PGC-1α and explored the therapeutic potential of the secretome released from these genetically modified PGC-ASCs in a mouse model of liver fibrosis. By leveraging the beneficial effects of PGC-1α–driven upregulation of mitochondrial proliferation, we hypothesize that the secretome of PGC-ASCs will exhibit improved antifibrogenic and regenerative properties compared to the secretome of unmodified ASCs. This investigation will provide valuable insights into the development of cell-based therapies for liver fibrosis and other fibrotic diseases, as well as contribute to the growing body of knowledge on the mechanisms underlying MSC-mediated tissue repair.

METHODS

Ethics statement

All experimental procedures involving animals were approved by the Institute for Laboratory Animal Research at the Catholic University of Korea (No. CUMC-2020-0125-03). The study is reported in accordance with ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. All methods were carried out in accordance with relevant guidelines and regulations.

Cell culture

A nontumorigenic mouse hepatocyte cell line, AML12 (CRL-2254), was purchased from American Type Culture Collection, and LX2 human hepatic stellate cells (HSCs) were kindly donated by Dr. Won-il Jeong of KAIST Biomedical Research of Korea and cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin. Human ASCs were donated by Hurim BioCell Inc. (IRB number 700069-201407-BR-002-01) and cultured in low-glucose DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin. Cells were incubated at 37 °C in a CO_2 incubator (Thermo) [8].

Preparation of PGC-secretome

ASCs were grown in 100-mm cell dishes (Corning Glass Works). After reaching 70%–80% confluence, ASCs were transiently transfected with 4-μg pcDNA-PGC-1α. Twenty-four hours later, 1.0×10^6 ASCs were cultured in 7-mL serum-free low-glucose DMEM for 24 hours. The conditioned media were concentrated 25-fold using ultrafiltration units with a 3-kDa molecular weight cutoff (Amicon Ultra-PL 3) to obtain 0.28 mL of secretome from 1.0×10^6 ASCs. Mice were injected with 0.1 mL of secretome and PGC-1α–boosted secretome (PGC-Sec), equivalent to the secretome obtained from 5×10^5 ASCs. The normal secretome was obtained from empty vector-transfected ASCs, while the PGC-Sec was obtained from pcDNA-PGC-1α–transfected ASCs.

Cell proliferation assay

Cell proliferation of LX2 and AML12 cells was evaluated using the EZ-Cytox Cell Proliferation Assay kit (Itsbio) according to the manufacturer's instructions [9].

Animal experiments

Seven-week-old male C57BL/6J mice (Central Laboratory Animal Inc.) were used. An *in vitro* model of liver fibrosis was generated using methionine/choline-deficient diets (MCD diet, ENVIGO). Mice freely consumed an MCD diet *ad libitum* for 2 weeks, which was continued until the 4th week of the

experiment. Mice were divided into 4 experimental groups: control (n = 5), MCD diet + saline injection (n = 8), MCD diet + control-secretome (Ctrl-Sec) injection (n = 8), and MCD diet + PGC-Sec (n = 8). After 14 days on the MCD diet, mice were administered intravenous injections of 0.1 mL of saline, Ctrl-Sec, or PGC-Sec 3 times per week for a duration of 2 weeks (totaling 6 injections). At the beginning of the 5th week, mice were euthanized for the collection of blood and liver samples.

Hepatic TG determination

Hepatic TG levels were determined by resuspending and homogenizing 100 mg of liver samples in 0.5 mL of a 5% NP-40/deionized distilled water solution using a Dounce homogenizer. The tissue homogenates were solubilized at 80 °C in a water bath for protein measurement. TG contents were quantified using a commercially available kit (Abcam) following the manufacturer's instructions.

Real-time PCR analysis

Total RNA was extracted from the samples using TRIzol reagent (Invitrogen) following the manufacturer's instructions [9]. Reverse transcription was performed using an RT-premix kit (Toyobo) with 1 μg of RNA as the template, according to the manufacturer's instructions. SYBR green real-time quantitative PCR was conducted using an Applied Biosystems Step One Plus Real-Time PCR system (Thermo) with primers specific to the target genes: human superoxide dismutase (SOD), mouse collagen type A1 (ColA1), mouse TGF-β1, and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used for real-time quantitative PCR were as follows: mTGF-β1-fwd: 5'-CAC CTG CAA GAC CAT CGA CA-3'; mTGF-β1-rev: 5'-GAG CCT TAG TTT GGA CAG GA-3', mCollagen1-fwd: 5'-CAA GAA CAG CAA CGA GTA CCG-3'; mCollagen1-rev: 5'-GTC ACT GGT CAA CTC CAG CAC-3'; mGAPDH-fwd: 5'-CGA CTT CAA CAG CAA CTC CCA CTC TTC C-3', mGAPDH-rev: 5'-TGG GTG GTC CAG GGT TTC TTA CTC CTT-3'. The expression levels of each target gene were calculated using the comparative threshold cycle method and normalized to the GAPDH gene. Data are presented as the mean ± standard deviation (SD) from 3 independent experiments.

Western blot analysis

Liver specimens from mice were lysed using the EzRIPA Lysis kit (ATTO Corp.) and protein concentrations were quantified using Bradford reagent (Bio-Rad). Proteins were analyzed by western blot using primary antibodies against tissue inhibitors of metalloproteinase (TIMP) 1 (Thermo Fisher Scientific), ColA1 (Novus Biologicals), and β-actin (Cell Signaling Technology) at a 1:1,000 dilution, followed by horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution) from Vector Laboratories. Specific immune complexes were detected using the Western BloT Hyper HRP Substrate (Millipore). [9].

Serology test and ELISA

Blood samples were collected from each mouse, and the concentrations of liver injury markers, including AST and ALT, were measured using an IDEXX VetTest Chemistry Analyzer (IDEXX Laboratories, Inc.). Mouse IL-6 and TNF-α concentrations were measured by sandwich ELISA (Abbkine) according to the manufacturer's instructions. Concentrations of human CD81 and human SOD were determined using a sandwich ELISA kit (Cusabio Technology LLC) following the manufacturer's instructions [10].

Immunohistochemistry and Sirius red staining

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated, and subjected to epitope retrieval using standard procedures. Antibodies against ColA1 (Santa Cruz Biotechnology), PGC-1α (Novus Biologicals), peroxisome proliferator-activated receptor alpha (PPAR-α; Cell Signaling Technology), and optic atrophy 1 (OPA1; Santa Cruz Biotechnology) were used for immunochemical staining. Sirius red staining was performed using the Sirius Red Staining Kit (Polysciences) according to the manufacturer's protocol. Samples were examined under a laser-scanning microscope (Eclipse TE300, Nikon) [10].

Quantification of mitochondrial superoxide by flow cytometry

Mitochondrial superoxide was detected in cells stained with MitoSOX (Thermo Fisher Scientific). After incubation for 10 minutes in the dark at 25 °C, cells were analyzed using an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific) [11].

Statistical analysis

Data were analyzed using SPSS ver. 11.0 (SPSS Inc.) and are presented as mean ± SD. Statistical comparisons among groups were determined using the Kruskal-Wallis test. The P-values of <0.05 were regarded as statistically significant.

RESULTS

Generation of PGC-Sec and *in vitro* determination of its effects

To evaluate the therapeutic potential of PGC-1α–overexpressing ASCs in liver fibrosis, we generated PGC-1α–overexpressing ASCs by transfecting a plasmid encoding PGC-1α (Fig. 1A). Subsequently, we harvested the PGC-Sec from these PGC-ASCs following an optimized protocol, involving centrifugation and filtration as described in the methods section. In comparison to Ctrl-Sec, PGC-Sec exhibited a 3.32-

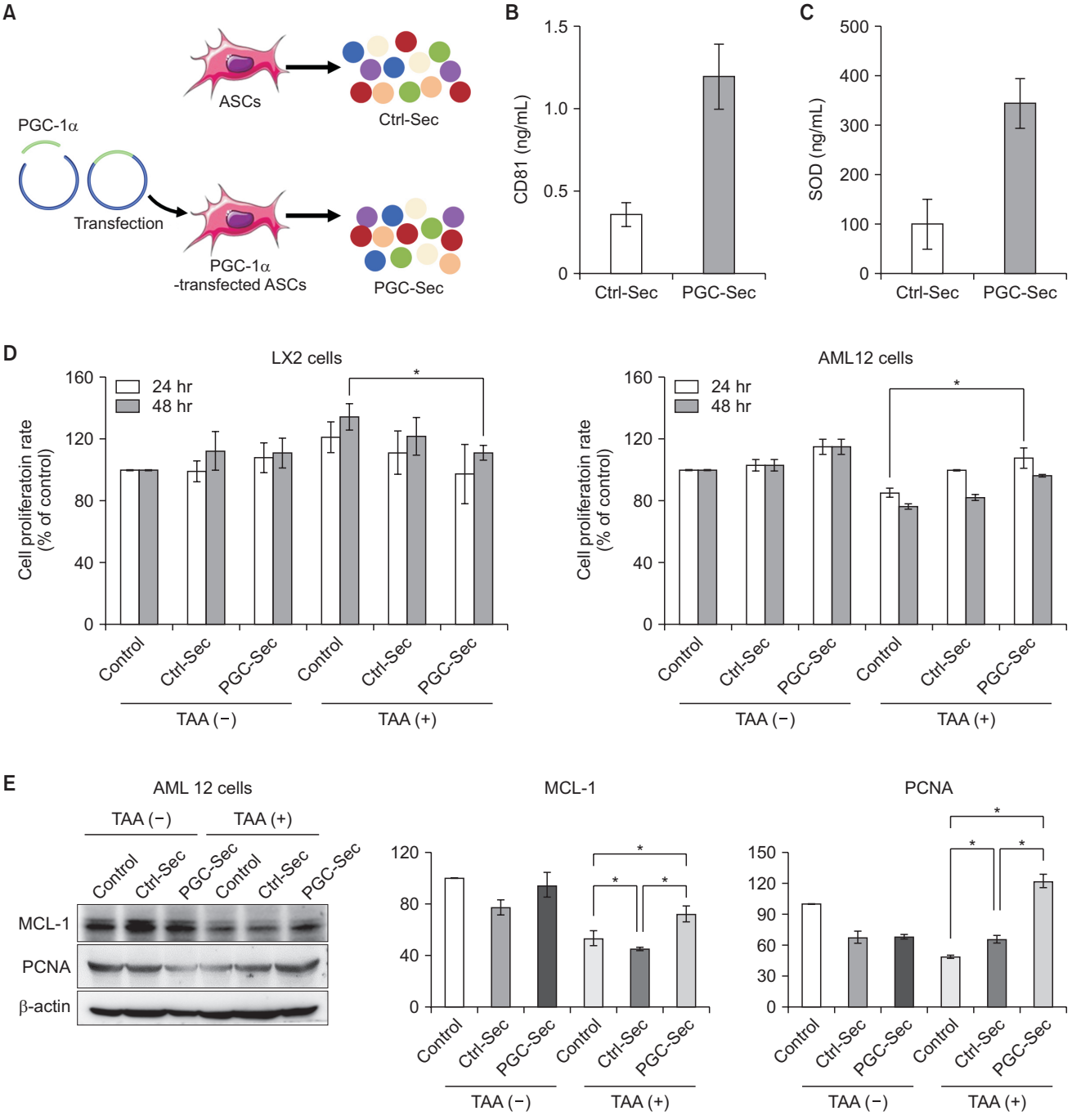

Fig. 1. Therapeutic effects of PGC-Sec on cell viability, exosome markers, and molecular markers in liver cells. (A) Schematic representation of PGC-1α–overexpressing ASCs generation by transfecting a plasmid encoding PGC-1α. (B) Comparison of CD81 expression, an exosome marker, in the secretome obtained from PGC-Sec and Ctrl-Sec. PGC-Sec exhibited a 3.32-fold higher secretion of CD81 compared to Ctrl-Sec. (C) ELISA showing the secretion of SOD mRNA in PGC-Sec and Ctrl-Sec. PGC-Sec displayed a 3.43-fold higher expression of SOD mRNA compared to Ctrl-Sec. (D) Viability of LX2 cells (human hepatic stellate cells) and AML12 cells (mouse hepatocytes) treated with Ctrl-Sec or PGC-Sec, with or without (TAA) treatment, as assessed by cell viability assay. PGC-Sec treatment resulted in the most significant decrease in TAA-treated LX2 cell viability and the most substantial increase in TAA-treated AML12 cell viability. (E) Effects of Ctrl-Sec and PGC-Sec on apoptosis and proliferation in AML12 cells. Expression of the antiapoptotic marker Bcl-xL and the proliferation marker PCNA decreased in AML12 cells upon TAA treatment. Treatment with Ctrl-Sec increased the expression of these markers, while treatment with PGC-Sec showed the most significant increase. Values are presented as mean ± standard deviation of 3 independent experiments. PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PGC-Sec, PGC-1α-boosted secretome; ASC, adipose-derived stem cell; SOD, superoxide dismutase; mRNA, messenger RNA; Ctrl-Sec, control-secretome; TAA, thioacetamide; MCL-1, myeloid cell leukemia 1; PCNA, proliferating cell nuclear antigen. *P < 0.05.

fold higher secretion of CD81, an exosome marker (Fig. 1B). Furthermore, the ELISA revealed that PGC-Sec showed a 3.43-fold higher secretion of SOD mRNA compared to Ctrl-Sec (Fig. 1C). These findings suggest that PGC-Sec may contain a higher abundance of exosomes and exhibit increased expression of SOD mRNA. We established an *in vitro* model of liver injury by treating LX2 cells (human HSC) and AML12 cells (mouse hepatocytes) with the potent hepatotoxin thioacetamide (TAA).

The impact of Ctrl-Sec and PGC-Sec on the viability of LX2 and AML12 cells, with or without TAA treatment, was investigated. Intriguingly, the secretome-treated groups displayed contrasting effects on cell viability; TAA-treated LX2 cells showed a decrease in cell viability, while TAA-treated AML12 cells exhibited an increase in cell viability upon secretome treatment. Furthermore, PGC-Sec treatment resulted in the most significant decrease in TAA-treated LX2 cell viability and the most substantial increase in TAA-treated AML12 cell viability ($P < 0.05$) (Fig. 1D).

Next, we investigated the effects of Ctrl-Sec and PGC-Sec on apoptosis and proliferation in AML12 cells (Fig. 1E). Upon treatment with TAA, the expression of the antiapoptotic marker Bcl-xL and the proliferation marker proliferating cell nuclear antigen (PCNA) decreased in AML12 cells. However, treatment with Ctrl-Sec resulted in increased expression of these markers, while treatment with PGC-Sec showed the most significant increase ($P < 0.05$). These findings suggest that PGC-Sec may exert a pronounced effect on the upregulation of Bcl-xL and PCNA, indicating its potential role in promoting antiapoptotic and proliferative processes in AML12 cells.

Effects of PGC-Sec on oxidative stress

Subsequently, we employed MitoSOX flow cytometry to investigate the role of PGC-secretome in modulating mitochondrial superoxide production (Fig. 2). In control AML12 cells, MitoSOX fluorescence remained unaltered among the no treatment, Ctrl-Sec treatment, and PGC-Sec treatment groups. Strikingly, following TAA-induced injury, both Ctrl-Sec and PGC-Sec treatments resulted in a significant decrease in MitoSOX fluorescence compared to the no treatment group ($P < 0.05$). Furthermore, the PGC-Sec treatment group exhibited

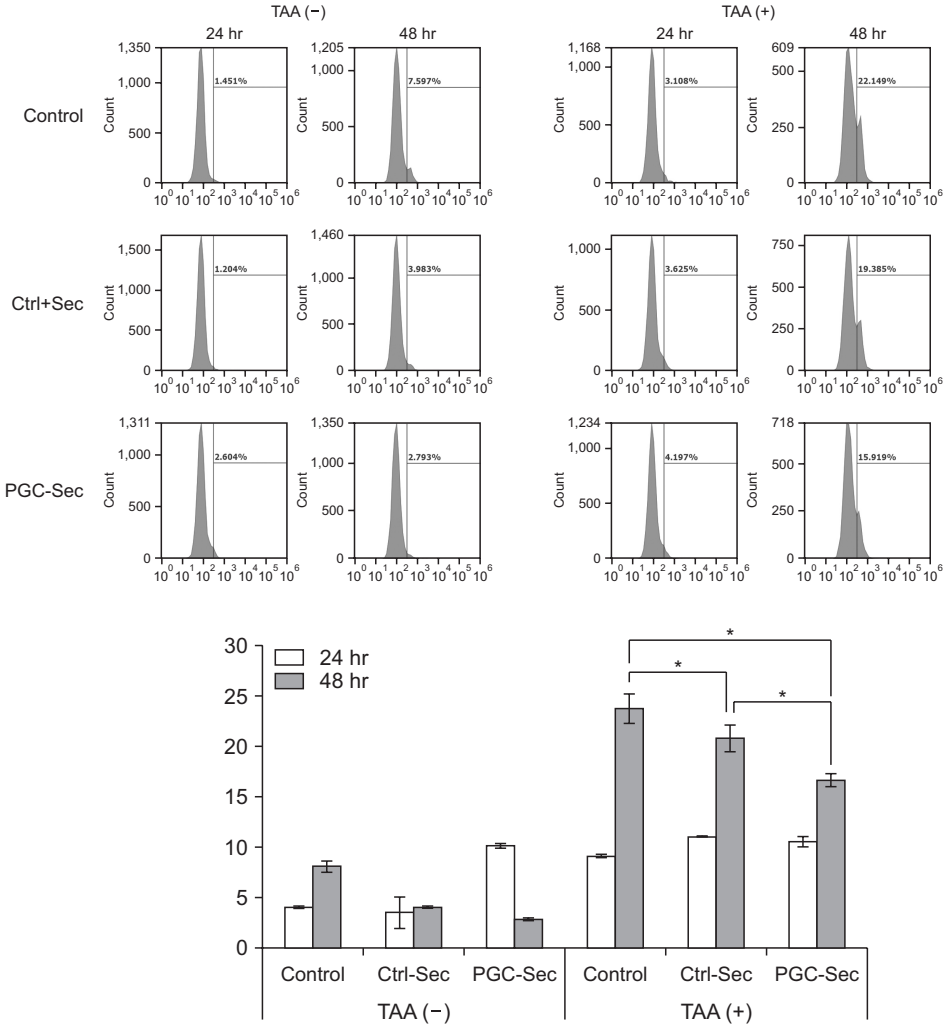

Fig. 2. Determination of effects of PGC-Sec on mitochondrial reactive oxygen species production. Mitochondrial superoxide indicator (MitoSOX) flow cytometry analysis of mitochondrial superoxide production in control AML12 cells and TAA-induced liver injury cells, indicating a significant reduction in MitoSOX fluorescence in both Ctrl-Sec and PGC-Sec treatment groups compared to the no treatment group, with a more pronounced reduction in the PGC-Sec treatment group. Values are presented as mean ± standard deviation of 3 independent experiments. Ctrl-Sec, Control-secretome, PGC-Sec, PGC-1α-boosted secretome; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TAA, thioacetamide; MitoSOX, mitochondrial superoxide indicator. *$P < 0.05$.

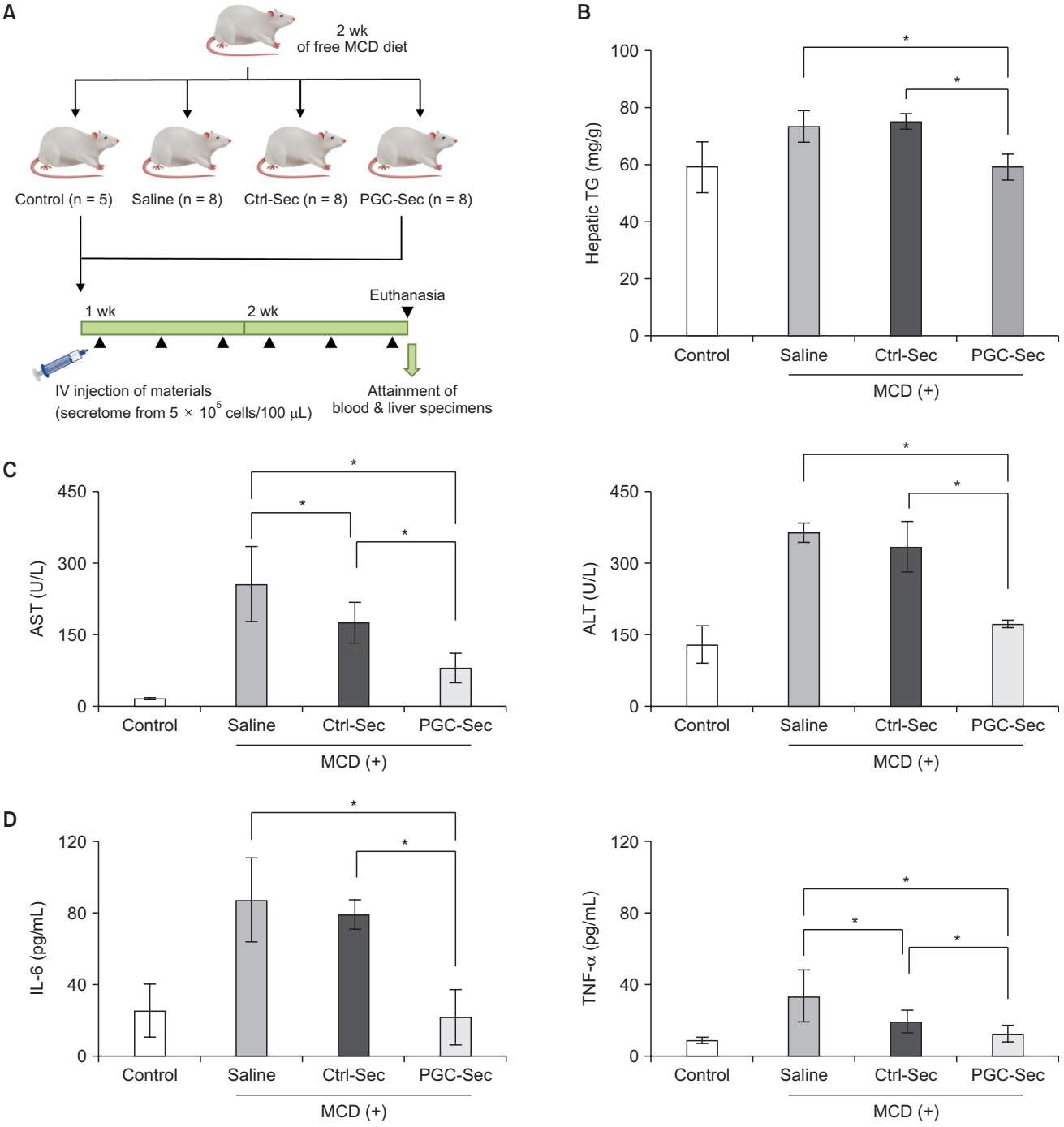

Fig. 3. *In vitro* effects of PGC-Sec on liver fibrosis; reduction in TG levels, liver enzymes, and proinflammatory cytokines. (A) Animal experimental design. (B) Assessment of TG levels in liver tissue samples from control mice, saline-injected mice, Ctrl-Sec–injected mice, and PGC-Sec–injected mice. Treatment with PGC-Sec significantly reduced TG levels, restoring them to levels comparable to the control group. (C) Measurement of serum liver enzyme levels, including AST and ALT, in the experimental groups. The PGC-Sec group showed significantly lower liver enzyme levels compared to the Ctrl-Sec group. (D) Evaluation of serum levels of proinflammatory cytokines, IL-6, and TNF-α in the experimental groups. The PGC-Sec group exhibited a significantly greater reduction in IL-6 and TNF-α levels compared to the Ctrl-Sec group. Values are presented as mean ± standard deviation of 3 independent experiments. IV, intravenous; MCD, methionine/choline-deficient; Ctrl-Sec, control-secretome; PGC-Sec, PGC-1α-boosted secretome; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha. *P < 0.05.

a significantly greater reduction in MitoSOX fluorescence compared to the Ctrl-Sec group ($P < 0.05$). Taken together, our findings reveal the remarkable potential of PGC-secretome in mitigating mitochondrial oxidative stress in an *in vitro* model of liver injury.

In vivo impact of PGC-Sec on hepatic enzyme activity and serum inflammatory cytokine concentrations

To investigate the antifibrogenic potential of PGC-Sec in liver fibrosis, we first established a mouse model of liver fibrosis by allowing mice to freely consume a MCD diet *ad libitum* for 2 weeks, which was continued until the fourth week of the experiment (Fig. 3A). The mice were divided into 4 groups: control mice ($n = 5$), saline injection ($n = 8$), Ctrl-Sec injection ($n = 8$), and PGC-Sec injection ($n = 8$). Each secretome intravenous injection contained 100 μL of secretome derived from 5×10^5 cells and was administered 3 times weekly, totaling 6 injections over a two-week period. At the beginning of the fifth week, mice were euthanized for the collection of blood and liver samples.

TG levels were assessed in liver tissue samples obtained from each experimental group. After the administration of the MCD diet, there was a noticeable elevation in TG levels. While treatment with Ctrl-Sec did not lead to a significant reduction in TG levels, treatment with PGC-Sec resulted in a significant decrease in TG levels, effectively restoring them to levels comparable to the control group ($P < 0.05$) (Fig. 3B). When examining serum liver enzyme levels, both AST and ALT demonstrated significant reductions in the secretome-treated groups (Ctrl-Sec and PGC-Sec) compared to the control group ($P < 0.05$) (Fig. 3C). Furthermore, the PGC-Sec group exhibited significantly lower liver enzyme levels than the Ctrl-Sec group ($P < 0.05$). We next assessed serum levels of proinflammatory cytokines, IL-6, and TNF-α (Fig. 3D). Both secretome-treated groups (Ctrl-Sec and PGC-Sec) displayed significant decreases in the blood levels of IL-6 and TNF-α compared to the control group ($P < 0.05$). Notably, the PGC-Sec group showed a significantly greater reduction in the blood levels of IL-6 and TNF-α relative to the Ctrl-Sec group ($P < 0.05$).

In vivo impact of PGC-Sec on the expression of fibrosis-related markers and liver histology

Liver specimens were obtained from each experimental group, and the expression of mRNAs associated with liver fibrosis was assessed using quantitative real-time PCR (Fig. 4A). Notably, the expression of profibrogenic markers, collagen and TGF-β mRNA, was significantly reduced in the secretome injection groups (Ctrl-Sec & PGC-Sec) compared to the saline injection group ($P < 0.05$). Furthermore, the PGC-Sec group displayed a more pronounced decrease in collagen and TGF-β mRNA expression relative to the Ctrl-Sec group ($P < 0.05$).

Subsequently, Western blot analysis was conducted to assess the protein levels of collagen type A1 (ColA1), an established marker of fibrogenesis, TIMP, a recognized marker of antifibrogenesis, and sterol regulatory element-binding protein 1 (SREBP-1), a key regulator of lipid biosynthesis (Fig. 4B). ColA1 expression was significantly lower in the secretome injection groups (Ctrl-Sec and PGC-Sec) than in the saline injection group ($P < 0.05$). Conversely, TIMP expression was significantly higher in the secretome injection groups (Ctrl-Sec and PGC-Sec) compared to the saline injection group ($P < 0.05$). Moreover, the PGC-Sec group exhibited a significantly increased TIMP expression relative to the Ctrl-Sec group ($P < 0.05$). Both Ctrl-Sec and PGC-Sec groups showed a decrease in SREBP-1 levels, with PGC-Sec exhibiting the most significant reduction ($P < 0.05$). This indicates that PGC-Sec possesses a pronounced capacity to suppress lipid biosynthesis.

Histological analysis was performed on liver specimens from each group using H&E staining (Fig. 4C). The secretome injection groups (Ctrl-Sec and PGC-Sec) demonstrated a significant reduction in liver fibrosis severity compared to the saline injection group, with the PGC-Sec group displaying the most substantial reduction ($P < 0.05$). This trend was further corroborated by Sirius red staining ($P < 0.05$) (Fig. 4D), a reliable method for visualizing and assessing liver fibrosis.

Immunohistochemistry validating the antifibrogenic effects of PGC-Sec

Next, we aimed to investigate the distinct effects of PGC-Sec using immunohistochemical staining. Initially, we employed PGC-1α immunohistochemistry to verify that PGC-1α expression was significantly elevated in the PGC-Sec group ($P < 0.05$) (Fig. 5A). The expression of PGC-1α was decreased following the MCD diet, slightly increased after Ctrl-Sec administration, but significantly increased after PGC-Sec administration ($P < 0.05$). This finding suggests that PGC-Sec administration significantly increases PGC-1α expression, demonstrating its potential to upregulate PGC-1α levels. Subsequently, we compared OPA1 expression, an indicator of mitochondrial function, across the different groups (Fig. 5B). Among the injection groups, the PGC-Sec group exhibited the highest OPA1 expression ($P < 0.05$). The notable overexpression of OPA1 in the PGC-Sec group is believed to stem from the remarkable enhancement of energy production and overall mitochondrial function, driven by the increased expression of PGC-1α in the PGC-Sec group. Moreover, immunohistochemistry revealed that the expression of the profibrogenic marker ColA1 was markedly reduced in the PGC-Sec group ($P < 0.05$) (Fig. 5C), while the expression of the antifibrogenic marker PPAR-α was significantly increased ($P < 0.05$) (Fig. 5D). Taken altogether, these results highlight the profound impact of PGC-Sec treatment on enhancing

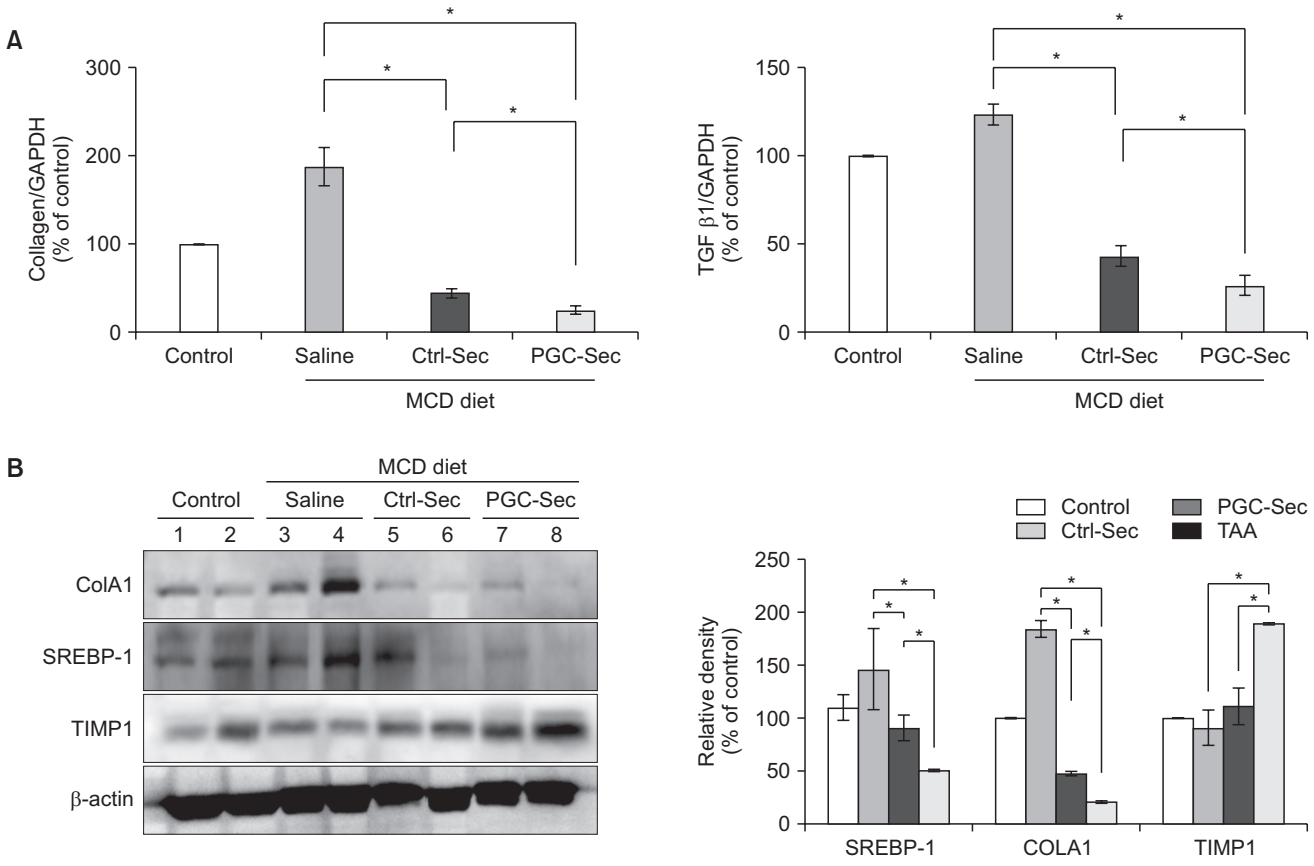

Fig. 4. *In vivo* effects of PGC-Sec on liver fibrosis; protein expression and histological improvement. (A) Quantitative real-time PCR analysis of liver fibrosis-associated messenger RNA (mRNA) expression, including collagen and TGF-β1, in liver specimens. Both secretome injection groups exhibited significantly reduced expression of collagen and TGF-β mRNA compared to the saline injection group. The PGC-Sec group displayed a more pronounced decrease in collagen and TGF-β1 mRNA expression compared to the Ctrl-Sec group. (B) Western blot analysis of protein levels of ColA1, TIMP1, and SREBP-1 in liver specimens. Secretome injection groups showed significantly lower ColA1 levels and higher TIMP levels compared to the saline injection group. The PGC-Sec group exhibited significantly increased TIMP expression compared to the Ctrl-Sec group. Both Ctrl-Sec and PGC-Sec displayed a decrease in SREBP-1 levels, with PGC-Sec showing the most significant reduction. (C) Histological analysis of liver fibrosis severity using H&E staining. The secretome injection groups demonstrated a significant reduction in liver fibrosis severity compared to the saline injection group, with the PGC-Sec group showing the most substantial improvement. (D) Evaluation of liver fibrosis using Sirius red staining, a reliable method for visualizing and assessing liver fibrosis. Both Ctrl-Sec and PGC-Sec groups exhibited a significant reduction in liver fibrosis compared to the saline injection group. Values are presented as mean ± standard deviation of 3 independent experiments. PGC-Sec, PGC-1α-boosted secretome; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ctrl-Sec, control-secretome; MCD, methionine/choline-deficient; ColA1, collagen type A1; SREBP, sterol regulatory element-binding protein; TIMP, tissue inhibitor of metalloproteinases; TAA, thioacetamide; SREBP-1, sterol regulatory element-binding protein 1; NAFLD, non-alcoholic fatty liver disease. *P < 0.05.

mitochondrial biogenesis and function, as well as promoting antifibrosis processes, collectively demonstrating its potential as a novel therapeutic strategy for liver fibrosis.

DISCUSSION

This study investigates the therapeutic potential of the secretome released from PGC-1α–overexpressing ASCs (PGC-Sec) in liver fibrosis treatment. Using *in vitro* and *in vivo* models, we demonstrate that PGC-Sec enhances cell viability, reduces mitochondrial oxidative stress, lowers serum liver enzyme levels, and attenuates proinflammatory cytokine concentrations. Furthermore, PGC-Sec significantly modulates the expression of fibrosis-related markers and improves liver histology. Immunohistochemistry confirms the upregulation of mitochondrial function markers, such as OPA1, in the PGC-Sec group, indicating a strong association between PGC-1α–driven mitochondrial proliferation and the antifibrogenic potential of the secretome. Collectively, our findings reveal the promise of PGC-Sec as a novel therapeutic strategy for liver fibrosis management.

PGC-1α is a transcriptional coactivator that plays a pivotal role

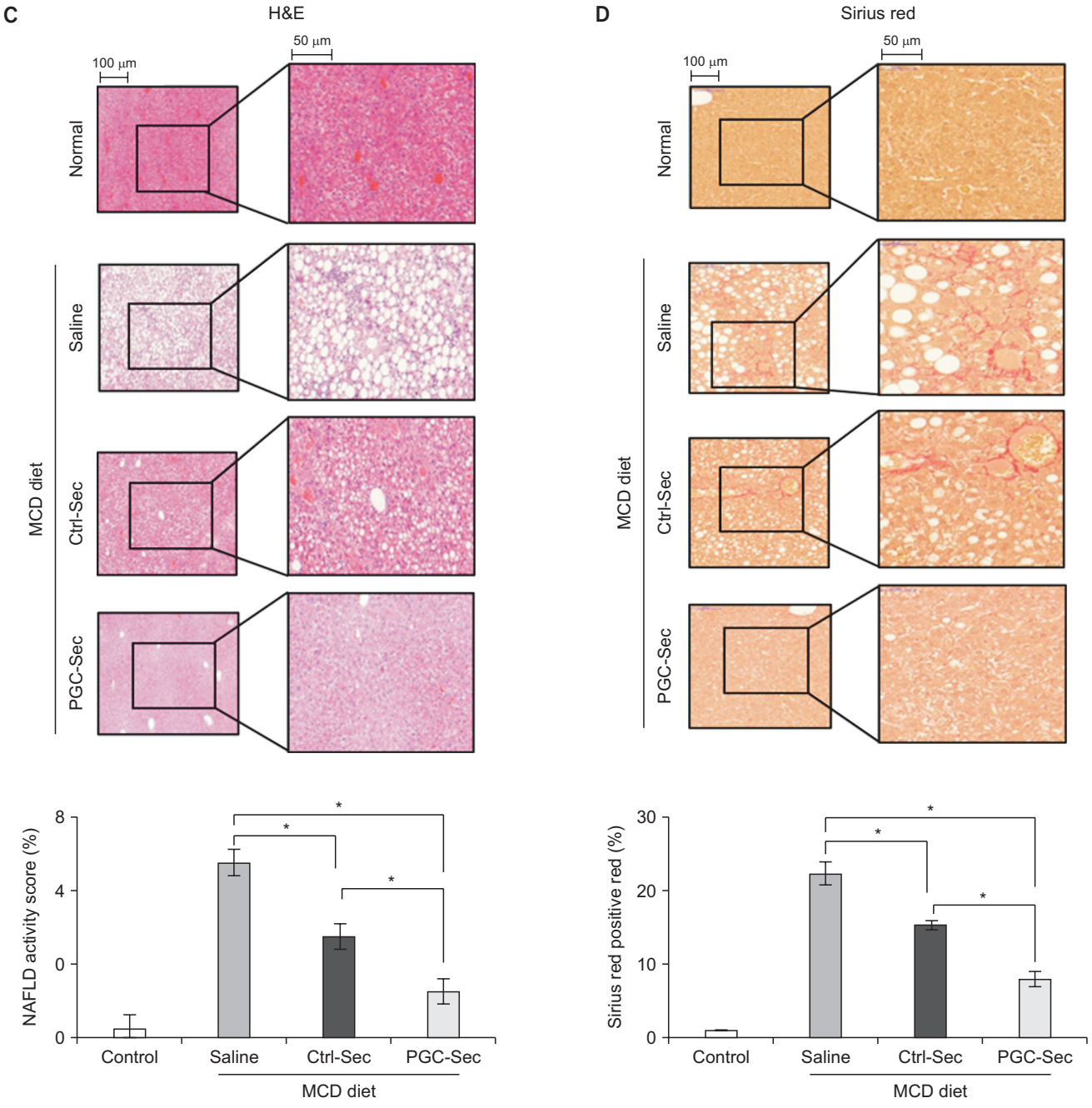

Fig. 4. Continued.

in regulating cellular energy metabolism, particularly in the context of mitochondrial biogenesis, oxidative phosphorylation, and fatty acid oxidation [6,7,12]. As a master regulator of energy homeostasis, PGC-1α is essential in maintaining metabolic flexibility in various tissues, such as the liver, skeletal muscle, and heart [13,14]. Its influence on mitochondrial function has profound implications for numerous physiological processes, including cellular respiration, thermogenesis, and overall energy balance [12]. Additionally, PGC-1α is implicated in various disease conditions, such as obesity, diabetes, and neurodegenerative disorders, where mitochondrial dysfunction and energy imbalance are key features [6]. Consequently, PGC-1α has garnered significant attention as a potential therapeutic target for a wide range of metabolic and degenerative diseases, highlighting its remarkable significance in the fields of cell biology, metabolism, and human health.

PGC-1α has emerged as a potential player in the context of liver fibrosis, a chronic liver disease characterized by excessive accumulation of extracellular matrix components, ultimately leading to impaired liver function [15,16]. As a key

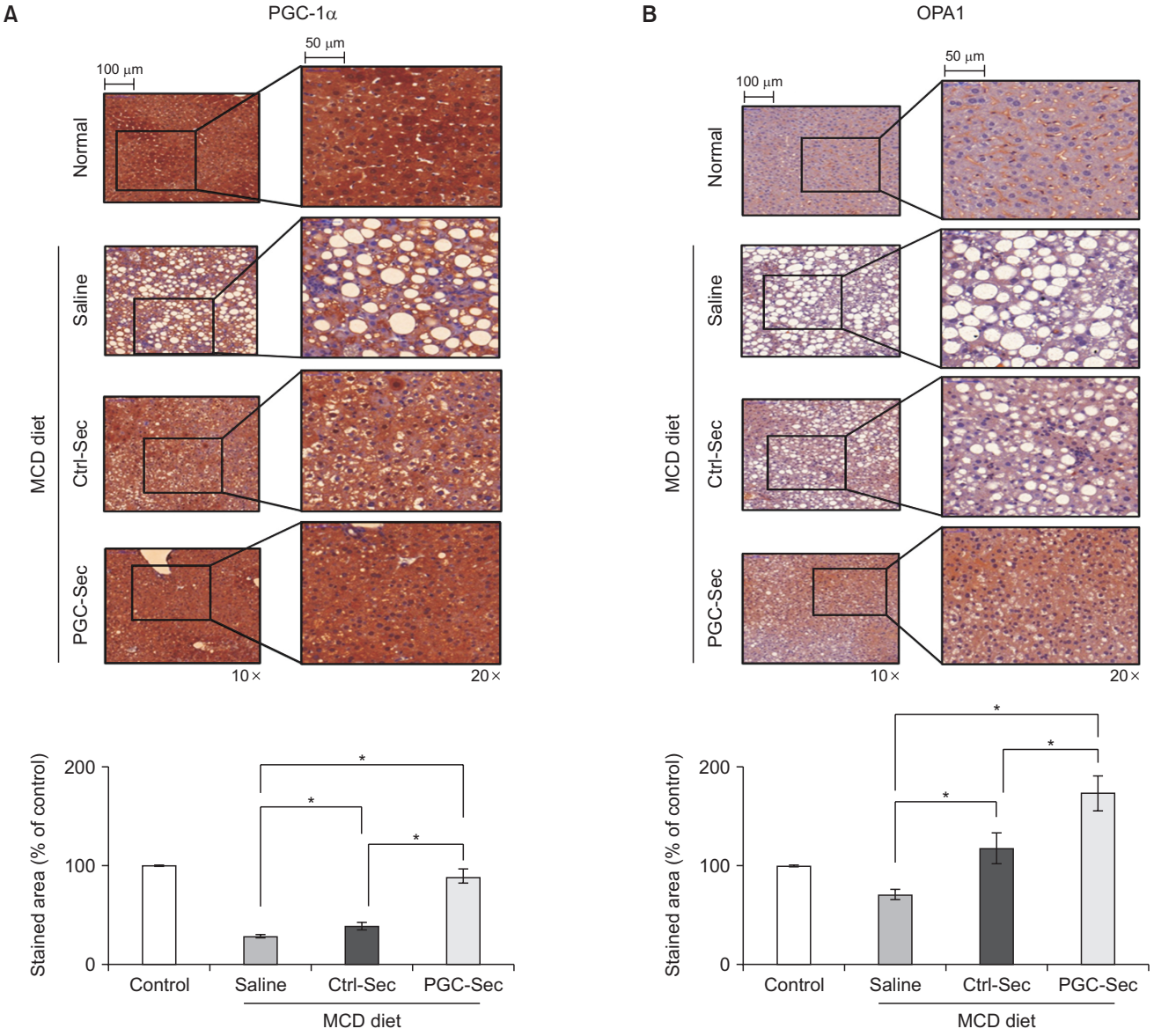

Fig. 5. Immunohistochemistry validating the antifibrogenic effects of PGC-Sec in a mouse model of liver fibrosis. (A) PGC-1α immunohistochemistry showing significantly elevated PGC-1α expression in the PGC-Sec group. (B) OPA1 expression comparison across different groups, with the highest OPA1 expression observed in the PGC-Sec group, indicating enhanced mitochondrial function. (C) ColA1 immunohistochemistry revealing markedly reduced expression of the profibrogenic marker in the PGC-Sec group. (D) PPAR-α immunohistochemistry showing significantly increased expression of the antifibrogenic marker in the PGC-Sec group. These findings emphasize the profound impact of PGC-Sec treatment on enhancing mitochondrial biogenesis and function, as well as promoting antifibrosis processes, collectively highlighting its potential as a novel therapeutic strategy for liver fibrosis. Percentages of immunoreactive areas were measured using ImageJ of the National Institutes of Health and expressed as relative values to those in normal livers. MCD, methionine/choline-deficient; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PGC-Sec, PGC-1α-boosted secretome; Ctrl-Sec, control-secretome; OPA1, optic atrophy 1; ColA1, collagen type A1; PPAR-α, peroxisome proliferator-activated receptor alpha. *P < 0.05.

regulator of mitochondrial biogenesis and function, PGC-1α has been implicated in modulating the processes associated with liver fibrosis progression and resolution. Dysregulated energy metabolism and mitochondrial dysfunction have been observed in fibrotic livers, with PGC-1α potentially serving as a therapeutic target to counteract these changes. By enhancing mitochondrial function, oxidative phosphorylation, and fatty acid oxidation, PGC-1α could alleviate inflammation, oxidative stress, and HSC activation, which are hallmarks of liver fibrosis [17,18]. Moreover, PGC-1α has been shown to regulate the expression of various fibrosis-related markers, further underscoring its potential in controlling the fibrogenic process

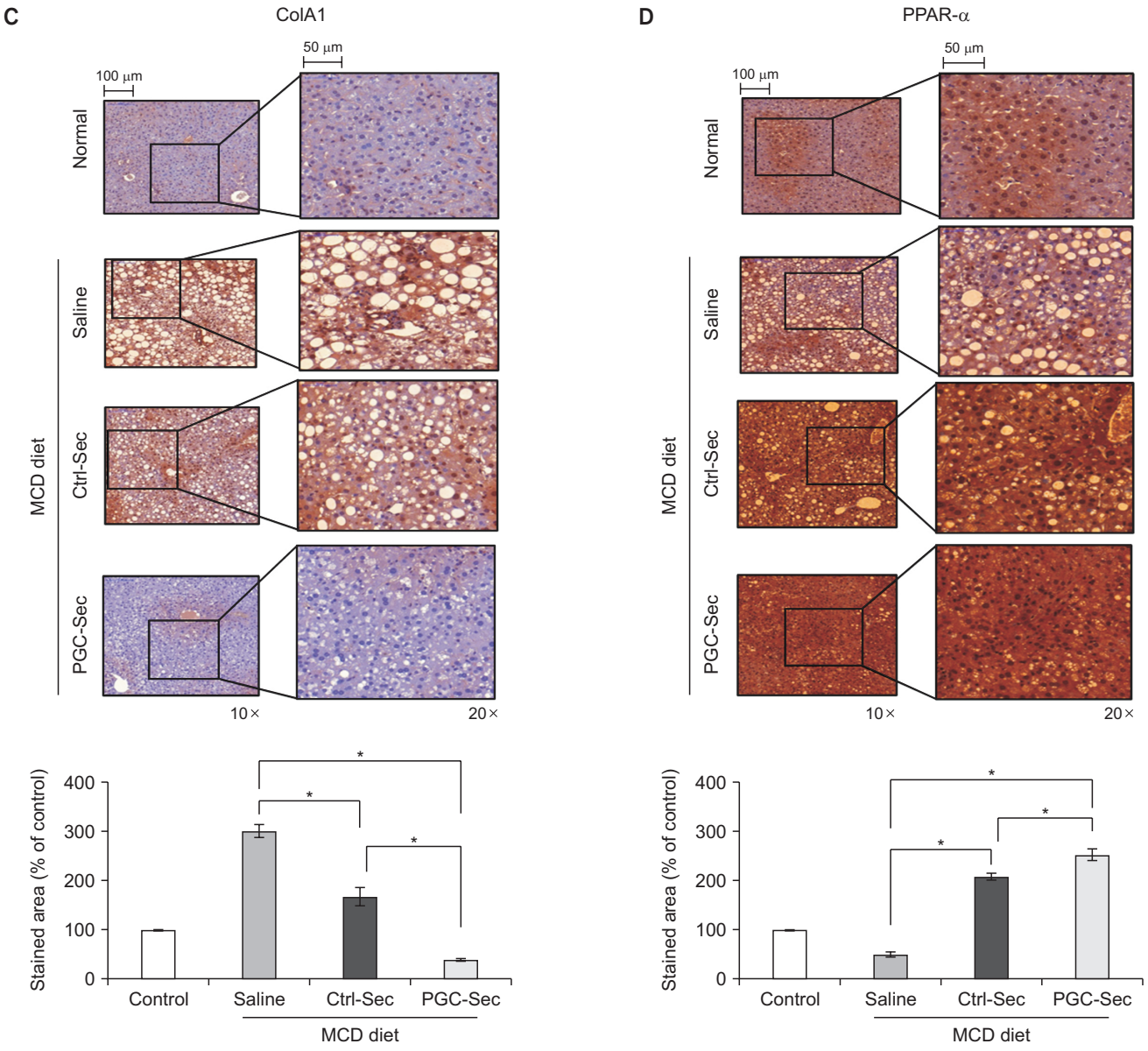

Fig. 5. Continued.

[19,20]. Therefore, a better understanding of PGC-1α's role in liver fibrosis may provide novel insights into the development of effective therapeutic strategies to manage and treat this debilitating liver disease.

The use of PGC-Sec presents several advantages over direct PGC-1α administration for the treatment of liver fibrosis. First, the secretome contains a rich array of bioactive molecules, including growth factors, cytokines, and extracellular vesicles, which can act in concert to promote tissue repair, modulate inflammation, and mitigate fibrosis [21,22]. This multifaceted approach may offer a more effective therapeutic outcome compared to targeting PGC-1α alone. Second, utilizing the secretome takes advantage of the paracrine effects of stem cells, circumventing potential challenges associated with stem cell engraftment, survival, and differentiation. Third, the use of PGC-Sec may reduce the risk of off-target effects or unintended consequences associated with direct PGC-1α manipulation. Lastly, PGC-Sec administration is a cell-free therapy, which may simplify production, storage, and transportation, ultimately expediting its translation to clinical settings. Collectively, these advantages suggest that PGC-Sec holds promise as a novel therapeutic strategy for the treatment of liver fibrosis, warranting further investigation into its efficacy and safety.

PGC-Sec is believed to contribute to the process of antifibrosis through several mechanisms (Fig. 6). Firstly, PGC-Sec activates mitochondrial biogenesis and oxidative phosphorylation, which enhances the functionality of mitochondria. This activation leads to an increase in energy production within the cells.

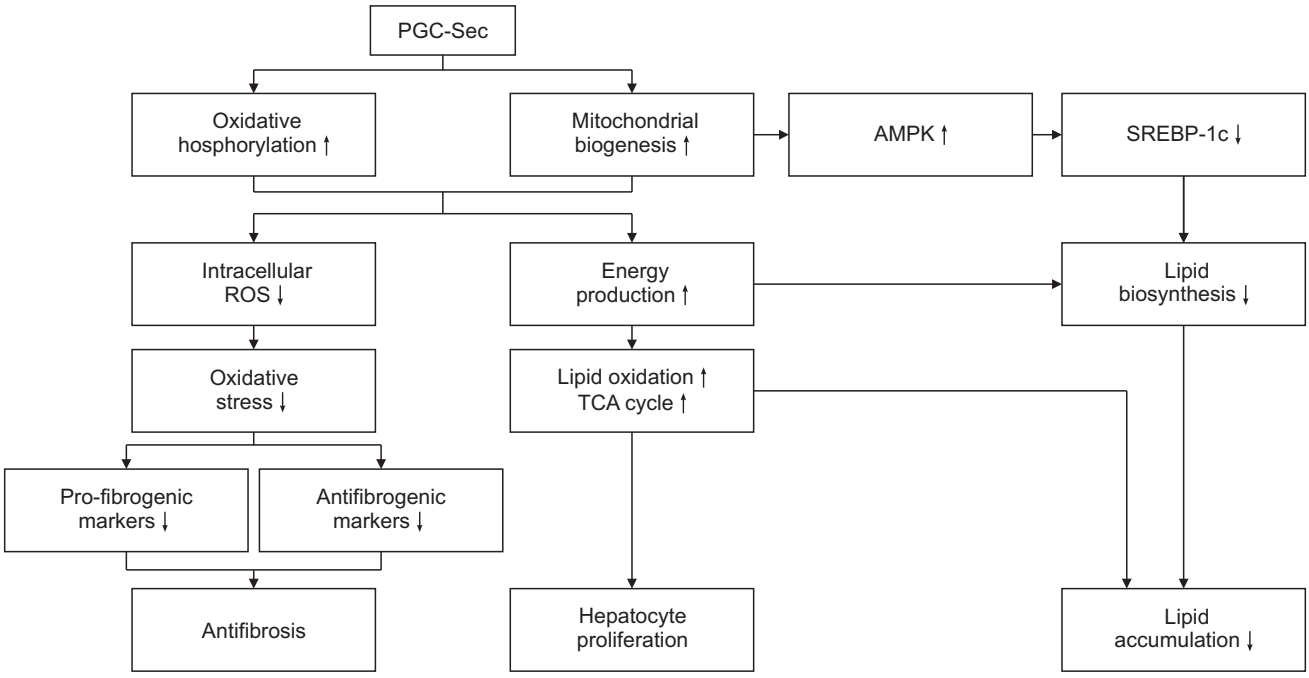

Fig. 6. The postulated mechanism by which PGC-Sec contributes to antifibrosis. PGC-Sec activates mitochondrial biogenesis and oxidative phosphorylation, leading to a decrease in intracellular ROS and oxidative stress. This reduction in oxidative stress results in decreased expression of profibrogenic markers and increased expression of antifibrosis markers, thereby promoting antifibrosis. PGC-Sec also enhances mitochondrial function, leading to increased energy production, which in turn promotes the proliferation of liver cells. Furthermore, it has been reported that increased mitochondrial biogenesis by PGC-Sec results in increased AMPK activation. This, as evidenced by our experimental results, leads to a decrease in SREBP-1c, a key regulator of lipid biogenesis, and consequently a reduction in lipid accumulation within hepatocytes. PGC-Sec, PGC-1α-boosted secretome; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, reactive oxygen species; AMPK, AMP-activated protein kinase; SREBP, sterol regulatory element-binding protein; TCA, tricarboxylic acid.

The increased energy availability promotes the proliferation of hepatocytes, which is beneficial for tissue repair and regeneration. Additionally, the activation of mitochondrial biogenesis by PGC-Sec has been reported to result in increased AMP-activated protein kinase (AMPK) levels [23,24]. AMPK is a cellular energy sensor and a key regulator of various metabolic processes. Its activation has been associated with a decrease in the expression of SREBP-1c, which is a crucial regulator of lipid biogenesis [25,26]. The reduction in SREBP-1c expression triggered by PGC-Sec-mediated AMPK activation leads to a decrease in lipid accumulation within hepatocytes [27]. This is significant because excessive lipid accumulation in the liver can contribute to the development of fibrosis. By reducing lipid accumulation, PGC-Sec helps mitigate the profibrogenic environment in the liver and promotes an antifibrotic state. Furthermore, the activation of mitochondrial biogenesis by PGC-Sec leads to a decrease in intracellular ROS and oxidative stress [28,29]. This reduction in oxidative stress has a direct impact on fibrosis development, as oxidative stress is known to play a critical role in promoting fibrotic processes [30]. The decreased oxidative stress resulting from PGC-Sec activity leads to a decrease in the expression of profibrogenic markers, which are molecules involved in the fibrotic cascade, and an increase in the expression of antifibrosis markers.

Despite the significant findings of this study, several limitations should be acknowledged. Firstly, the specific mechanisms underlying the effects of PGC-Sec on liver fibrosis were not fully elucidated in this study. Although we provided evidence of its impact on various indicators of fibrosis, further investigations are required to unravel the precise molecular pathways involved. Specifically, elucidation is needed as to whether the exact source of the antifibrotic effect observed originates directly from PGC-1α or through the modulation of downstream proteins in the PGC-1α pathway within transfected ASCs. To determine the exact mechanisms of PGC-Sec, comparative experiments using Ctrl-Sec and PGC-Sec from non-ASC cells (e.g., HEK293 cells) would be informative. Additionally, comparing the composition of Ctrl-Sec and PGC-Sec through techniques such as liquid chromatography-mass spectrometry could help identify differentially expressed proteins and determine their individual roles in antifibrosis. Although the precise mechanisms were not fully elucidated in this study, it is noteworthy that this research has determined the role of PGC-1α–overexpressing secretome, which is involved

in mitochondrial biogenesis and function, as a potential antifibrogenic strategy.

In conclusion, our findings reveal the secretome from PGC-1α–overexpressing ASCs (PGC-Sec) as a promising therapeutic candidate for liver fibrosis treatment, by decreasing proliferation of activated HSC cells, reducing oxidative stress, modulating fibrosis-related markers, and improving liver histology. Furthermore, the observed upregulation of mitochondrial function markers, such as OPA1, highlights the critical role of PGC-1α–driven mitochondrial proliferation in contributing to the antifibrogenic potential of the secretome. Collectively, these results underscore the promise of PGC-Sec as a novel and effective therapeutic strategy for liver fibrosis management, paving the way for future research and clinical applications.

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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