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The mechanisms of fibrotic disorders and pharmacological therapies

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The mechanisms of fibrotic disorders and pharmacological therapies

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Knockout of C1q/tumor necrosis factor-related protein-9 aggravates cardiac fibrosis in diabetic mice by regulating YAP-mediated autophagy

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Introduction: Diabetic cardiomyopathy (DCM) is predominantly distinguished by impairment in ventricular function and myocardial fibrosis. Previous studies revealed the cardioprotective properties of C1q/tumor necrosis factor-related protein 9 (CTRP9). However, whether CTRP9 affects diabetic myocardial fibrosis and its underlying mechanisms remains unclear.

Methods: We developed a type 1 diabetes (T1DM) model in CTRP9-KO mice via streptozotocin (STZ) induction to examine cardiac function, histopathology, fibrosis extent, Yes-associated protein (YAP) expression, and the expression of markers for autophagy such LC3-II and p62. Additionally, we analyzed the direct impact of CTRP9 on high glucose (HG)-induced transdifferentiation, autophagic activity, and YAP protein levels in cardiac fibroblasts.

Results: In diabetic mice, CTRP9 expression was decreased in the heart. The absence of CTRP9 aggravated cardiac dysfunction and fibrosis in mice with diabetes, alongside increased YAP expression and impaired autophagy. *In vitro*, HG induced the activation of myocardial fibroblasts, which demonstrated elevated cell proliferation, collagen production, and α-smooth muscle actin (α-SMA) expression. CTRP9 countered these adverse effects by restoring autophagy and reducing YAP protein levels in cardiac fibroblasts. Notably, the protective effects of CTRP9 were negated by the inhibition of autophagy with chloroquine (CQ) or by YAP overexpression through plasmid intervention. Notably, the protective effect of CTRP9 was negated by inhibition of autophagy caused by chloroquine (CQ) or plasmid intervention with YAP overexpression.

Discussion: Our findings suggest that CTRP9 can enhance cardiac function and mitigate cardiac remodeling in DCM through the regulation of YAP-mediated autophagy. CTRP9 holds promise as a potential candidate for pharmacotherapy in managing diabetic cardiac fibrosis.

KEYWORDS

diabetic cardiomyopathy, CTRP9, fibrosis, autophagy, YAP, fibroblasts

1 Introduction

DCM is a prevalent diabetic macrovascular complication, marked primarily by diffuse myocardial fibrosis and impairment in cardiac function (Luo et al., 2022). Fibroblasts, the most populous nonmyocyte cell type within the heart, are central to the pathophysiology of DCM (Pesce et al., 2023). When subjected to stress, these fibroblasts become activated and transdifferentiate into myofibroblasts, as evidenced by increased alpha-smooth muscle actin (α-SMA) level and heightened secretion of collagen-rich extracellular matrix (ECM), as well as enhanced cell proliferation capacity (Tallquist, 2020; Ko et al., 2022). This process results in increased cardiac wall stiffness and consequent deterioration of cardiac function. Therefore, conducting a detailed investigation into the mechanisms driving the activation and transdifferentiation of cardiac fibroblasts is critical to deepening our understanding of DCM pathogenesis and unveiling new therapeutic targets.

Recent evidence has revealed that impaired autophagy and activated Yes-associated protein (YAP) are significant contributors to cardiac fibrosis (Miyamoto, 2019; Zhang Q. et al., 2022). Autophagy, a vital cellular process for degrading and recycling damaged organelles and macromolecules via lysosomes, is indispensable for maintaining cardiac homeostasis (Klionsky et al., 2021). In diabetic models, impaired autophagy has been observed, marked by the excessive buildup of the autophagic substrate p62 and increasing levels of LC3II (Qiao et al., 2022). Autophagy-promoting drugs exhibit therapeutic efficacy against fibrosis and ventricular dysfunction in DCM (Shen et al., 2021; Zhang L. et al., 2023). These observations imply a likely involvement of autophagy in the pathogenesis of DCM. In addition, transforming growth factor-β (TGF-β), a primary fibrosis driver, has been shown to exert its effects through YAP, a downstream effector within the Hippo pathway, reinforcing YAP's importance in fibrosis (Zhang T. et al., 2022; Weng et al., 2023). YAP is also implicated in fibrosis in other organs; its upregulation in hepatic stellate cells is linked to sustained myofibroblasts activation and increased extracellular matrix deposition in liver fibrosis (Xiang et al., 2020; Mia and Singh, 2022). Similarly, YAP overexpression in renal mesangial cells is correlated with excessive collagen production, which contributes to renal fibrosis (Choi et al., 2023). Additionally, the results showed that YAP was closely related to autophagy. Increased YAP expression in mouse proximal tubular epithelial cells inhibited autophagy, exacerbating diabetic nephropathy (Claude-Taupin et al., 2024). Recent findings also underscore the implications of YAP activation in ventricular remodeling and cardiac dysfunction in diabetic mice, suggesting that targeting YAP-mediated autophagy could be a strategic focus for DCM treatment (Ikeda et al., 2019).

C1q/tumor necrosis factor-related protein 9 (CTRP9) is a recently discovered adipokine within the CTRP superfamily, playing a pivotal role in regulating glycolipid metabolism and providing cardioprotection (Zhao et al., 2018; Guan et al., 2022). Clinical investigations have unveiled a correlation between lower CTRP9 levels and metabolic syndrome, and insulin resistance in diabetic individuals (Hwang et al., 2014; Jia et al., 2017; Moradi et al., 2018). Additionally, research has indicated that CTRP9 alleviates myocardial fibrosis postinfarction and improves fibrotic conditions in diabetic nephropathy (Hu et al., 2020; Lee et al., 2022). Nevertheless, its potential for attenuating diabetic myocardial fibrosis is not fully understood, highlighting the need for in-depth mechanistic research to clarify its therapeutic role.

This study aimed to investigate how CTRP9 ma y suppress the transdifferentiation of cardiac fibroblasts and mitigate diabetic myocardial fibrosis. We focused on determining whether CTRP9 achieves this effect by regulating the YAP-mediated autophagy pathway, which could offer a novel approach to preventing and treating DCM. To this end, a comprehensive array of *in vivo* and *in vitro* experiments was conducted.

2 Methods and materials

2.1 Animals and protocols

CTRP9 knockout (on a C57BL/6J background) mice, were generated by Shanghai Biomodel Organism Science & Technology Development Co., Ltd. STZ (MCE, USA, 55 mg/KG) in citrate buffer was injected intraperitoneally for five consecutive days to induce type 1 diabetes mellitus (T1DM) (Meng et al., 2023). Fasting glucose values above 16.7 mM were considered to be diabetes. After the mice were anesthetized with Pentobarbital Sodium (70 mg/kg, IP), animal tissues were retained for subsequent experiments. The Ethics Committee of Qiluhospital of Shandong University reviewed (KYLL-2022(ZM)-1300) and authorized all animal procedures performed with the Guide for the Care and Use of Laboratory Animals.

2.2 Echocardiography

Mice were anesthetized with the inhalational anesthetic isoflurane and underwent echocardiography. The induction anesthetic dose was 2%, 0.2 mg/10 g, 400 mg/kg, and the maintenance anesthetic dose was 1.2%, 0.2 mg/10 g, 240 mg/kg. The operator is unaware of the animal grouping. The left ventricle function was evaluated using a Vevo2100 imaging system (VisualSonics, Toronto, Canada). The echocardiography parameters included left ventricular end-diastolic internal diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF), fraction shortening (FS), early-to-late diastolic mitral flow velocities (E/A), and the ratio of early diastolic mitral inflow to mitral annular velocity (E/e').

2.3 Immunohistochemistry

Hearts were prepared as 4 μm paraffin sections for subsequent staining procedures, including hematoxylin and eosin (H&E) staining as well as Masson's trichrome staining. Anti-Collagen I (Abcam, ab34710, 1:200), Anti-Collagen III (Abcam, ab7778, 1:200), Anti-α-SMA (HUABIO, ET1607-53, 1:5000), and Anti-YAP (ABclonal, A19134, 1:200), Anti-CTRP9 (NOVUSBIO, NBP2-46834, 1:200) were applied to the paraffin sections at 4°C. Subsequent treatment included exposure to secondary antibodies (Gene Tech, GK600505) and hematoxylin staining. Three fields of

view were selected for each sample, and the mean optical density was measured and then averaged. The resulting data represents one biological replicate. The results were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics Inc., USA).

2.4 Cell culture

Primary cardiac fibroblasts were isolated from the hearts of 3- to 5-day-old C57BL/6J mice. The neonatal mouse hearts were removed and sliced into tissue fragments in pre-cooled D-Hank's solution. The fragments were then transferred into conical flasks containing type II collagenase (Solarbio) and incubated overnight at 4°C on a shaker. The next day, the fragmented heart tissue was digested in a 37°C water bath using an EDTA-free trypsin (Solarbio) digestion solution. The collected supernatant was inoculated into flasks for cell culture. After an incubation period of 2 h at 37°C with 5% CO_2, the fibroblasts attached to the flasks and the complete medium was subsequently substituted.

During the experiment, only cardiac fibroblasts of one to three generations were used. Primary cardiac fibroblasts were grown in complete DMEM (Gibco). The cells were preincubated with recombinant gCTRP9 (1 μg/mL) (Lei et al., 2021) for 2 h before they were exposed to a high-glucose environment (33.3 mM). Following a 48-h incubation period, the cells were harvested for analysis. CQ (1 mM) was administered 12 h before the end of the experiment to inhibit autophagy.

2.5 Cell transfection

Transfection of cells with the YAP overexpression plasmid (Shandong Gene & Bio Co., Ltd.) (1,000 ng/mL) and the control plasmid was conducted using Lipofectamine™ 3000 reagent (Invitrogen) in Opti-MEM™ reduced serum medium (Gibco). Complete medium was added to replace the medium 8 hours after transfection.

To knock down CTRP9, small interfering RNA (siRNA) (shandong Gene&Bio) was transfected into CFS using Lipofectamine™3000 reagent (Invitrogen) in Opti-MEM™ reduced serum medium (Gibco). The medium was replaced with complete medium 6–8 h later, and small interference was screened by detecting the mRNA expression of CTRP9 24 h later.

2.6 Western blotting

Extracted protein samples were underwent separation through SDS-PAGE, followed by transfer onto PVDF membrane (Millipore). Then, the membranes underwent overnight with antibodies against Collagen I (Proteintech, 66761-Ig, 1:1000), Collagen III (Abcam, ab184993, 1:1000), α-SMA (HUABIO, ET1607-53, 1:5000), GAPDH (Proteintech, 60004-1-Ig, 1:10,000), p62 (Abcam, ab109012, 1:1000), LC3B (CST, 3868S, 1:1000), and YAP (ABclonal, A19134, 1:1000), CTRP9 (NOVUSBIO, NBP2-46834, 1:500). Subsequently, secondary antibodies (HUABIO, HA1006 and HA1001) were applied to the membranes, followed by visualization using an Amersham Imager 680.

2.7 Immunofluorescence staining

Cardiac fibroblasts were sequentially treated with methanol on ice and 5% BSA. Subsequently, the cells were exposed to anti-α-SMA (HUABIO, ET1607-53,1:500) or anti-LC3B (CST, 3868S, 1:200) primary antibodies and left to incubate overnight. The coverslips were subjected to secondary antibody incubation, followed by staining with DAPI. Images were captured using a fluorescence microscope (Nikon Eclipse TE2000-S) or a Zeiss confocal laser scanning microscope (LSM 710, Carl Zeiss, Germany). Three fields of view were selected for each sample, and the fluorescence intensity was measured and averaged. The results obtained represented a biological replication. The ImageJ software was used for analysis.

2.8 qRT-PCR

FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme Biotech Co., Ltd.) extracted total RNA from fibroblasts and determined its concentration, followed by HiScript II Q RT SuperMix (Vazyme Biotech Co., Ltd.) and ChamQ universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) for reverse transcription and PCR quantification. The primer sequences used are as follows: Collagen I-F, CCCTGGTCCCTCTGGAAATG, Collagen I-R, GGACCtttgccccCTTCTTCTTT; Collagen III-F, TGACTGTCCCACGTAAGCAC, Collagen III-R, GAGGGCCAT AGCTGAACTGA; α-SMA-F, TTCGTGACTACTGCCGAGC, α-SMA-R, GTCAGGCAGTTCGTAGCTCT; p62-F, CCTCAGCCC TCTAGGCATTG, p62-R, TTCTGGGGTAGTGGGTGTCA; LC3B-F, AGAGCGATACAAGGGGGAGA, LC3B-R, TGCAAG CGCCGTCTGATTA; ATG-7-F, CCTTCTGGAGCAGTCAGC AA, ATG-7-R, AGGAGCATGGGGTTTTCGAG; YAP1-F, TCC AACCAGCAGCAGCAAAT, YAP1-R, CCTGTTGTTTCAACC GCAGTC; CTRP9-F, GTGCCCAAGAGTGCTTTCAC, CTRP9-R, AACTTCCCCGTCGCTACATT; GAPDH-F, TGTCTCCTG CGACTTCAACA, GAPDH-R, GGTGGTCCAGGGTTTCTTACT.

2.9 Cell proliferation assay

The proliferation of cardiac fibroblasts was detected using Cell-Light EdU DNA Cell Proliferation Apollo567 Kit (RiBoBio). The treated cardiac fibroblasts were incubated with 10 nm EdU (5-ethynyl-2'-deoxyuridine) for 16 h, followed by subsequent experimental steps according to the instructions. Images were captured using a Zeiss fluorescence microscope, and the percentage of EdU-positive cells was analyzed using the ImageJ software.

2.10 Statistical analysis

The data are presented as mean ± SEM. Shapiro-Wilk test was used to evaluate the normality of the data distribution before data analysis. Statistical analyses involved Student's t-test for comparing two groups and one-way ANOVA for assessing differences among

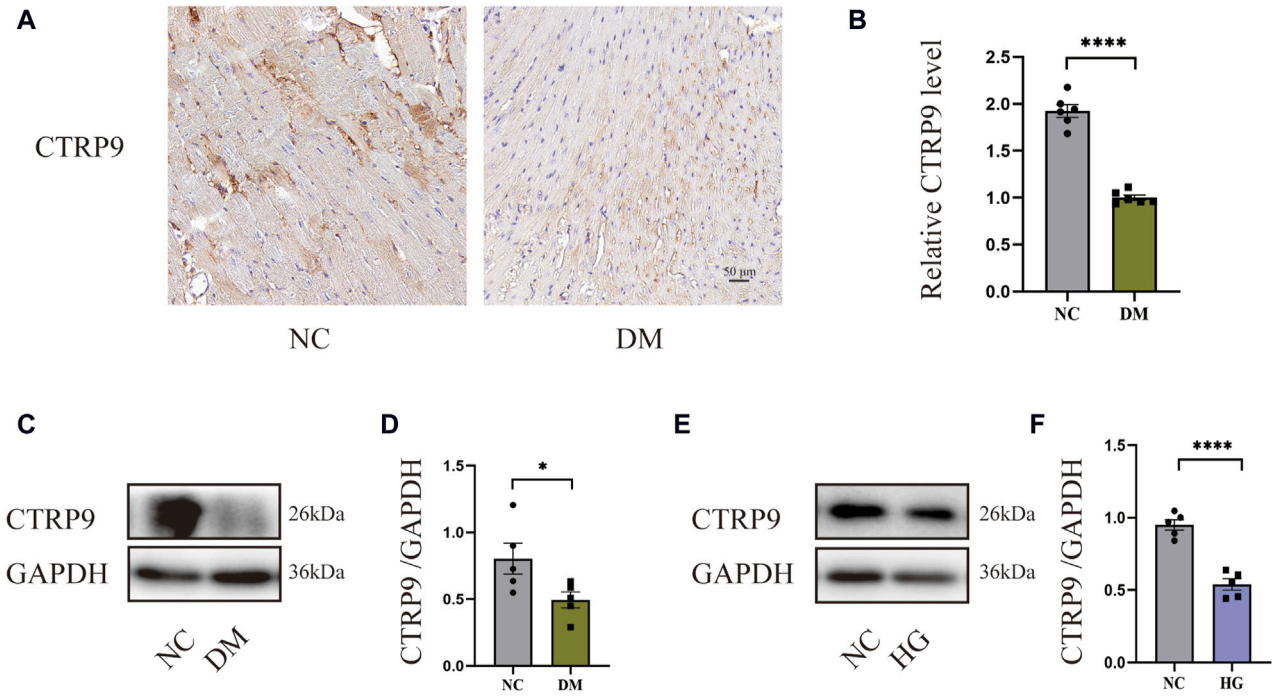

FIGURE 1
CTRP9 expression was diminished within the cardiac tissue of diabetic mice. **(A)** Illustrative immunohistochemistry images of CTRP9 in two groups. **(B)** Assessment of CTRP9 in two groups. **(C)** Illustrative Western blot of CTRP9 in animals. **(D)** Assessment of CTRP9 in two groups. **(E)** Illustrative Western blot of CTRP9 in cardiac fibroblasts. **(F)** Measurement of CTRP9 in two groups. Scale bar = 50 μm. The data are depicted as the mean ± SEM (n = 5–6). *p < 0.05, ****p < 0.0001.

three or more groups. Statistical analysis was performed using GraphPad Prism version 8, with significance defined as $p < 0.05$.

3 Results

3.1 CTRP9 expression was diminished within the cardiac tissue of diabetic mice

To demonstrate the involvement of CTRP9 in cardiac fibrosis, immunohistochemistry and Western blot analysis were conducted. The findings demonstrated a marked decrease in CTRP9 expression within the hearts of diabetic mice (Figures 1A–D). *In vitro*, CTRP9 expression was identified by Western blot following the stimulation of primary cardiac fibroblasts with high glucose (HG). Fibroblast CTRP9 expression was significantly lower in the HG group compared with the group without added high glucose (Figure 1E, F).

3.2 CTRP9 knockout worsened cardiac dysfunction in diabetic mice

To elucidate the influence of CTRP9 deficiency on cardiac function, we performed echocardiograms prior to anesthesia (Figure 2A). In contrast to the NC group, the DM group demonstrated marked impairment in cardiac function, with a notable reduction in LVEF, LVFS, and E/A. These impairments were further accompanied by elevated E/e', increased LVEDD and LVESD (Figure 2B–G). Notably, CTRP9 knockout diabetic mice exhibited greater deterioration in LVEF, LVFS, and E/A. Additionally, they exhibited increased E/e', LVEDD, and LVESD values (Figure 2B–G). Furthermore, diabetic mice in the CTRP9 deficiency group displayed significantly higher blood glucose levels and decreased body weights than those in the DM group (Figure 2H, I). In addition, in the absence of diabetes, there were no notable disparities observed in cardiac function, body weight, or blood glucose levels between the CTRP9 knockout mice and the control mice. These findings validated the successful establishment of the T1DM mouse model and demonstrated that CTRP9 deletion aggravated cardiac dysfunction in diabetic mice.

3.3 CTRP9 knockout exacerbated cardiac fibrosis in diabetic mice

To elucidate the impact of CTRP9 knockout on fibrosis within the myocardium of diabetic mice, we performed pathological staining and immunohistochemistry. Hematoxylin and eosin (H&E) staining showed significant myocardial disarray within the DM group relative to the NC group. The disruption was more severe in CTRP9 knockout mice (Figure 3A). Moreover, Masson's trichrome staining further displayed augmented collagen accumulation within the DM group, which intensified with CTRP9 gene deletion (Figure 3A, B). Immunohistochemical

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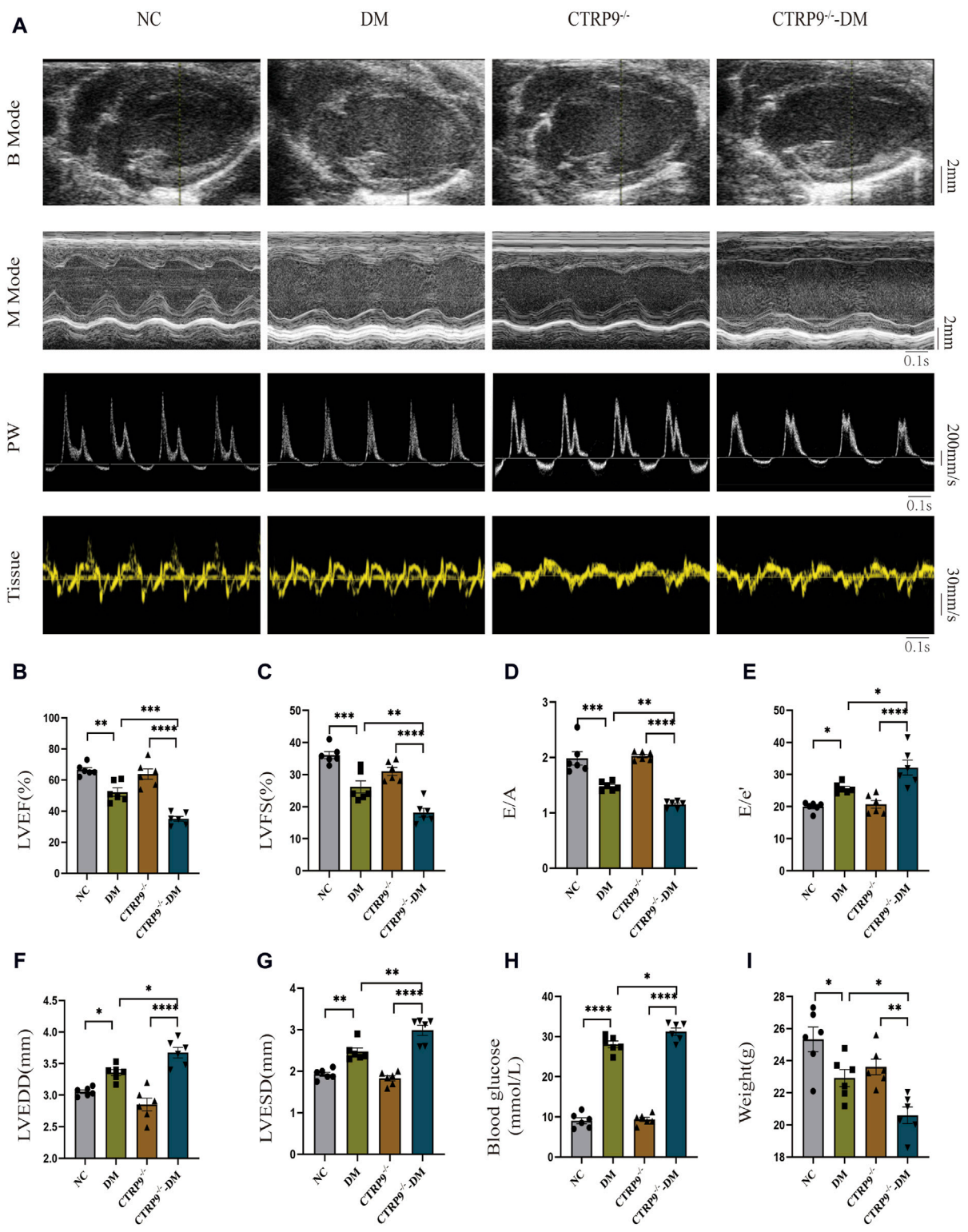

FIGURE 2
CTRP9 knockout worsened cardiac dysfunction in the mice of diabetes. **(A)** Representative B-mode (scale bar in mm), M-mode (scale bar in mm and time stamp in seconds), PW (scale bar in mm/s and time stamp in seconds) and Tissue images (scale bar in mm/s and time stamp in seconds) in four groups. **(B)** Assessment of LVEF in four groups. **(C)** Assessment of LVFS in four groups. **(D)** Assessment of E/A in four groups. **(E)** Assessment of E/e' in four groups. **(F)** Assessment of LVEDD in four groups. **(G)** Assessment of LVESD in four groups. **(H)** Blood glucose in four groups. **(I)** Body weights in four groups. The data are depicted as the mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

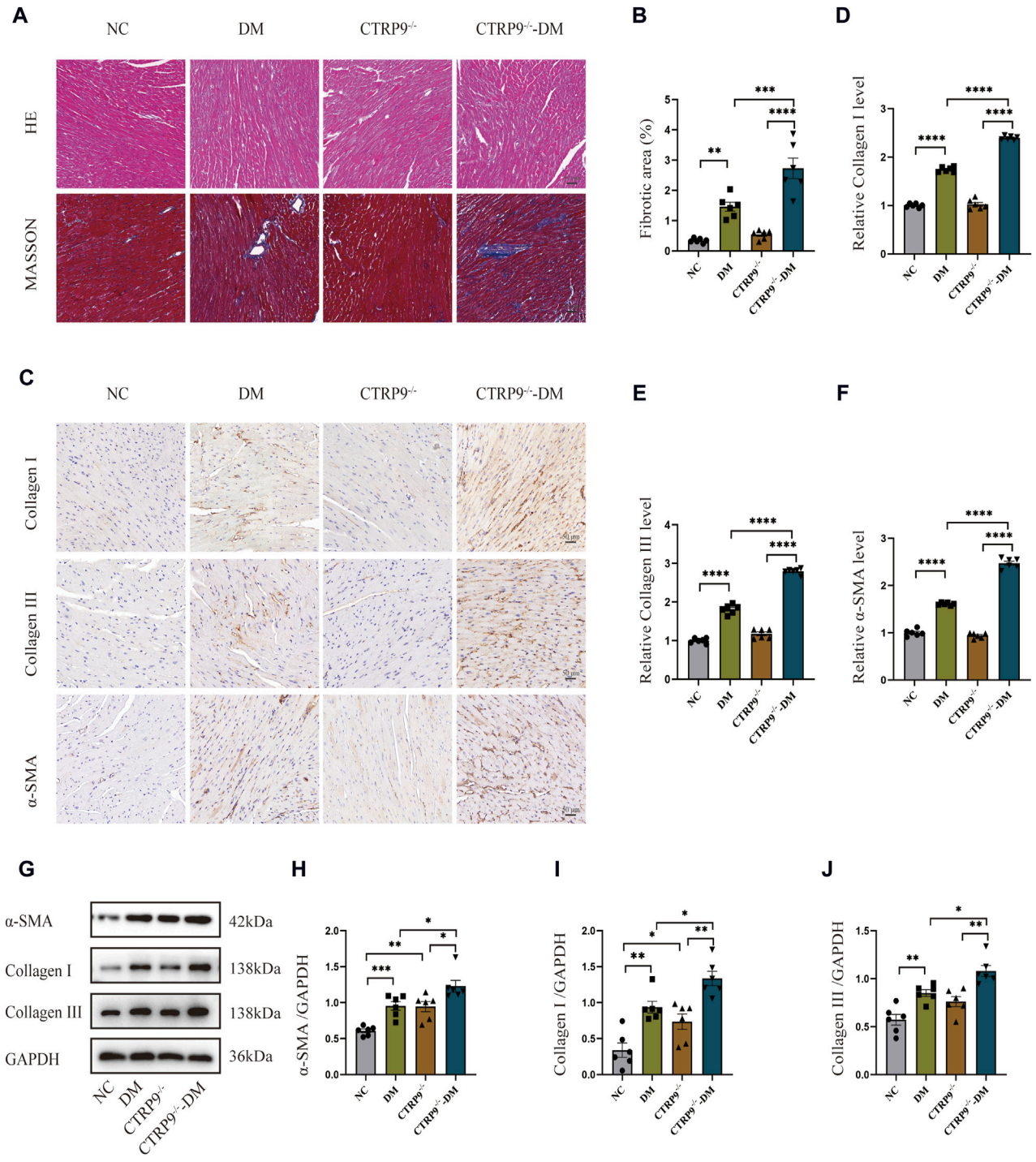

FIGURE 3
CTRP9 deletion worsened cardiac fibrosis. **(A)** Representative images of hematoxylin and eosin (H&E) and Masson's trichrome staining in four groups. Scale bar, 50 μm. **(B)** Evaluation of the fibrotic area within four groups. **(C)** Illustrative immunohistochemistry images of Collagen I, Collagen III, and α-SMA in four groups. **(D)** Evaluation of Collagen I in four group. **(E)** Measurement of Collagen III in four group. **(F)** Relative α-SMA level in four group. **(G)** Representative blot images of α-SMA, Collagen I and Collagen III within animals. **(H)** Assessment of α-SMA in four groups. **(I)** Assessment of Collagen I within four groups. **(J)** Assessment of Collagen III in four groups. The data are depicted as the mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

analysis revealed that collagen I, collagen III, and α-SMA was significantly elevated within the DM group. This increase was even more pronounced in the absence of CTRP9, indicating a heightened fibrotic response (Figure 3C–F). Western blot analysis provided additional support for these findings, showing similar trends in protein expression (Figure 3G–J). Collectively, these results suggested that CTRP9 deletion exacerbates myocardial fibrosis in diabetic mice.

3.4 CTRP9 knockout aggravated autophagy inhibition and upregulated YAP expression in diabetic mice

Previous studies have shown a strong link between suppressed autophagy, activated YAP and the advancement of myocardial fibrosis in DCM (Ikeda et al., 2019; Wang et al., 2022). Building upon this foundation, our study examined the expression levels of p62 and LC3-II, alongside YAP, recognized for its critical involvement in organ fibrosis. Western blot analysis of cardiac tissues demonstrated a dramatic increase of p62 and LC3-II with the DM group relative to the NC group, with CTRP9 deletion exacerbating this trend further (Figure 4A–C). In parallel, YAP protein expression exhibited a significant upregulation in the DM group in comparison to the NC group, a disparity that was further amplified by CTRP9 deletion (Figure 4A, D). Immunohistochemical analysis confirmed the upregulation of YAP in the DM group, especially in the absence of CTRP9 (Figure 4E, F). These findings demonstrated that CTRP9 knockout exacerbated autophagy inhibition and upregulated YAP protein expression in diabetic mice.

3.5 CTRP9 treatment inhibited HG-induced myofibroblast activation

Examining the influence of CTRP9 on fibroblasts activation triggered by HG, we pretreated primary cardiac fibroblasts isolated from mice with exogenous CTRP9, followed by stimulation with a 33.3 mM HG solution. Western blot analysis revealed that after 48 h of HG stimulation, myofibroblast marker α-SMA, as well as collagen level, significantly elevated within the HG group in contrast to both the NC and HO (high osmotic control) groups, whereas treatment with CTRP9 notably reduced the expression levels of these fibrosis markers (Figure 5A–D). Similar results were obtained in the RT-PCR experiment (Supplementary Figures S1A–C). Immunofluorescence staining for α-SMA further confirmed the inhibitory effect of CTRP9 on fibroblast activation (Figure 5E, F). Meanwhile, the results of the cell proliferation assay also showed that CTRP9 treatment caused a significant reduction in the level of HG-induced fibroblast proliferation (Figure 5G, H). These results suggested that CTRP9 treatment significantly inhibited myofibroblasts activation and the extracellular matrix accumulation induced by HG.

3.6 CTRP9 treatment inhibited HG-induced cardiac fibroblast activation by improving autophagy inhibition

This research sought to investigate the influence of autophagy on myofibroblast activation. Western blot analysis revealed significant upregulation of p62 and LC3-II in the HG group, suggesting possible impairment of autophagy. Notably, exogenous CTRP9 administration reduced the expression of these markers, indicating the restoration of autophagic activity (Figure 6A–C). The results of RT-PCR were consistent with the above results (Supplementary Figures S1F–G). Confocal microscopy further revealed a decrease in the number of LC3B puncta upon CTRP9 treatment, indicating a partial reversal of autophagy inhibition under HG conditions (Figure 6D, E). To investigate whether the CTRP9-mediated inhibition of cardiac fibroblast activation was autophagy dependent, cardiac fibroblasts were exposed to CQ, an autophagy inhibitor. Western blot analysis demonstrated a notable elevation of α-SMA, collagen I, and collagen III in the HG + CTRP9+CQ group compared to the HG + CTRP9 group (Figure 6F–I). The same results were obtained in the RT-PCR experiment (Supplementary Figures S2A–C). Immunofluorescence staining also revealed similar results (Figure 6J, K). In addition, the cell proliferation level in HG + CTRP9+CQ group was significantly higher than that in HG + CTRP9 group (Figure 6L, M). These combined results suggest that CTRP9 can suppress fibroblast activation and extracellular matrix secretion through restoring autophagy disrupted by high-glucose conditions.

3.7 CTRP9 treatment inhibited HG-induced myofibroblast activation through the YAP-mediated autophagy pathway

Given that YAP is closely associated with fibrosis (Francisco et al., 2020), we elucidated its role in cardiac fibroblast activation. Western blot revealed notable YAP increase within the high glucose (HG) group relative to both the NC and HO groups, and the addition of exogenous CTRP9 led to a significant suppression of YAP expression (Figure 7A, B). The mRNA change level of YAP was consistent with the protein level (Supplementary Figure S1D). To determine the impact of YAP on autophagy and its potential role in mediating the regulatory effect of CTRP9 on cardiac fibroblast activation, we overexpressed YAP in primary mouse cardiac fibroblasts via plasmid-mediated transfection (Figure 7C–E). We observed that overexpression of YAP eliminated CTRP9-mediated autophagy recovery (Figure 7F–J). The experimental results of RT-PCR also support the above results (Supplementary Figures S3E, F). Additionally, overexpressing YAP abolished the inhibitory effect exerted by CTRP9 toward myofibroblast activation. In the group overexpressing YAP, the levels of fibrosis markers and collagen were significantly elevated compared to the group treated with HG + CTRP9+NT/pcDNA3.1(Figure 7K–N). The results of RT-PCR were consistent with the above results (Supplementary Figures S3A–C). Immunofluorescence staining for α-SMA provided additional confirmation of these effects (Figure 7O–P). Overexpression of YAP significantly increased the proliferation of cardiac fibroblasts treated with high glucose and CTRP9 (Figure 7Q–R). Based on the observations, CTRP9 suppresses high glucose-induced cardiac fibroblast activation and extracellular matrix deposition through YAP-mediated autophagy.

4 Discussion

In this study, we observed that CTRP9 knockout aggravated cardiac fibrosis and dysfunction in diabetic mice, indicating its potential protective role against cardiac complications associated with diabetes. This exacerbation was accompanied by a marked reduction in cardiac autophagy and an increase in YAP protein

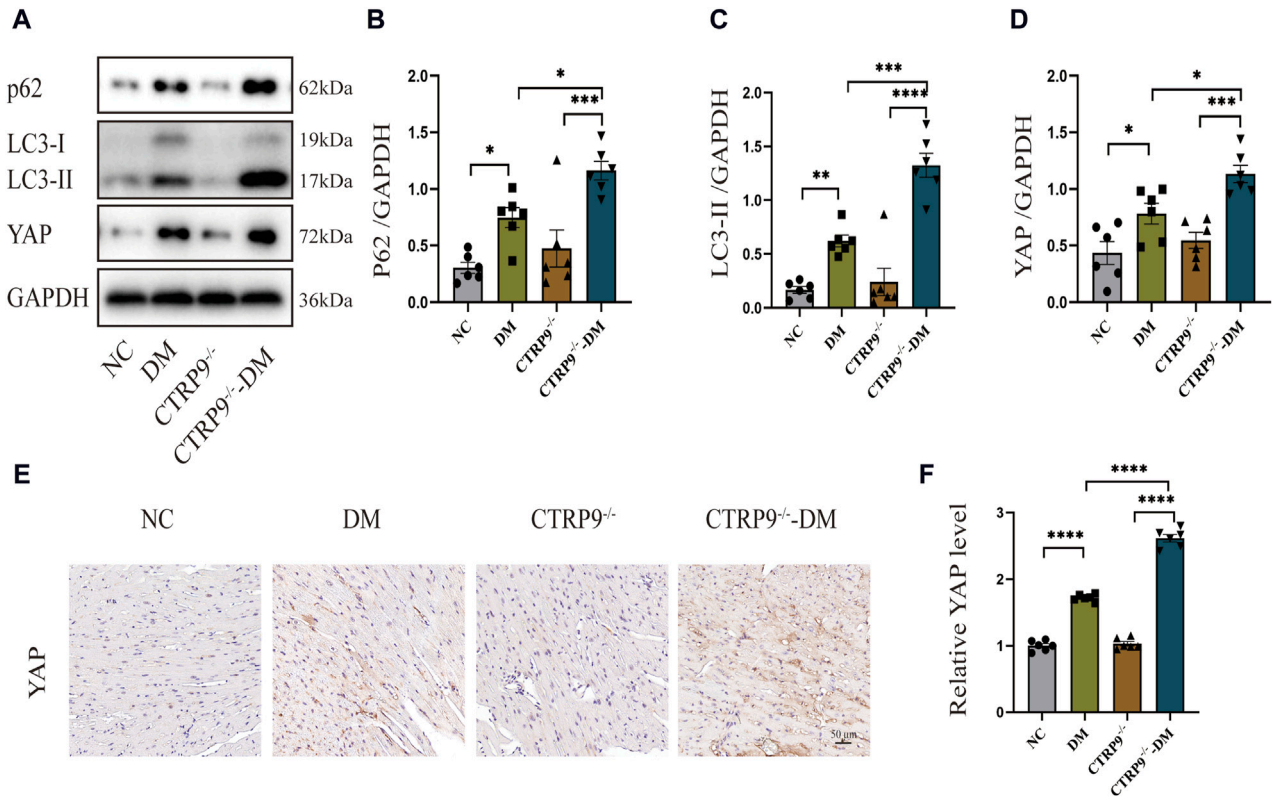

FIGURE 4
CTRP9 knockout aggravated autophagy inhibition and upregulated YAP expression in diabetic heart. **(A)** Illustrative blot images of p62, LC3-II and YAP within four groups. **(B)** Assessment of p62 in four groups. **(C)** Assessment of LC3-II in four groups. **(D)** Assessment of YAP in four groups. **(E)** Illustrative immunohistochemistry images of YAP in four groups. **(F)** Assessment of YAP in four groups. Scale bar, 50 μm. The data are depicted as the mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

levels, suggesting a regulatory imbalance in diabetic conditions due to CTRP9 deficiency (Figure 8). Our *in vitro* experiments further demonstrated that supplementation with CTRP9 attenuated cardiac fibroblast activation and improved their fibrotic profile, primarily by restoring autophagy, which was impaired by HG exposure. Moreover, we observed that CTRP9 supplementation effectively suppressed the expression of YAP, a protein closely linked to the fibrotic process, whereas YAP overexpression counteracted the autophagy-restoring and antifibrotic effects of CTRP9. Together, these findings indicated that CTRP9 mitigated diabetic cardiac fibrosis through the regulation of YAP-mediated autophagy.

Autophagy, a key homeostatic pathway highly conserved in cells for degrading and recycling macromolecules and damaged organelles, serves as an important guardian of quality control in cardiac cells (Dewanjee et al., 2021). In the context of diabetes, however, autophagy often becomes dysfunctional in the heart. (Zang et al., 2020). Previous studies have demonstrated that sustained hyperglycemia impairs cardiomyocyte autophagy, resulting in elevated cardiac collagen deposition and cardiac dysfunction in diabetic mice (Xue et al., 2022). Conversely, Metrnl, by activating the AMPK pathway, has been found to restore suppressed autophagy in the heart, providing a safeguard against DCM (Lu et al., 2023). However, the exact contribution of autophagy to cardiac health in DCM is subject to debate, as contrasting evidence exists. Some findings suggest that certain diabetic hearts exhibit autophagic hyperactivation, which has been shown to have a detrimental effect on the heart (Guo et al., 2020; Jiang et al., 2022). Such disparate conclusions could arise from differences in diabetes types, stages, severity, and concurrent medical conditions across studies. In our current work, we developed a T1DM mouse model using successive low-dose intraperitoneal injections of STZ. Our observations revealed markedly reduced autophagic activity in the hearts of mice with diabetes, which was further exacerbated by CTRP9 knockout. This finding was supported by *in vitro* evidence indicating that CTRP9 pretreatment can mitigate HG-induced autophagy inhibition in cardiac fibroblasts and improve their fibrotic state. In contrast, autophagy suppression by CQ counteracted the beneficial effects of CTRP9. These findings elucidated the critical role of inhibited autophagy in diabetic myocardial fibrosis and suggested that autophagy restoration by CTRP9 represents a promising therapeutic avenue for DCM.

YAP, a pivotal constituent within the Hippo pathway, subjects to negative regulation within this pathway (Ibar and Irvine, 2020). Stimulating the Hippo pathway prompts kinases such as mammalian sterile 20-like protein kinase 1/2 (Mst1/2) and large tumor suppressor (Lats1/2) to phosphorylate YAP, causing its sequestration in the cytoplasm and subsequent degradation (Choi et al., 2024). During Hippo pathway inactivation, YAP *undergoes* dephosphorylation and translocates to the nucleus, where it activates genes that drive its biological roles (Kiang et al., 2024). Elevated YAP

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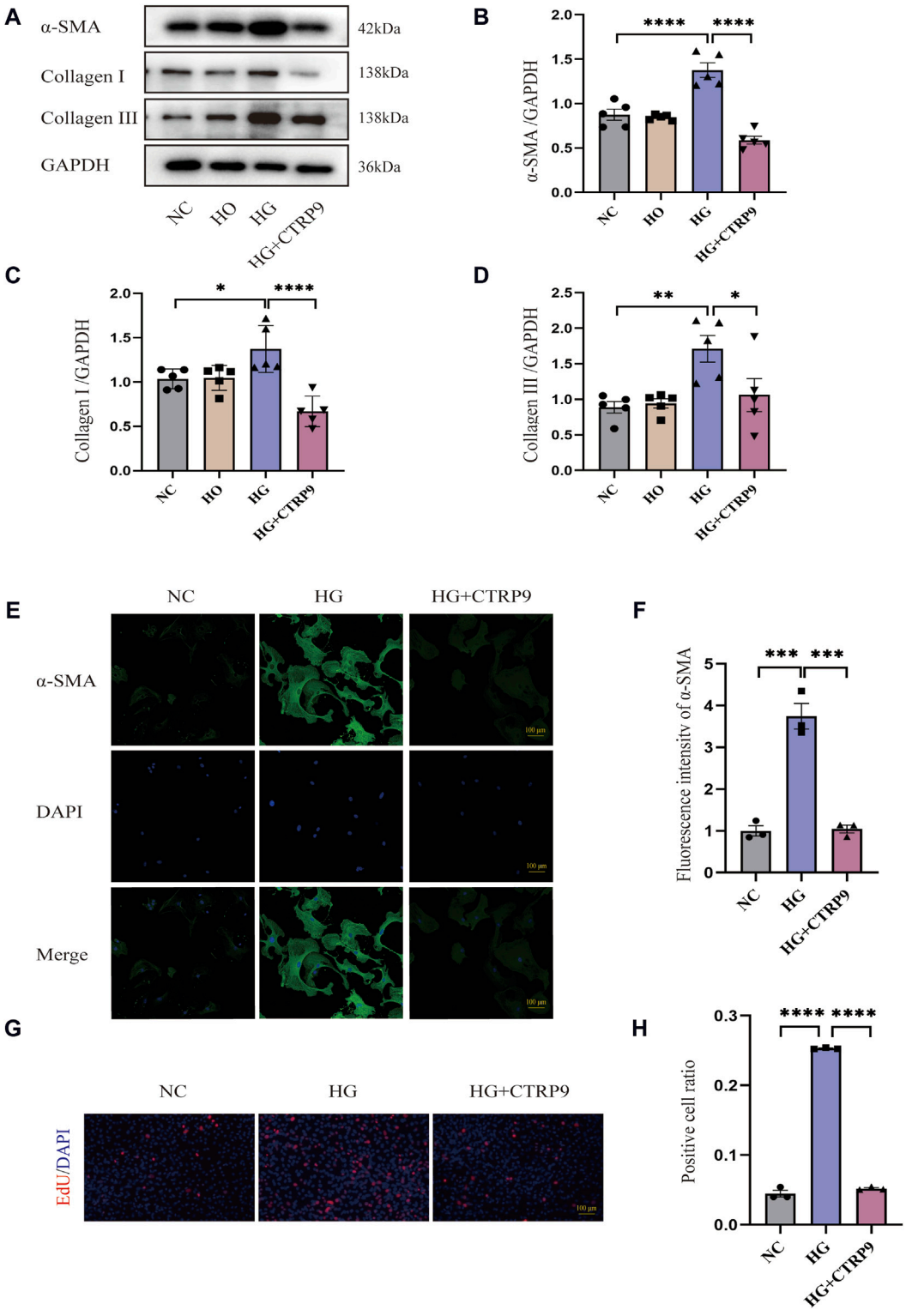

FIGURE 5
CTRP9 treatment inhibited HG-induced cardiac fibroblast activation. **(A)** Illustrative blot images of α-SMA, Collagen I and Collagen III within four groups. **(B)** Assessment of α-SMA in four groups. **(C)** Assessment of Collagen I within four groups. **(D)** Assessment of Collagen III in four groups. **(E)** Illustrative immunofluorescence images of α-SMA in three groups. **(F)**. Assessment fluorescence intensity of α-SMA in three groups. **(G)** Representative images of EdU detecting cell proliferation in three groups. **(H)** Quantification of the proportion of EdU positive cells. Scale bar, 100 μm. The data are depicted as the mean ± SEM (n = 3–5). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

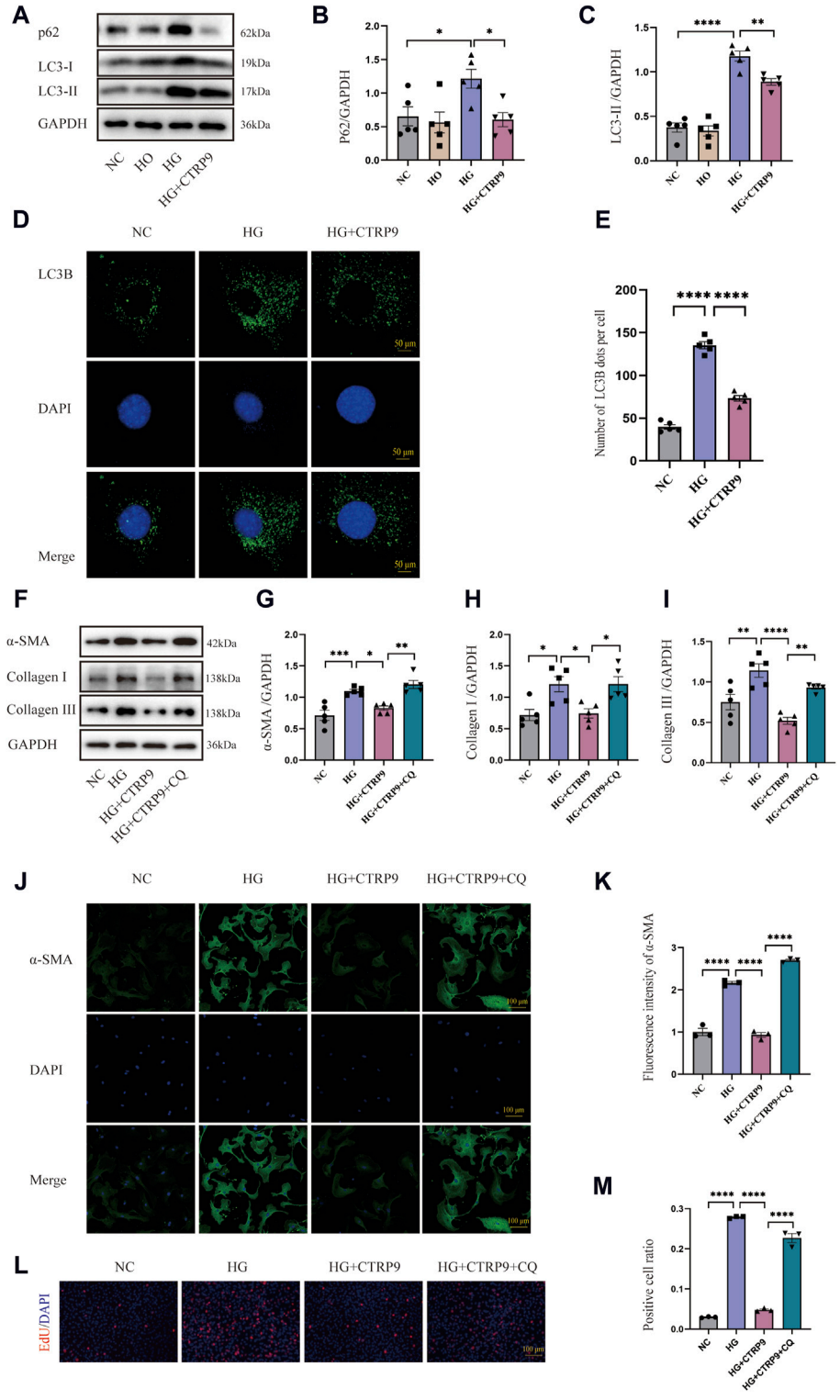

FIGURE 6
CTRP9 treatment inhibited myofibroblast activation induced by HG through improving autophagy inhibition. **(A)** Illustrative Western blot of p62 and LC3-II within four groups of cells. **(B)** Assessment of p62 in four groups. **(C)** Assessment of LC3-II in four groups. **(D)** Representative confocal microscopy images of LC3B in three groups of cells. Scale bar, 50 μm. **(E)** Quantification of LC3B puncta in three groups. **(F)** Illustrative Western blot of α-SMA, Collagen I and Collagen III within four groups of cells. **(G)** Assessment of α-SMA in four groups. **(H)** Assessment of Collagen I within four groups. **(I)** Assessment of Collagen III in four groups. **(J)** Representative immunofluorescence images of α-SMA in four groups of cells. Scale bar, 100 μm. **(K)** Quantification of fluorescence intensity of α-SMA in four groups. **(L)** Representative images of EdU detecting cell proliferation in four groups. **(M)** Quantification of the proportion of EdU positive cells. The data are depicted as the mean ± SEM (n = 3–5). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

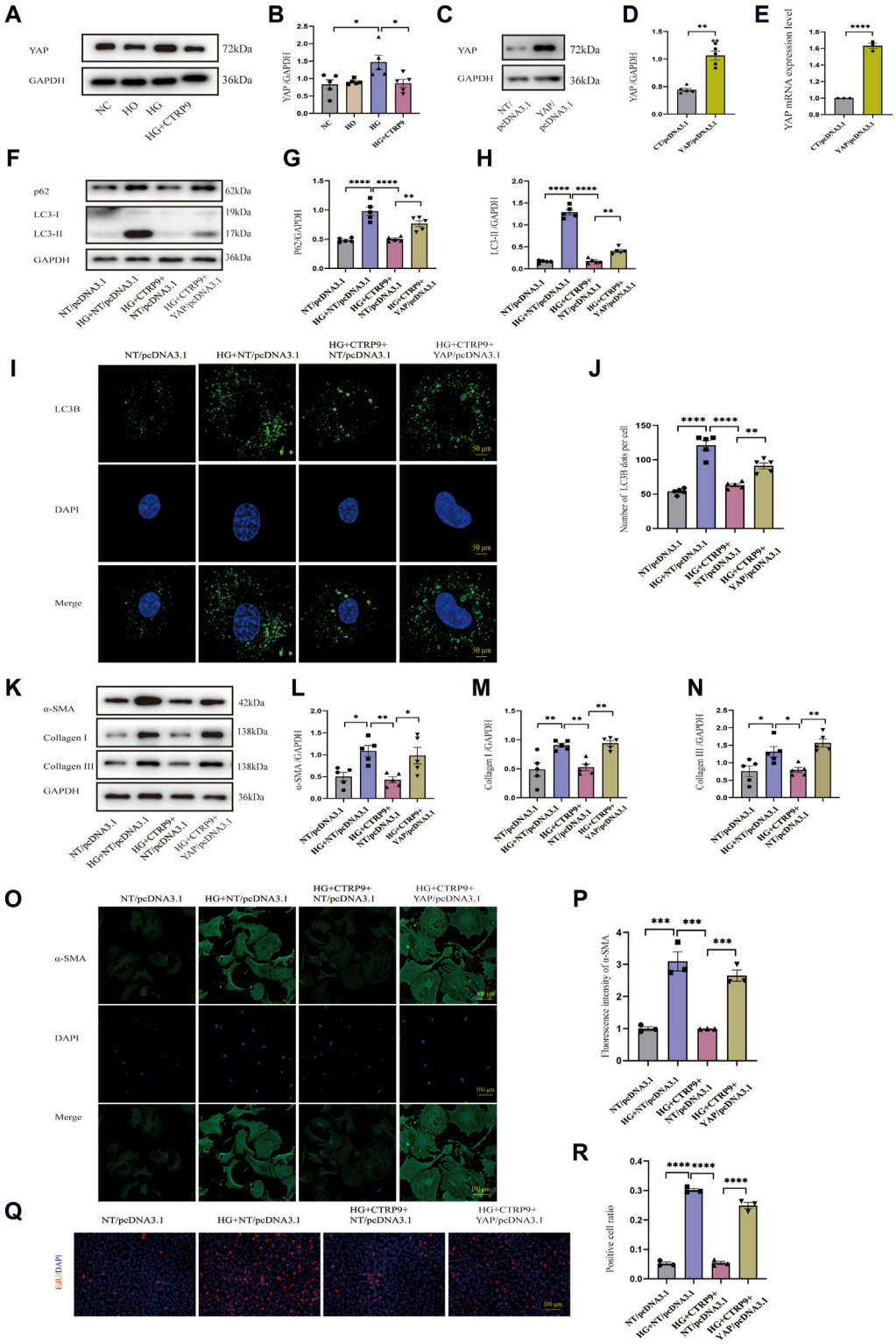

FIGURE 7
CTRP9 treatment inhibited HG-induced activation through the YAP-mediated autophagy pathway. **(A)** Illustrative Western blot of YAP in four groups of cells. **(B)** Assessment of YAP in four groups. **(C, D)** Illustrative Western blot images of YAP overexpression and their quantification in two groups. **(E)** Quantification YAP mRNA expression level in two groups. **(F)** Illustrative Western blot of p62 and LC3-II with four groups. **(G)** Assessment of p62 in four groups. **(H)** Assessment of LC3-II in four groups. **(I)** Representative confocal microscopy images of LC3B in four groups of cells. Scale bar, 50 μm. **(J)** Quantification of LC3B puncta in four groups. **(K)** Illustrative Western blot of α-SMA, Collagen I and Collagen III within four groups of cells. **(L)** Assessment of α-SMA in four groups. **(M)** Assessment of Collagen I within four groups. **(N)** Assessment of Collagen III in four groups. **(O)** Representative immunofluorescence images of α-SMA in four groups of cells. Scale bar, 100 μm. **(P)** Quantification of fluorescence intensity of α-SMA in four groups. **(Q)** Representative images of EdU detecting cell proliferation in four groups. Scale bar, 100 μm. **(R)** Quantification of the proportion of EdU positive cells. The data are presented as the mean ± SEM (n = 3–5). *p < 0.05, **p < 0.01, ***p < 0.001, ****P < 0.0001.

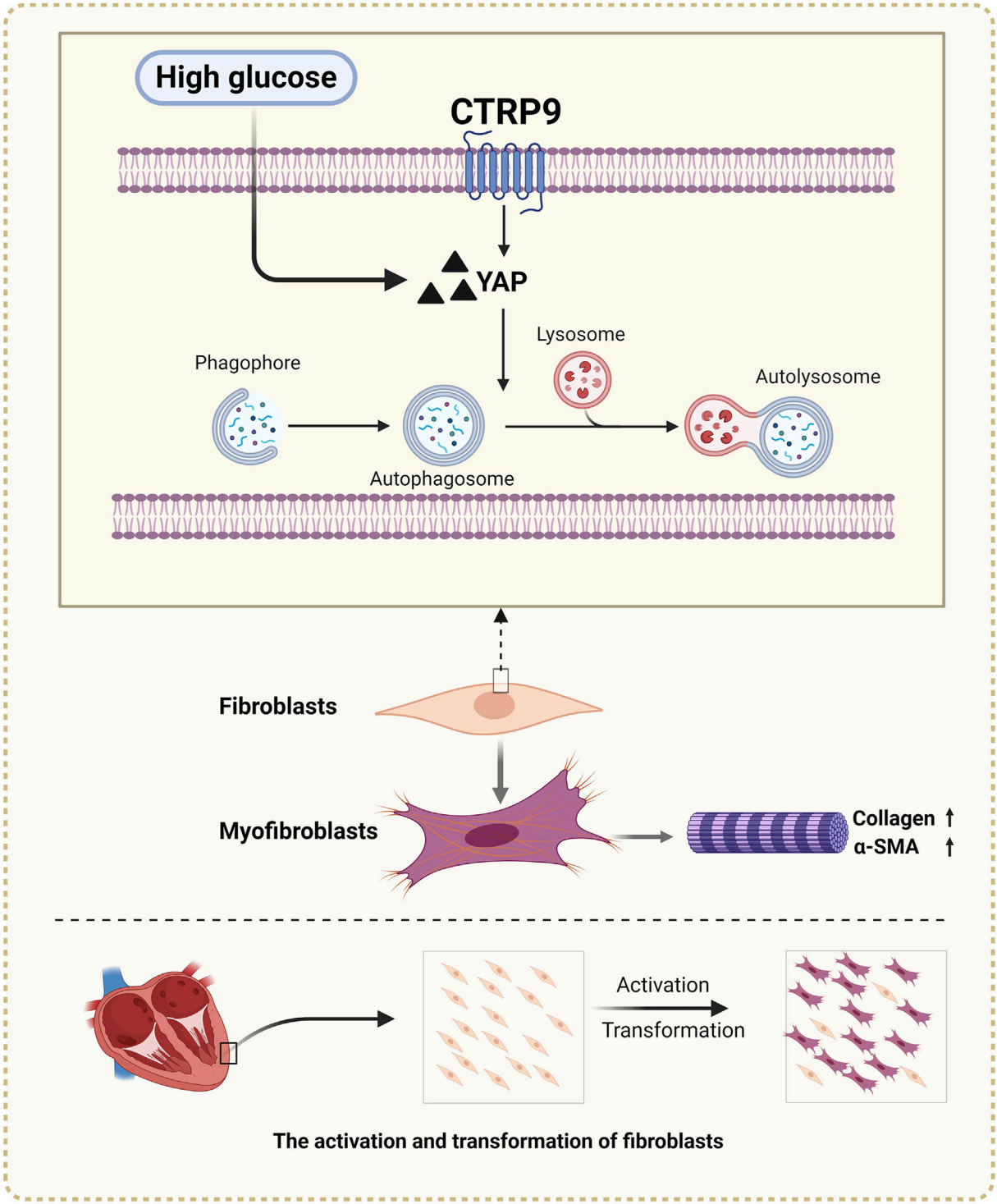

FIGURE 8
Diagram of CTRP9 attenuating cardiac fibrosis in diabetic mice though affecting YAP-mediated autophagy pathway. Created with BioRender.com.

expression is a common feature of fibrosis in various organs, including pulmonary, hepatic, and renal fibrosis, and has been increasingly associated with cardiac pathologies (Stancil et al., 2021; Zhang J. et al., 2023; Chitturi et al., 2023). YAP activation results in myocardial hypertrophy and fibrosis (Garoffolo et al., 2022; Kashihara et al., 2022). Moreover, YAP is activated in resident cardiac fibroblasts postmyocardial infarction, leading to adverse remodeling and even heart failure (Mia et al., 2022). Our study showed that absence of the CTRP9 gene resulted in increased YAP expression within the diabetic heart. *In vitro*, the addition of CTRP9 to cardiac fibroblasts inhibited the HG-induced upregulation of YAP. To further elucidate the impact of YAP

and CTRP9 on ameliorating HG-induced cardiac fibroblast activation, we overexpressed YAP with plasmids to counteract the inhibitory effect of CTRP9 on HG-triggered cardiac fibroblast transdifferentiation and collagen secretion. Furthermore, growing evidence suggests that YAP may regulate autophagy levels as an upstream mechanism. Activation of YAP can lead to autophagy inhibition (Claude-Taupin et al., 2023; Wu et al., 2024). However, there is also evidence that autophagy controls YAP expression. When autophagy is active, YAP interacts with the receptor protein p62 of the autophagy pathway and is degraded by autophagic lysosomes (Hao et al., 2024). Our *in vitro* experiments showed that overexpression of YAP counteracted the autophagy restoring effect of CTRP9.

However, our study is not without its limitations. First, we established an STZ-induced T1DM mouse model, as T1DM mice develop both diastolic and systolic dysfunction, which closely resemble the cardiac impairments observed in clinical diabetic patients. Type 2 diabetes mellitus primarily presents with diastolic dysfunction, and creating type 2 diabetic animal models necessitates more intricate environmental interventions, such as high-fat diets and a lack of physical activity, potentially leading to highly variable experimental outcomes. Second, while the current literature acknowledges the multifaceted interaction between YAP and autophagy, our research did not explore this relationship in depth. Delving into the nuanced interplay between YAP and autophagy is a primary aim of subsequent investigations.

In conclusion, we demonstrated that CTRP9 knockout exacerbated diabetic myocardial fibrosis by inhibiting autophagy and upregulating YAP expression. Our investigation provides valuable mechanistic understanding regarding the therapeutic implications of CTRP9 in diabetic myocardial fibrosis, laying a foundation for the advancement of novel CTRP9-based pharmaceutical interventions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by The Ethics Committee of Qilu hospital of Shandong University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SR: Conceptualization, Methodology, Writing–original draft. JL: Conceptualization, Writing–original draft. SL: Writing–review and editing. SZ: Formal Analysis, Validation, Writing–original draft. DX: Data curation, Writing–review and editing. AZ: Supervision, Writing–review and editing. LL: Software, Writing–original draft. YG: Funding acquisition, Project administration, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1407883/full#supplementary-material

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Emerging roles of non-coding RNAs in fibroblast to myofibroblast transition and fibrotic diseases

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The transition of fibroblasts to myofibroblasts (FMT) represents a pivotal process in wound healing, tissue repair, and fibrotic diseases. This intricate transformation involves dynamic changes in cellular morphology, gene expression, and extracellular matrix remodeling. While extensively studied at the molecular level, recent research has illuminated the regulatory roles of non-coding RNAs (ncRNAs) in orchestrating FMT. This review explores the emerging roles of ncRNAs, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), in regulating this intricate process. NcRNAs interface with key signaling pathways, transcription factors, and epigenetic mechanisms to fine-tune gene expression during FMT. Their functions are critical in maintaining tissue homeostasis, and disruptions in these regulatory networks have been linked to pathological fibrosis across various tissues. Understanding the dynamic roles of ncRNAs in FMT bears therapeutic promise. Targeting specific ncRNAs holds potential to mitigate exaggerated myofibroblast activation and tissue fibrosis. However, challenges in delivery and specificity of ncRNA-based therapies remain. In summary, ncRNAs emerge as integral regulators in the symphony of FMT, orchestrating the balance between quiescent fibroblasts and activated myofibroblasts. As research advances, these ncRNAs appear to be prospects for innovative therapeutic strategies, offering hope in taming the complexities of fibrosis and restoring tissue equilibrium.

KEYWORDS

non-coding RNAs, fibroblast, myofibroblast, fibrosis, therapies

1 Introduction

Fibroblast to myofibroblast transition (FMT) is a fundamental process that holds immense significance in various physiological contexts such as wound healing, tissue repair, and the pathogenesis of fibrotic diseases (Zhang et al., 2016; Usher et al., 2019; Blessing et al., 2021; Wang et al., 2023). This intricate transition is characterized by profound changes in cellular phenotype, encompassing alterations in cellular morphology, gene expression profiles, and the synthesis of extracellular matrix components (Michalik et al., 2018). These modifications collectively culminate in substantial tissue remodeling, which is essential for restoring tissue integrity and function following injury or damage (D'Urso and Kurniawan, 2020).

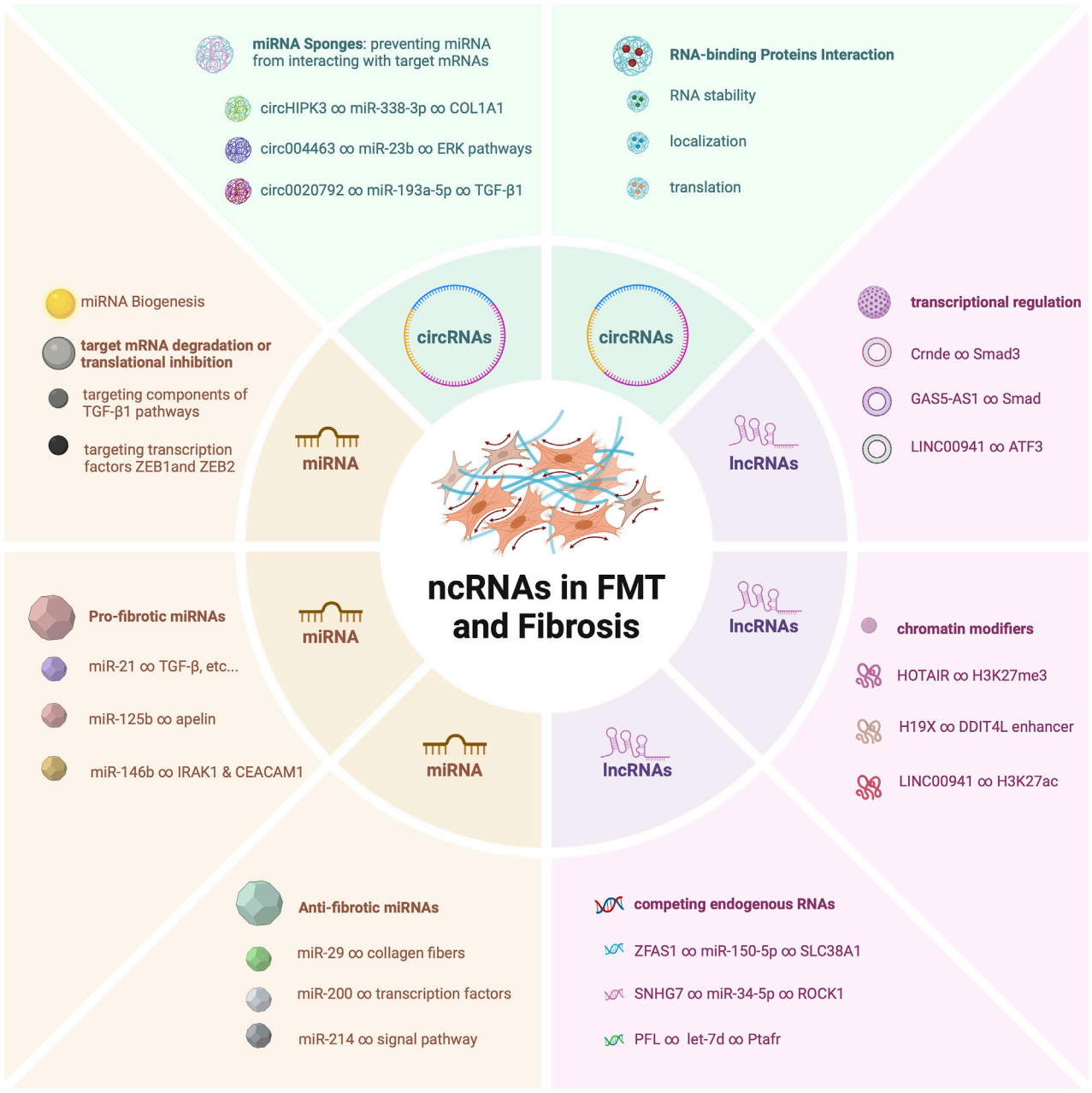

FIGURE 1
Mechanistic Roles of ncRNAs in Fibroblast-Myofibroblast Transition and Fibrosis.

The process of FMT can be broadly divided into four stages (Wynn and Ramalingam, 2012). Initially, quiescent fibroblasts are activated in response to injury or stress signals, leading them to start proliferating. Following this, activated fibroblasts differentiate into myofibroblasts, characterized by the expression of alpha-smooth muscle actin (α-SMA) and increased production of extracellular matrix (ECM) components. Myofibroblasts then play a crucial role in extracellular matrix remodeling, depositing collagen and other ECM proteins to repair tissue. Normally, myofibroblasts undergo apoptosis once the tissue is repaired. However, in pathological conditions, myofibroblasts persist, leading to fibrosis. Several key signaling pathways regulate FMT (Zhang et al., 2023), including the Transforming Growth Factor-beta (TGF-β) pathway, which is a major driver of FMT, promoting myofibroblast differentiation and ECM production. The MAPK pathway is involved in fibroblast activation and differentiation, while the PI3K/Akt pathway plays a role in cell survival and proliferation during FMT.

The exploration of the molecular intricacies governing FMT has been a subject of extensive research, driven by the imperative to comprehend the underlying mechanisms that drive tissue repair and fibrosis (Li et al., 2015). In this context, recent scientific exploration has evaluated the pivotal role of non-coding RNAs (ncRNAs) as indispensable orchestrators of the FMT process (Zhang et al., 2023). Traditionally overlooked due to their lack of protein-coding capacity

(Ilieva and Uchida, 2022), ncRNAs are now recognized as key players in shaping the delicate equilibrium between quiescent fibroblasts and their activated myofibroblast counterparts during FMT (Creemers and van Rooij, 2016).

The ensemble of ncRNAs, including microRNAs (miRNAs) (Lu and Rothenberg, 2018), long non-coding RNAs (lncRNAs) (Fernandes et al., 2019), and circular RNAs (circRNAs) (Li et al., 2018a), showcases a multifaceted array of regulatory molecules that converge to finely tune the transition from fibroblasts to myofibroblasts (Wang et al., 2014; Fan et al., 2021; Su et al., 2021). This cascade of molecular events encompasses miRNAs that function as fine-tuners (Miao et al., 2018), lncRNAs that orchestrate complex gene expression networks (Dong et al., 2022), and circRNAs that act as dynamic sponges and orchestrators of intricate interactions (Yang et al., 2022). These ncRNAs are far from being bystanders; rather, they intricately interweave with signaling pathways, transcription factors, and epigenetic modulators to steer the gene expression programs that govern FMT (Zhou et al., 2018; Niu et al., 2022; Hertig et al., 2023).

The pivotal roles of these ncRNAs do not exist in isolation (Figure 1). Rather, they synergistically contribute to a complex regulatory network that dictates the fine balance between fibroblast quiescence and myofibroblast activation (Wang et al., 2020). Dysregulation of these ncRNAs has been found to be a common thread linking to the development of pathological fibrosis across diverse tissues (Tao et al., 2016; Tarbit et al., 2019; Senavirathna et al., 2020). Their dysregulated expression levels or altered interactions can have profound implications, leading to exaggerated myofibroblast activation (Wasson et al., 2020a), excessive extracellular matrix deposition (Zhang et al., 2018), and ultimately tissue fibrosis (Lino Cardenas et al., 2013).

In this review, our primary emphasis will be on elucidating the involvement of ncRNAs in both FMT process and fibrotic diseases, highlighting their significant therapeutic promise. Insights into their roles not only deepen our comprehension of fibrotic processes but also offer potential avenues for therapeutic interventions aimed at mitigating the excessive activation of myofibroblasts and inhibiting the progression of fibrosis.

2 MicroRNAs (miRNAs) in FMT

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules, typically about 22 nucleotides in length, that play crucial roles in post-transcriptional gene regulation. MiRNAs exert their regulatory effects by binding to the 3'untranslated region (UTR) of target messenger RNAs (mRNAs), leading to mRNA degradation or translational repression. In the context of fibrotic diseases, miRNAs are significantly altered (Selman et al., 2016). Emerging evidence highlights the substantial impact of miRNAs in modulating FMT dynamics. MiRNAs play intricate roles in both promoting and inhibiting FMT, making them key regulators of this transition.

Several miRNAs have been identified as promoters of FMT by targeting key regulators of the transition. Notably, miR-21 has emerged as a potent inducer of FMT (Liu et al., 2010; Yao et al., 2011; Liang et al., 2012; Wang et al., 2012; Bullock et al., 2013; Glowacki et al., 2013; Gong et al., 2014; Hedbäck et al., 2014; Lorenzen et al., 2015; Cui et al., 2018; Xu et al., 2018; Li et al., 2019; Kilari et al., 2019; Schipper et al., 2020; Wang et al., 2021; Nonaka et al., 2021; Ramanujam et al., 2021; Yang et al., 2021; Liao et al., 2022) (Figure 2). Its impact on FMT is primarily mediated through its ability to regulate the transforming growth factor-beta (TGF-β) signaling pathway (Liu et al., 2010; Yao et al., 2011; Liang et al., 2012; Cui et al., 2018; Nonaka et al., 2021; Yang et al., 2021). TGF-β is a pivotal cytokine that plays a central role in fibrotic processes (Fernandez and Eickelberg, 2012; Yousefi et al., 2020). MiR-21 achieves this regulatory effect by targeting TGF-β receptor inhibitors, leading to their downregulation. This downregulation results in an increased responsiveness of fibroblasts to TGF-β signaling, effectively priming them for myofibroblast differentiation. MiR-21 also promotes the expression of various extracellular matrix (ECM) components, such as collagens (Liang et al., 2012; Cui et al., 2018; Nonaka et al., 2021) and fibronectin (Cui et al., 2018), thereby contributing to the phenotypic shift of fibroblasts into myofibroblasts. This induction of ECM components strengthens the fibrotic matrix, leading to tissue remodeling and fibrosis development. Additionally, miR-146b have been shown to facilitate FMT by targeting interleukin 1 receptor-associated kinase 1 (IRAK1) and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) that inhibit myofibroblast activation (Liao et al., 2021). The research revealed that miR-146b led to increased proliferation and migration of fibroblasts, the conversion of fibroblasts into myofibroblasts, and disrupted signaling among macrophages. Likewise, miR-125b contributes to FMT by downregulating apelin that would otherwise repress the activation of fibroblasts into myofibroblasts (Nagpal et al., 2016). This downregulation effectively removes barriers that restrain the transition process, resulting in enhanced myofibroblast formation. Collectively, these miRNAs exemplify the intricate regulatory landscape of FMT. Their effects extend beyond singular pathways, intertwining with the TGF-β, WNT, and PI3K/AKT signaling pathways (Zhang et al., 2023), ultimately driving the progression of fibrosis.

Conversely, certain miRNAs act as suppressors of FMT. These miRNAs play a crucial role in counteracting the signals and factors that drive fibroblasts towards myofibroblast differentiation, ultimately contributing to the maintenance of tissue homeostasis and preventing excessive fibrosis. The miR-29 family stands out as a group of miRNAs that counteract FMT by targeting collagen synthesis and deposition, essential processes in fibrosis (Kwiecinski et al., 2011; Wang et al., 2021; Yu et al., 2021; Yang et al., 2022; Xi et al., 2023). miR-29 directly targets and downregulates the expression of various collagens, including collagen type I (Yu et al., 2021), III (Wang et al., 2019), and IV (Kwiecinski et al., 2011), as well as other extracellular matrix components (Zhang et al., 2023). This regulatory mechanism orchestrated by miR-29 efficiently dampens the excessive accumulation of collagen fibers, which is a hallmark of fibrotic tissue remodeling. By inhibiting collagen production, miR-29 acts as a protection against the pathological transformation of fibroblasts into myofibroblasts, thus preventing the progression of fibrosis. Moreover, the miR-200 family members counteract FMT by targeting transcription factors ZEB1 (Bhome et al., 2022) and ZEB2 (Liao et al., 2018), which are integral to the epithelial-mesenchymal transition. By inhibiting ZEB1 and ZEB2 expression, miR-200 miRNAs effectively impede the

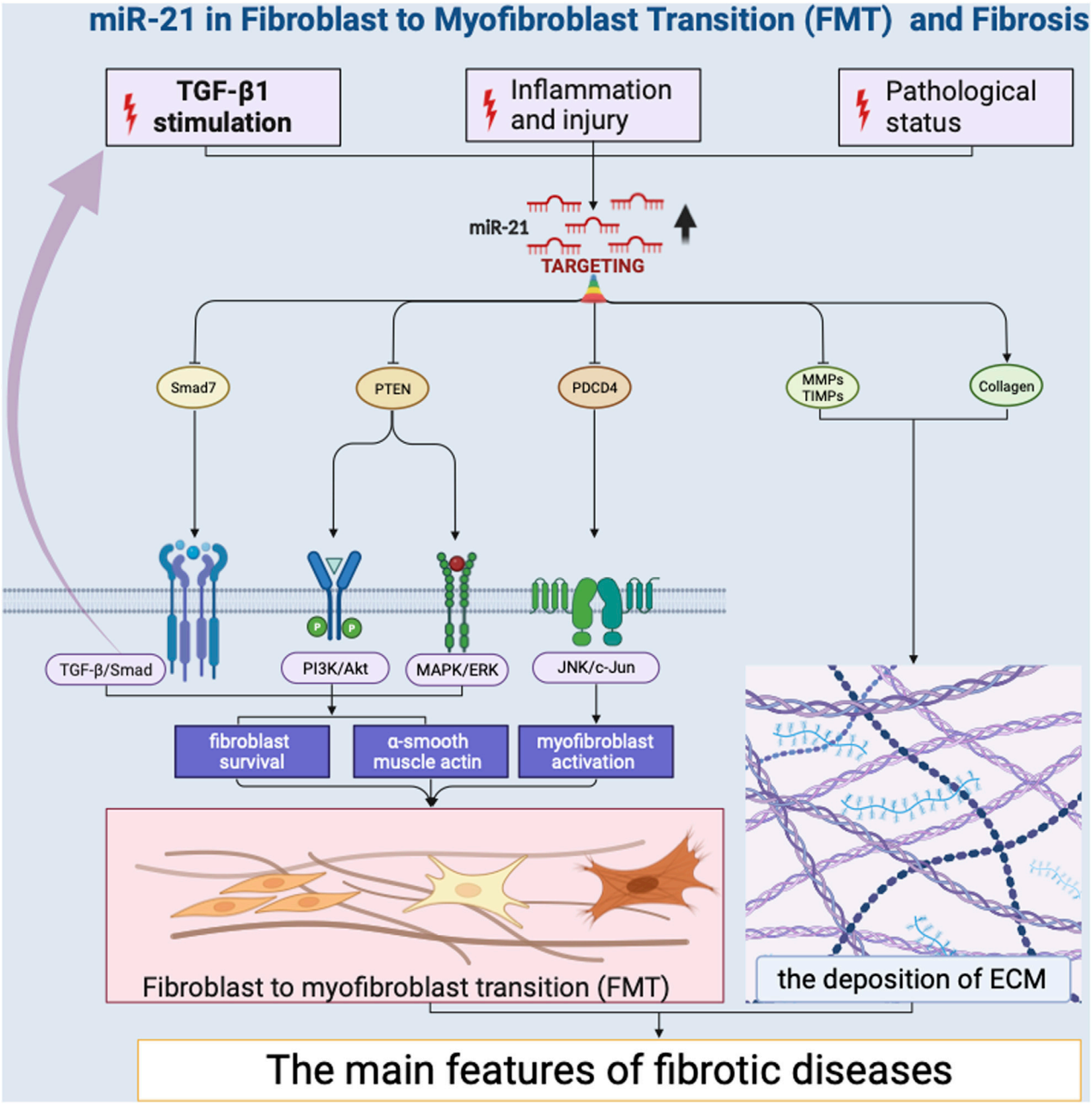

FIGURE 2
miR-21 in Fibroblast to Myofibroblast Transition (FMT) and Fibrosis.

transition of fibroblasts into myofibroblasts, contributing to the maintenance of the fibroblast phenotype and preventing fibrotic tissue remodeling. Similarly, miR-214 plays a role in inhibiting FMT by targeting factors that repress the activation of myofibroblasts (Izawa et al., 2015; Zhu et al., 2016; Yang et al., 2019). By suppressing these inhibitory elements, miR-214 helps tilt the balance in favor of myofibroblast differentiation. In brief, the balanced interplay between miRNAs that promote and those that suppress fibroblast to myofibroblast transition is crucial for maintaining tissue integrity and preventing pathological fibrosis. The opposing actions of these miRNAs create a finely tuned regulatory network that governs the dynamic equilibrium between fibroblasts and myofibroblasts.

In fibrotic conditions, miRNAs undergo various modifications that affect their expression and function. These modifications include changes in miRNA transcription, processing, and stability. Fibrotic signals such as TGF-β can induce or repress the transcription of specific miRNAs (Selman et al., 2016). Additionally, alterations in miRNA processing enzymes, such as Drosha and Dicer, can impact miRNA maturation and stability (Mishra et al., 2009; Cho et al., 2020). Epigenetic modifications, including DNA methylation and histone modifications, also play a role in regulating miRNA expression in fibrotic tissues (Yang et al., 2015). These mechanisms collectively contribute to the dysregulation of miRNAs in fibrosis, influencing their ability to modulate gene expression during FMT.

The regulatory roles of miRNAs in FMT are far from linear, as many miRNAs participate in intricate regulatory networks. MiRNAs often target multiple genes and pathways simultaneously, influencing the balance between pro-fibrotic and anti-fibrotic processes. This phenomenon allows miRNAs to fine-tune the overall outcome of FMT by modulating the expression of various genes that are involved in different stages of the transition. One key feature of miRNA-mediated regulation in FMT is the concurrent targeting of multiple genes within the same or related signaling pathways (Kwiecinski et al., 2011; Gong et al., 2014; Lorenzen et al., 2015; Nagpal et al., 2016; Wang et al., 2021; Medzikovic et al., 2023). This results in a synergistic impact on the cellular processes associated with FMT. This multi-targeting capacity enables miRNAs to exert a more potent and coordinated influence on FMT compared to a linear one-to-one relationship between miRNA and target gene. Cross-talk between miRNAs and other non-coding RNAs, such as lncRNAs(Li et al., 2018b; Wang et al., 2019) and circRNAs(Zhang et al., 2020; Ma et al., 2023), further complicates the regulatory landscape. The interplay between miRNAs, target genes, and other ncRNAs collectively constitutes a systems-level regulatory network that governs FMT. This network-based perspective highlights the interconnectedness and interdependence of various components in shaping the outcome of FMT.

MiRNAs play pivotal roles in orchestrating fibroblast to myofibroblast transition. Their dual nature as promoters and inhibitors of FMT underscores their complex regulatory functions in fibrosis. As our understanding of the roles of miRNAs in FMT continues to evolve, the prospects for innovative therapeutic strategies in fibrotic diseases become increasingly promising. The ability to manipulate miRNAs to finely tune the fibrotic response offers a level of precision that was previously unimaginable.

3 Long non-coding RNAs (lncRNAs) in FMT

Long Non-Coding RNAs (lncRNAs) constitute a diverse group of RNA molecules exceeding 200 nucleotides in length that lack protein-coding capacity but exert critical regulatory roles across various cellular processes. Within the intricate processes of FMT, a recent focus has emerged on lncRNAs as key regulatory elements. These lncRNAs establish their presence within the framework of FMT by orchestrating complex molecular interactions. They serve as regulators, directing the delicate interplay among chromatin modifiers, transcription factors, and a competing endogenous RNA (ceRNA) that govern gene expression patterns critical to FMT. These orchestrated activities assume a crucial role in the transformation of fibroblasts into myofibroblasts, a pivotal event in the development of tissue fibrosis. A diverse group of lncRNAs, including notable examples such as MALAT1 (Wu et al., 2015), H19X (Pachera et al., 2020), ZFAS1 (Yang et al., 2020), and SAFE (Hao et al., 2019), have garnered attention for their role as promoters of myofibroblast differentiation. Their contributions add a novel layer of regulatory intricacy to the evolving narrative surrounding FMT.

lncRNAs have emerged as key regulators of chromatin remodeling in the process of myofibroblast differentiation. These lncRNAs act as guides, directing chromatin modifiers to specific genomic loci that are strategically poised to undergo transformation. Through their interaction with chromatin-modifying complexes, these lncRNAs initiate a cascade of epigenetic changes that play a central role in the activation of genes critical for FMT. For example, HOTAIR and H19X have important effects on chromatin. Their strategic interaction with chromatin modifiers, including histone methyltransferases (Wasson et al., 2020b; Wang et al., 2023) and chromatin accessibility (Pachera et al., 2020), initiates the unwinding of the tightly packed chromatin structure. This allows for increased accessibility of transcription factors, such as GLI2, and other regulatory molecules to the gene promoters that drive myofibroblast differentiation. As chromatin remodeling takes place under the guidance of these lncRNAs, a series of events unfold that culminate in the activation of genes pivotal to FMT. These activated genes include those encoding extracellular matrix components, cytoskeletal proteins, and signaling molecules that are characteristic of the myofibroblast phenotype. The orchestrated chromatin changes initiated by lncRNAs lead to the establishment of a permissive transcriptional environment that favors the expression of genes essential for myofibroblast differentiation.

Through their intricate interplay with transcription factors smad, lncRNAs wield significant influence over the gene expression landscape that guides fibroblasts through the intricate process of myofibroblast differentiation. In zheng's study (Zheng et al., 2019), Smad3 activated the expression of Crnde, revealing insights into the molecular process. Intriguingly, Crnde also suppressed Smad3's transcriptional activation of target genes, thus blocking the expression of myofibroblast-specific marker genes in cardiac fibroblasts. Lin's research demonstrated that GAS5-AS1 levels were significantly reduced in oral submucous fibrosis tissues and fibrotic buccal mucosal fibroblasts (Lin et al., 2018). Furthermore, increasing GAS5-AS1 expression led to inhibition of both p-Smad expression and myofibroblast markers. Their presence ensures the coordination, precision, and fidelity of gene expression programs essential for driving FMT. By functioning as transcriptional regulators, lncRNAs contribute to orchestrating a complex series of molecular events culminating in the acquisition of the myofibroblast phenotype.

In the realm of gene expression regulation, transcription factors assume the role of master regulators, directing the intricate sequence of molecular events that govern cellular differentiation. However, this role is not undertaken in isolation. lncRNAs serve as adept collaborators, guiding the transformative process of FMT. LncRNAs emerge as crucial co-regulators in this complex transcriptional symphony, intricately woven into the regulatory landscape to ensure the precise execution of gene expression programs that steer fibroblasts along the path of myofibroblast differentiation. Through specific interactions with transcription factors, they play a role beyond conventional transcriptional regulation. LINC00941 act as co-regulators, interacting transcription factors ATF3 and histone 3 lysine 27 acetylation to play its pro-fibrotic role (Zhang et al., 2022). This coordinated collaboration guarantees the timely and accurate activation of the genes necessary for driving the transformation of fibroblasts into myofibroblasts.

Furthermore, the co-regulator role of lncRNAs extends beyond mere guidance; LncRNA Airn actively participate in modulating the

development of cardiac fibrosis via IMP2-p53 axis in an m6A dependent manner (Peng et al., 2022). Serving as molecular scaffolds, lncRNA H19X create a conducive environment for the assembly of complexes, thereby influencing the accessibility of target gene enhancer (Pachera et al., 2020). This interaction fine-tunes transcriptional activity, either amplifying or attenuating the expression of genes involved in FMT.

The incorporation of lncRNAs into the narrative of FMT introduces a fresh and intricate layer to the multifaceted story of fibrosis. These elusive molecules, previously overshadowed by protein-coding genes, now emerge as critical protagonists, orchestrating the delicate balance between fibroblast quiescence and the transformative process into myofibroblasts. By seamlessly integrating themselves into the complex molecular choreography of FMT, lncRNAs exert their influence in previously unforeseen ways. Their roles as guides, regulators, and network architects has provided new insights into our understanding of fibrosis, suggesting their potential as therapeutic targets for the benefit of patients afflicted with fibrotic conditions.

4 Circular RNAs (circRNAs) in FMT

Circular RNAs (circRNAs) are a class of non-coding RNAs (ncRNAs) characterized by their covalently closed loop structure. Unlike linear RNAs, circRNAs lack 5′caps and 3′polyadenylated tails, making them resistant to exonucleases. This unique structure imparts remarkable stability, allowing circRNAs to persist longer in cells compared to their linear counterparts (Kristensen et al., 2019). These stable molecules are involved in various cellular processes by acting as miRNA sponges, interacting with RNA-binding proteins, and influencing gene expression. In the intricate landscape of FMT, circRNAs have emerged as pivotal players, wielding their regulatory influence through multifaceted mechanisms that are now elucidated by recent studies.

One of the prominent roles that circRNAs play in FMT is that of miRNA sponges, implying that circRNAs have sequences that can bind to and interact with miRNAs, preventing them from carrying out their usual regulatory functions on other messenger RNAs. CircRNAs possess a remarkable ability to sequester miRNAs, small regulatory RNAs that modulate gene expression by binding to mRNA targets and suppressing their translation or promoting their degradation (Patop et al., 2019). By acting as miRNA sponges, circRNAs effectively titrate miRNAs away from their mRNA targets, thus preventing their inhibitory effects. This intricate regulation allows circRNAs to regulate gene expression programs that are crucial for FMT (Zhu et al., 2019; Hu et al., 2022; Zou et al., 2023). Notably, circRNAs like circHIPK3 have been identified as potent regulators of FMT-associated genes (Zhang et al., 2019). By binding to miR-338-3p, circHIPK3 prevents the miR-338-3p from interacting with their intended mRNA targets. As a result, the expression of target gene SOX4 and COL1A1, is spared from miRNA-mediated suppression, leading to the enhancement of fibroblast activation. This mechanism underscores the pivotal role circRNAs play in modulating gene expression patterns that drive the transition of fibroblasts into myofibroblasts.

Beyond their role as miRNA sponges, circRNAs also interact with RNA-binding proteins, adding another layer of complexity to their regulatory functions. For example, Circ-sh3rf3 (circular RNA SH3 domain containing Ring Finger 3) interacts with RNA-binding protein GATA-4 to promote the expression of miR-29a, thereby inhibiting FMT and myocardial fibrosis (Ma et al., 2023). These interactions can impact RNA stability, localization, and translation, further expanding the repertoire of mechanisms through which circRNAs influence FMT. Through their interactions with both miRNAs and RNA-binding proteins, circRNAs wield a dynamic and multifaceted influence on the regulatory networks that govern FMT.

Recent studies have also shed light on circRNAs' role in modulating signaling pathways critical for FMT. CircTTN, for instance, has been implicated in the PI3K/AKT pathway, a key signaling cascade in myofibroblast differentiation. By sponging miR-432, circTTN regulates the expression of genes like IGF2, thereby influencing the activation of the PI3K/AKT signaling pathway (Wang et al., 2019). This regulation demonstrates how circRNAs can modulate specific signaling pathways, affecting the cellular transitions in fibrosis.

Furthermore, circRNAs like circ004463 have been found to interact with AKT/ERK pathways. Circ004463 sponges miR-23b, which targets the mRNA of AKT and ERK. By regulating CADM3 and MAP4K4 expression, circ004463 plays a significant role in promoting fibroblast proliferation and collagen type I synthesis (Zou et al., 2023). Another notable circRNA is hsa_circ_0020792, which acts as a sponge for miR-193a-5p, thereby regulating the expression of pro-fibrotic genes such as TGF-β1 (Hu et al., 2022). This interaction is crucial in the context of fibrosis, as TGF-β1 is a key cytokine driving fibrogenesis, and collagen type I is a major component of the extracellular matrix.

The intricate regulatory function of circRNAs within the context of FMT suggests their significance in shaping cell fate. Their capacity to sponge miRNAs and interact with RNA-binding proteins underscores their ability to modulate gene expression programs, thus determining whether fibroblasts remain in their quiescent state or transition into myofibroblasts. As ongoing research unravels the intricacies of these regulatory mechanisms, circRNAs hold the promise of becoming not only diagnostic markers but also potential therapeutic targets for mitigating the progression of fibrotic diseases.

5 Role of non-coding RNAs (ncRNAs) in fibrotic diseases

Recent research reveals a substantial exploration into the contribution of dysregulated ncRNAs to the intricate landscape of pathological fibrosis. These ncRNAs have emerged as critical players in driving the development and progression of fibrotic diseases across diverse tissues. MiRNAs exhibit a multifaceted role in pathological fibrosis. Pro-fibrotic miRNAs, exemplified by miR-21, facilitate fibrosis by augmenting fibroblast responsiveness to profibrotic stimuli and promoting extracellular matrix deposition. Conversely, anti-fibrotic miRNAs like miR-133a (Wei et al., 2019), counteract fibrosis by targeting multiple components of TGF-β1 profibrogenic pathways. LncRNAs exert significant influence on pathological fibrosis. Pro-fibrotic lncRNAs such as HOTAIR and H19X contribute to myofibroblast differentiation by engaging with chromatin modifiers, transcription factors, and regulatory

TABLE 1 ncRNAs spectrum of diverse fibrotic diseases.

	miRNAs	lncRNAs	circRNAs
cardiac fibrosis	miR-9 (Wang et al., 2016c), miR-21 (Liang et al., 2012; Lorenzen et al., 2015; Nonaka et al., 2021; Ramanujam et al., 2021), miR-22 (Zhang et al., 2018b), miR-23a-3p (Su et al., 2022), miR-29 b (Horii et al., 2023), MiR-32–5p (Shen et al., 2019), miR-34a/miR-93 (Zhang et al., 2018a), miR-101a (Zhou et al., 2018), miR-125 b (Nagpal et al., 2016; Dufeys et al., 2021), miR-130a (Li et al., 2017a; Feng et al., 2022), miR-133a (Matkovich et al., 2010), miR-135a (Wei et al., 2020), miR-142–3p (Wang et al., 2016d; Cai et al., 2020a), miR-150 (Deng et al., 2016), miR-152–3p (Xu et al., 2021b), miR-155 (Zhang et al., 2016b; Wei et al., 2017), miR-195–3p (Carvalho et al., 2023), miR-214–3p (Zhu et al., 2016; Yang et al., 2019), miR-216a (Qu et al., 2019), miR-327 (Ji et al., 2018), miR-331 (Yousefi et al., 2021), miR-338–3p (Huang et al., 2022), miR-369–5p (Tao et al., 2018), miR-409–3p (Wang et al., 2022), miR-433 (Tao et al., 2016b), miR-451a (Deng et al., 2022), miR-486 (Chen et al., 2022), miR-574–5p (Cui et al., 2020)	Airn (Peng et al., 2022) Crnde (Zheng et al., 2019) Gm41724 (Kong et al., 2023) PFL (Liang et al., 2018) RMST (Ma et al., 2023b) Safe (Hao et al., 2019) SRA1(Zhang et al., 2019c) SNHG7(Wang et al., 2020b) TUG1 (Zhu et al., 2018)	circNFIB(Zhu et al., 2019) circHRCR (Wang et al., 2016b) circ-sh3rf3 (Ma et al., 2023a) circSMAD4 (Jeong et al., 2023)
pulmonary fibrosis	let-7 (Elliot et al., 2019; Thakur et al., 2022; Xu et al., 2022), miR-7 (Zhang et al., 2020b), miR-9-5p (Fierro-Fernández et al., 2015), miR-19a (Fujita et al., 2023), miR-21 (Yamada et al., 2013; Cui et al., 2018; Wang et al., 2021a), miR-22 (Kuse et al., 2020), miR-24 (Ebrahimpour et al., 2019), miR-26a (Liang et al., 2014), miR-27a-3p (Cui et al., 2016), miR-29 (Herrera et al., 2018), miR-30c (Kanno et al., 2021), miR-30d (Zhao et al., 2018), miR-34a (Cui et al., 2017; Bulvik et al., 2020), miR-34b-5p (Hu et al., 2019), miR-96 (Nho et al., 2014), miR-124 (Lu et al., 2019), miR-133a (Wei et al., 2019), miR-144–3p (Bahudhanapati et al., 2019), miR-145 (Yang et al., 2013), miR-155 (Artlett et al., 2017), miR-199a-5p (Lino Cardenas et al., 2013; Yi et al., 2018), miR-200 (Chilosi et al., 2017), miR-338–3p (Rackow et al., 2022), miR-424 (Xiao et al., 2015; Huang et al., 2020), miR-375 (Zhang et al., 2020c), miR-449a (Han et al., 2016), miR-497–5p (Chen et al., 2017), miR-541–5p (Ren et al., 2017), miR-627 (Li et al., 2019a), miR-877–3p (Wang et al., 2016a), miR-7219–3p (Niu et al., 2022)	CTD-2528L19.6 (Chen et al., 2021a) DNM3OS(Savary et al., 2019) GAS5 (Wang et al., 2023b) H19 (Xiao et al., 2021) ITPF(Song et al., 2019) LINC00941(Zhang et al., 2022) LOC344887(Liu et al., 2021) LOC103691771(Cai et al., 2020b) PFI(Sun et al., 2021) PFAL(Li et al., 2018b) SNHG1(Wu et al., 2021) SNHG20(Cheng et al., 2021) ZFAS1(Yang et al., 2020)	circ0044226 (Zhang et al., 2020a) circHIPK3(Zhang et al., 2019b; Xu et al., 2021a)
renal fibrosis	miR-34a (Saito et al., 2023), miR-132 (Bijkerk et al., 2016), miR-335–5p (Qiu et al., 2022), miR-378a-5p (Zhang et al., 2023b)	Rian and Miat (Bijkerk et al., 2019)	—
hepatic fibrosis	miR-16 (Pan et al., 2020), miR-19 b (Brandon-Warner et al., 2018), miR-29 (Kwiecinski et al., 2011; Kwiecinski et al., 2012), miR-132 (Mann et al., 2010), miR-214 (Izawa et al., 2015)	MALAT1 (Wu et al., 2015)	—
dermal fibrosis	miR-130a (Zhang et al., 2019a), miR-192 (Li et al., 2017b; Li et al., 2021), miR-196b-5p (Baral et al., 2021)	HOTAIR (Wasson et al., 2020b)	circAMD1 (Su et al., 2021)
oral submucous fibrosis	miR-10 b (Fang et al., 2020), miR-21 (Yang et al., 2021; Liao et al., 2022), miR-29c (Yang et al., 2022a), miR-200 b (Liao et al., 2018)	GAS5-AS1 (Lin et al., 2018) HOTTIP(Lee et al., 2021) H19 (Yu et al., 2021)	—
musculoskeletal tissues	miR-29a (Millar et al., 2015),miR-214–3p (Arrighi et al., 2021)	—	circTTN (Wang et al., 2019b)

molecules. This interaction modulates gene expression profiles and drives fibroblasts towards the myofibroblast phenotype. In contrast, certain lncRNAs such as PFI (Sun et al., 2021) and LOC344887 (Liu et al., 2021) act as suppressors of fibrosis, impeding myofibroblast activation and promoting tissue equilibrium. CircRNAs, with their circular structure, introduce an additional layer of complexity to the fibrotic scenario. Operating as miRNA sponges and interacting with RNA-binding proteins, circRNAs regulate gene expression patterns with precision. CircRNAs like circHIPK3 exemplify this role by sequestering miRNAs targeting key genes involved in fibrotic processes, thereby modulating gene expression profiles that underpin fibrosis. In summary, prior studies underscore the integral roles of dysregulated ncRNAs in driving pathological fibrosis. These ncRNAs impact the equilibrium between fibroblast activation and tissue health.

The formation and expression of ncRNAs are tightly regulated processes that are often altered during disease conditions. ncRNAs are transcribed by RNA polymerase II and III, and their maturation involves complex processing steps, including splicing, editing, and modifications. For example, primary miRNAs (pri-miRNAs) are processed by Drosha and Dicer enzymes to generate mature miRNAs that can bind to target mRNAs (Herrera et al., 2018; Cho et al., 2020). Similarly, lncRNAs undergo splicing and modifications that influence their stability and function (Hao et al., 2019). The expression of ncRNAs is tightly regulated under normal conditions but can become dysregulated during fibrosis. This dysregulation plays a crucial role in the pathological progression of fibrosis by affecting the balance between fibroblast quiescence and myofibroblast activation. In kidney fibrosis, the upregulation of miR-21 correlates with increased kidney stiffness and fibrosis severity, indicating its role in disease progression (Glowacki et al., 2013). Similarly, reduced levels of miR-449a are observed in fibrotic lung tissues and correlate with the severity of lung lesions induced by silica, suggesting its involvement in the Silicosis (Han et al., 2016). Understanding the correlation between ncRNA expression and fibrosis progression provides valuable

insights into the molecular mechanisms underlying fibrotic diseases. These insights highlight the potential of ncRNAs as biomarkers for disease diagnosis and prognosis and as therapeutic targets for modulating fibrotic processes and restoring tissue homeostasis.

Notably, ncRNAs exhibit their multifaceted roles across a diverse spectrum of fibrotic conditions, ranging from cardiac fibrosis, hepatic fibrosis, pulmonary fibrosis, renal fibrosis, dermal fibrosis, and musculoskeletal tissues (Table 1). This broad influence underscores the significance of ncRNAs as central regulators of fibrotic processes across diverse tissues and organs. In particular, arthrofibrosis is a common and debilitating complication that can occur following knee surgery (Lee et al., 2022). Abdel et al. have identified differentially expressed genes associated with arthrofibrosis by comparing tissue samples from fibrotic and non-fibrotic human knee joints using RNA sequencing (Bayram et al., 2020). Further, Chen et al. carried out further bioinformatics analysis and reported new biomarkers for diagnosing arthrofibrosis, shedding light on the role of transforming growth factor-beta receptor 1 (TGFBR1) (Chen et al., 2021). These data provide further insight into the role of ncRNAs in the regulation of joint fibrosis.

ncRNAs exhibit both ubiquitous and tissue-specific functions, which together shape the initiation and progression of fibrosis. Ubiquitous ncRNAs, such as miR-21, are widely expressed across different tissues and play a central role in fibrosis by modulating common fibrogenic pathways. miR-21 enhances fibroblast activation and extracellular matrix deposition by targeting multiple genes involved in the TGF-β signaling pathway, including SMAD7 and PTEN, thus promoting fibrosis in various organs (Glowacki et al., 2013; Li et al., 2019; Wang et al., 2021; Nonaka et al., 2021; Liao et al., 2022). In contrast, tissue-specific ncRNAs are expressed in particular organs and contribute to localized fibrotic processes. For instance, lncRNA MALAT1 is prominently expressed in the liver and contributes to hepatic fibrosis by interacting with the silent information regulator 1(SIRT1) and promoting the expression of pro-fibrotic genes such as COL1A1 and α-SMA (Wu et al., 2015). Similarly, circNFIB is predominantly expressed in the heart and, where it activates the TGF-β–Smad3 signaling pathway and is crucial in cardiac fibrosis (Zhu et al., 2019).

ncRNAs can exert paracrine effects, influencing cells beyond their origin and contributing to multi-organ fibrosis. These ncRNAs can be secreted into the extracellular environment and transported to distant cells and tissues through extracellular vesicles (EVs), such as exosomes and microvesicles. This capability allows ncRNAs to participate in intercellular communication and influence various physiological and pathological processes across different organs.

In the context of fibrosis, ncRNAs can be secreted by fibroblasts or other cell types and taken up by neighboring cells, thereby modulating their behavior. For example, miR-21, a well-known pro-fibrotic miRNA, can be packaged into EVs and transferred from myofibroblasts to adjacent endothelial cells. This transfer can induce a pro-angiogenic process of endothelial cells, a process contributing to the fibrotic response (Li et al., 2019). Similarly, miR-200, another miRNA implicated in fibrosis, can be secreted by endothelial cells and taken up by fibroblasts, influencing fibroblast heterogeneity in colorectal cancer (Bhome et al., 2022).

NcRNAs can enter the systemic circulation, allowing them to travel to distant organs and exert their effects. Circulating miRNAs, for instance, have been detected in blood, urine, and other body fluids, serving as biomarkers for various diseases (De Guire et al., 2013). These circulating ncRNAs extend their impact beyond the local tissue environment, affecting distant organs and contributing to the pathology of multi-organ diseases. For instance, miR-29, which regulates extracellular matrix production, is involved in cutaneous, prostate, cardiac and oral submucous fibrosis. Its dysregulation in one organ can have implications for fibrotic processes in others.

LncRNAs also exhibit multi-organ effects. LncRNA H19, known for its role in pulmonary fibrosis, can influence fibrotic buccal mucosal myofibroblast activities, such as collagen gel contractility and migration ability when dysregulated, highlighting its potential impact on both pulmonary and oral submucous tissues. Similarly, the lncRNA GAS5, which modulates fibrotic pathways in the skin, can have systemic effects, potentially affecting other fibrotic conditions in organs like the lung.

Understanding the paracrine and multi-organ effects of ncRNAs is crucial for developing therapeutic strategies targeting fibrotic diseases. Therapies designed to modulate ncRNA levels in one organ might have beneficial effects on fibrosis in other organs, offering a systemic approach to treating multi-organ fibrotic conditions. For example, therapeutic inhibition of miR-21 has shown promise in reducing fibrosis in both the heart and lung, demonstrating the potential of ncRNA-targeted therapies to address multi-organ fibrosis.

ncRNAs may have distinct impacts on acute versus chronic diseases, reflecting their roles in immediate injury responses versus long-term maladaptive processes. During acute injury, the rapid and transient changes in ncRNA expression are crucial for the immediate response to cellular damage and stress. For instance, miR-101a is rapidly upregulated following myocardial infarction (MI) and plays a critical role in promoting cardiac fibroblast activation and fibrosis to stabilize the injured tissue (Zhou et al., 2018). In contrast, chronic conditions and aging involve sustained ncRNA dysregulation, contributing to persistent fibrosis and organ dysfunction. For example, miR-34A is consistently dysregulated in chronic liver and renal fibrosis, leading to sustained extracellular matrix production and fibrogenesis (Cui et al., 2017; Saito et al., 2023). Understanding the distinct roles of ncRNAs in acute and chronic conditions can inform the development of targeted therapies. In acute injury, therapeutic strategies may aim to modulate ncRNAs to enhance tissue repair and limit damage. In chronic diseases and aging, ncRNA-based therapies could focus on reversing maladaptive gene expression patterns and reducing fibrosis and inflammation.

In short, the regulatory influence of various ncRNAs extends across diverse fibrotic diseases. The pervasive presence of these ncRNAs within the fibrotic milieu underscores the need for a comprehensive understanding of their intricate functions. Unraveling the precise molecular mechanisms through which ncRNAs exert their regulatory effects could pave the way for the development of targeted therapeutic strategies. By targeting these ncRNAs or modulating their interactions with key regulatory molecules, it might be possible to attenuate fibrosis progression and restore tissue homeostasis in a range of fibrotic diseases.

6 Therapeutic implications

The intricate involvement of ncRNAs in FMT has opened new avenues for therapeutic interventions in fibrotic diseases. These regulatory molecules have been identified as critical players in fine-tuning gene expression programs that govern the delicate balance between fibroblast quiescence and myofibroblast activation. By deciphering the precise roles of ncRNAs in regulating this transition, researchers have uncovered potential targets that could be manipulated to mitigate the excessive activation of myofibroblasts and slow the progression of fibrosis.

Therapeutic strategies involving miRNAs typically include miRNA mimics to restore the function of downregulated miRNAs or miRNA antagonists (antagomirs) to inhibit the function of upregulated miRNAs. For instance, MiR-29 family mimics exhibit antifibrotic effects across various tissues by targeting collagen synthesis and extracellular matrix remodeling. A completed open-label phase 2 RCT clinical trial (Clinical Trial Number: NCT03601052) has defined the efficacy, safety, and tolerability of Remlarsen (MRG-201), which is designed to mimic the activity of miR-29 that may be an effective therapeutic to prevent cutaneous fibrosis. This study demonstrated that administering high doses of this miR-29 mimic could effectively decrease fibrosis (Gallant-Behm et al., 2019). It is worth noting that the dosage utilized in this research was excessively high for practical use in human patients. Nevertheless, these findings provided encouraging evidence for investigators working towards the development of microRNA mimics as potential therapeutics for fibrosis. Anti-miR oligonucleotides, designed to inhibit the function of pro-fibrotic miRNAs, also show potential; for example, targeting miR-21, a pro-fibrotic miRNA, has shown promise in reducing fibrosis in preclinical models. Anti-miR-21 therapies aim to decrease fibroblast responsiveness to pro-fibrotic stimuli and reduce extracellular matrix deposition.

LncRNA-based therapeutics involve targeting pro-fibrotic lncRNAs, such as ASLNCS5088 (Chen et al., 2019) and Gm41724 (Kong et al., 2023), to mitigate fibrosis by disrupting their interactions with RNA-binding proteins, and M2 macrophage modulation. By preventing these interactions, it is possible to modulate gene expression profiles that drive fibroblast activation and myofibroblast differentiation. Additionally, boosting the expression of anti-fibrotic lncRNAs like GAS5 can help inhibit myofibroblast activation and fibrogenesis through suppressing TGF-β/Smad3 signaling (Tang et al., 2020). Therapeutic strategies may involve gene therapy approaches to deliver these lncRNAs or small molecules that enhance their endogenous expression.

CircRNA-based therapeutics focus on the unique abilities of circRNAs to act as miRNA sponges or interact with RNA-binding proteins. CircHIPK3 serves as a prime example, as it influences myofibroblast differentiation by sponging miR-338-3p that target SOX4 and COL1A1 (Zhang et al., 2019). Designing synthetic circRNA sponges can regulate miRNA activity and modulate gene expression patterns involved in fibrosis. Additionally, modulating circRNA-protein interactions can impact the regulatory networks driving fibrosis. For example, Circ-sh3rf3 can bind to GATA-4 proteins and decrease their expression, which prevents GATA-4 from suppressing miR-29a expression. As a result, miR-29a expression is increased, leading to the inhibition of fibroblast-to-myofibroblast differentiation and myocardial fibrosis. Targeting these ncRNAs might offer a means to disrupt the regulatory networks that drive fibroblast activation. Such precision-based approaches could revolutionize the treatment landscape for fibrotic diseases, allowing for tailored interventions that target the underlying molecular mechanisms.

While the potential of ncRNA-based therapies for fibrosis is exciting, several challenges must be navigated for successful translation into clinical applications. One significant hurdle is the delivery of ncRNA-based therapeutics to target tissues. Ensuring efficient and specific delivery remains a key obstacle. Strategies such as viral vectors (Tang et al., 2020), nanoparticle-mediated delivery (Zahir-Jouzdani et al., 2018), or organ-targeted liposomes (Yan et al., 2023) are being explored to address this challenge. Additionally, the specificity of ncRNA-targeting therapies is crucial to avoid off-target effects and unintended consequences (Yan et al., 2023). Ensuring that therapies selectively target the dysregulated ncRNAs while preserving the physiological functions of others is essential for clinical success. The stability and bioavailability of ncRNA-based therapeutics are critical factors for their effectiveness. Chemical modifications, such as locked nucleic acids (LNAs) and phosphorothioate backbones, can enhance the stability and resistance of ncRNA-based therapeutics to degradation. These modifications improve the pharmacokinetic properties and therapeutic efficacy of ncRNA-based treatments (Ali Zaidi et al., 2023). ncRNA-based therapeutics, particularly those involving viral vectors, may elicit immune responses. Strategies to minimize immunogenicity include optimizing vector design, using tissue-specific promoters, and developing non-viral delivery systems (Awan et al., 2017). Furthermore, the complex regulatory networks involving ncRNAs add another layer of complexity. Many ncRNAs participate in intricate crosstalk with other regulatory molecules, such as transcription factors and signaling pathways, leading to a network of interdependencies. Designing therapies that effectively modulate these networks requires a deep understanding of the molecular interactions and their consequences.

Future directions and prospects in ncRNA-based therapies for fibrotic diseases include combination therapies, personalized medicine, advancements in delivery systems, and robust translational research efforts. Combining ncRNA-based therapies with existing antifibrotic drugs or other therapeutic modalities may enhance efficacy and overcome resistance mechanisms. Personalized approaches tailored to individual patients' specific ncRNA expression profiles can improve treatment outcomes and minimize adverse effects. Ongoing advancements in delivery systems, such as exosome-based delivery and tissue-specific nanoparticles, hold promise for improving the targeted delivery of ncRNA-based therapeutics. Collaborative efforts between academia, industry, and regulatory agencies can accelerate the development and approval of ncRNA-based therapies.

The emerging roles of ncRNAs in FMT offer novel avenues for therapeutic intervention in fibrotic diseases. By targeting specific ncRNAs, it is possible to intervene in the processes that drive myofibroblast activation and tissue fibrosis. However, the journey from bench to bedside requires the successful resolution of delivery challenges, the mitigation of off-target effects, and in depth understanding of the complex regulatory networks involved. As research in this field advances, the development of effective and

precise therapies holds the promise of transforming the landscape of fibrotic disease treatment.

7 Conclusion

In summary, recent research highlights the crucial involvement of ncRNAs in the complex process of FMT. These ncRNAs, including miRNAs, lncRNAs, and circRNAs, collectively constitute a regulatory ensemble that finely modulates the equilibrium between quiescent fibroblasts and their activated myofibroblast counterparts. Recent studies have meticulously unraveled the multifaceted mechanisms by which these ncRNAs exert their influence.

The narrative begins with miRNAs, which play a central role by targeting key regulators of FMT. MiR-21 assumes a prominent position as a potent inducer of FMT, primarily by inhibiting TGF-β receptor inhibitors. This action sensitizes fibroblasts to TGF-β signaling, thereby promoting myofibroblast differentiation and subsequent fibrosis. MiR-146b and miR-125b also contribute to FMT by targeting factors that otherwise restrain myofibroblast activation. Conversely, miRNAs such as miR-29 and the miR-200 family act as suppressors of FMT, counteracting excessive collagen synthesis and inhibiting myofibroblast differentiation through their targeting of related genes.

LncRNAs act as pivotal regulators of FMT. Notable lncRNAs like H19X and GAS5 emerge as regulators in the FMT process. They engage with chromatin modifiers, transcription factors, and regulatory molecules, facilitating chromatin remodeling, reprogramming of gene expression, and the orchestration of transcriptional forces that guide fibroblasts toward the myofibroblast lineage. LncRNAs further their influence by fostering crosstalk among regulatory molecules, perpetuating essential signaling cascades crucial for FMT progression.

Simultaneously, circRNAs embrace their role as miRNA sponges, intricately fine-tuning gene expression during FMT. Notable circRNAs like circHIPK3 demonstrate their ability to sequester miRNAs targeting genes associated with FMT. In doing so, these circRNAs release these genes from miRNA-mediated suppression, ultimately enhancing the differentiation of fibroblasts into myofibroblasts. Moreover, the intricate interactions of circRNAs with RNA-binding proteins add an additional layer of complexity to their regulatory repertoire.

In a broader context, these ncRNAs collaboratively interweave their actions, constructing a complex network of regulatory interactions that modulate the transformation of fibroblasts into myofibroblasts. Their contributions extend beyond individual roles, creating a dynamic interplay that profoundly influences the delicate equilibrium between fibroblast quiescence and myofibroblast activation. Dysregulation of these ncRNAs has been closely linked to the development of pathological fibrosis in various tissues, underscoring their significance as potential therapeutic targets.

As we stand on the cusp of a new era in the treatment of fibrotic diseases, the emerging roles of ncRNAs in FMT offer substantial therapeutic promise. By deciphering the intricacies of ncRNA-mediated regulatory networks, researchers could uncover innovative therapeutic avenues that could effectively counteract the progression of fibrotic diseases. However, translating these insights into clinical applications presents challenges such as efficient delivery methods, specificity, and the potential for off-target effects. As the journey continues, the potential to harness the power of ncRNAs may illuminate a path toward restoring tissue health and function, offering renewed hope to those affected by these debilitating conditions.

In conclusion, the process of FMT occupies a central role in tissue repair and the pathogenesis of fibrotic diseases. The intricate interplay of cellular morphological changes, altered gene expression profiles, and extracellular matrix remodeling underscores its significance. With recent discoveries revealing the pivotal roles of ncRNAs, including miRNAs, lncRNAs, and circRNAs, in orchestrating FMT, a new chapter has opened in our understanding of tissue remodeling. These ncRNAs act as master regulators, shaping the symphony of FMT by influencing a diverse array of molecular players. Their regulatory capabilities extend across signaling cascades, transcriptional programs, and intricate interactions, and their dysregulation can lead to pathological fibrosis. As research continues to elucidate the precise mechanisms by which ncRNAs guide FMT, their therapeutic potential emerges as a promising frontier, offering novel strategies to combat fibrotic diseases and restore tissue health.

Author contributions

XX: Writing–original draft. SR: Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Human adipose mesenchymal stem cell-derived exosomes alleviate fibrosis by restraining ferroptosis in keloids

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Background: Keloid is a fibroproliferative disease with unsatisfactory therapeutic effects and a high recurrence rate. exosomes produced by adipose-derived mesenchymal stem cells (ADSC-Exos) have attracted significant interest due to their ability to treat fibrosis. However, the molecular mechanisms of ADSC-Exos in keloids remain inconclusive.

Objective: Our study revealed the relationship between ferroptosis and fibrosis in keloids. Subsequently, this study aimed to explore further the anti-fibrotic effect of ADSC-Exos on keloids through ferroptosis and the potential underlying mechanisms.

Methods: To investigate the impact of ferroptosis on keloid fibrosis, Erastin and ferrostatin-1 (fer-1) were utilized to treat keloid fibroblast. Keloid keloids treated with Erastin and fer-1 were cocultured with ADSC-Exos to validate the impact of ferroptosis on the effect of ADSC-Exos on keloid anti-ferrotic protein, peroxidase 4 (GPX4) and anti-fibrotic effects *in vivo* and *in vitro* by Western blot, as well as variations in iron metabolite expression, malondialdehyde (MDA), liposomal peroxidation (LPO) and glutathione (GSH) were analyzed. The effect of solute carrier family 7-member 11 (SLC7A11) silencing on ADSC-Exo-treated keloid fibroblast was investigated.

Results: Iron metabolite dysregulation was validated in keloids. Fibrosis progression is enhanced by Erastin-induced ferroptosis. The anti-fibrotic effects of ADSC-Exos and fer-1 are related to their ability to prevent iron metabolism. ADSC-Exos effectively suppressed keloid fibrosis progression and increased GSH and GPX4 gene expression. Additionally, the use of Erastin limits the effect of ADSC-Exos in keloids. Furthermore, the effect of ADSC-Exos on keloids was associated with SLC7A11-GPX4 signaling pathway.

Conclusion: We demonstrated a new potential mechanism by which anti-ferroptosis inhibits the progression of keloid fibrosis and identified an ADSC-Exo-based keloid therapeutic strategy. Resisting the occurrence of ferroptosis and the existence of the SLC7A11-GPX4 signaling pathway might serve as a target for ADSC-Exos.

KEYWORDS

adipose-derived mesenchymal stem cells, fibrosis, ferritic, extracellular vesicles, GPx4

1 Introduction

Keloids can lead to physical discomfort, functional difficulties, and aesthetically pleasing problems, all of which can trigger psychological discontent (Jeschke et al., 2023). The traditional treatment methods for keloids mainly include ionizing beams, hormone injection, and cryosurgery. However, effective treatment methods are needed to guarantee the recurrence rate after treatment (Kadunc and Brunner, 2024). Therefore, identifying a treatment method that can target the pathogenesis of keloids is the key to solving this problem.

Ferroptosis is a recently discovered process that regulates cell necrosis (Jiang et al., 2021). It has been scientifically linked to several diseases. Changes in iron homeostasis, for example, have been associated with an increased probability of end-stage renal disease (ESKD) (Yu et al., 2020; Cai et al., 2023), atherosclerotic cardiovascular disease (Fang et al., 2023), and diabetes (Hoy et al., 2021). Preventing ferroptosis can substantially reduce the number of myofibroblast-like cells, which leads to less fibrosis. Ferroptosis controls fibroblast apoptosis and fibrosis in a complex and tissue-specific manner (Du et al., 2023). The role of ferroptosis in keloids is currently unclear. In our previous study, we compared the expression of ferroptosis genes in keloid fibroblast (KF) and normal fibroblast. The results showed that keloid fibrosis was associated with ferroptosis.

MSC-derived extracellular vesicles are innovative cell-free therapeutics for immunomodulation and regenerative purposes (Xiao et al., 2021). Human adipose-derived stem cells (ADSCs) are a vital source of stem cells because of their simplicity of utilization, self-renewal ability, minimum immunogenicity, high proliferation rate, and capacity to undergo differentiation into different lineages (Hoang et al., 2022). Exosomes, one of the most common types of extracellular vesicles, function in intercellular communication (Baumann, 2021). Some investigators believe that exosomes from human adipose-derived mesenchymal stem cells (ADSC-Exos) potentially restrict excessive collagen formation in fibroblasts. By activating the PI3K/AKT/mTOR signaling pathway, ADSC-Exos inhibited the expression of profibrogenic proteins and epithelial-to-mesenchymal transition (EMT). (Zhang et al., 2023). Furthermore, certain investigators have achieved unique medicinal properties. Excessive scar formation can be remediated by employing ADSC-Exos as transport carriers for pharmaceuticals and noncoding RNAs (Zhu et al., 2020; Li et al., 2021; Yuan et al., 2021). Consequently, it is crucial to elucidate the potential mechanism underlying the inhibitory effect of ADSC-Exos on the progression of keloid fibrosis.

ADSC-Exos inhibits ferroptosis induced by excessive inflammation and upregulates the expression of glutathione peroxidase 4 (GPX4) in human brain microvascular endothelial cells (Wu et al., 2024). ADSC-Exos can effectively improve the neurobehavior of mice and improve ferroptosis-related outcomes (Wang Y. et al., 2023). In this study, we identified an innovative approach in which ADSC-Exos inhibited the myofibroblast differentiation of KF by decreasing ferroptosis in keloids.

2 Materials and methods

2.1 Tissue and cell sources

The First Affiliated Hospital of Harbin Medical University's Ethics Committee approved the collection of human tissue samples, and the study was conducted in accordance with the 2013 Declaration of Helsinki (No. 2023IIT115). For every tissue biopsy, informed consent was obtained. Samples from mature keloids and adipocytes were collected from plastic surgery patients. Professional dermatologists and plastic surgeons test the clinical nature of keloids.

ADSCs were isolated from subcutaneous adipose tissue of patients who underwent lipoplastic surgery and were freely available. Human keloid and standard skin fibroblast samples were obtained from patients who underwent surgery to eliminate a keloid and its surrounding normal skin or from the same patient's normal skin from the donor location of skin graft surgery; 16 different patients were included in this study. Under low glucose conditions, Dulbecco's modified Eagle's medium (DMEM, Gibco, United States), keloid fibroblast (KF), normal skin fibroblasts, and ADSCs were cultivated. The medium also included 100 IU/mL penicillin, 10 mg/mL streptomycin, and 10% fetal bovine serum (FBS, BI, United States). The medium was replaced every 3 days. The cells were passaged once they reached confluence. These cells progress through three to four growth stages. Fourth-generation ADSCs, which were obtained from different individuals, contained the cells required for exosome extraction.

2.2 Adipogenic and osteogenic differentiation of ADSCs

Following prior methods, ADSCs at passage three were divided into osteogenic and adipogenic lineages. Briefly, ADSCs were grown for 2 weeks in a complete osteogenic medium supplemented with 10 mM b-glycerophosphate (Sigma), 0.1 mM dexamethasone (Sigma), and 0.05 mM ascorbic acid (Sigma). Differentiated cells were then stained with alizarin red. ADSCs were grown for 3 weeks and exhibited an adipogenic phenotype when stained with Oil Red O after adipogenic induction.

2.3 Characterization of ADSCs

In passage three, ADSCs were identified following earlier protocols. Flow cytometric examination of the cell immunophenotype verified the presence of ADSCs. The ADSC surface markers examined included CD29, CD34, CD44, CD45, CD14, and CD105, which were all PE-labeled.

2.4 Concentration and characterization of ADSC-Exos

Exosomes were purified as previously described. Adipose-derived stem cells from the fourth passage were fused, and the

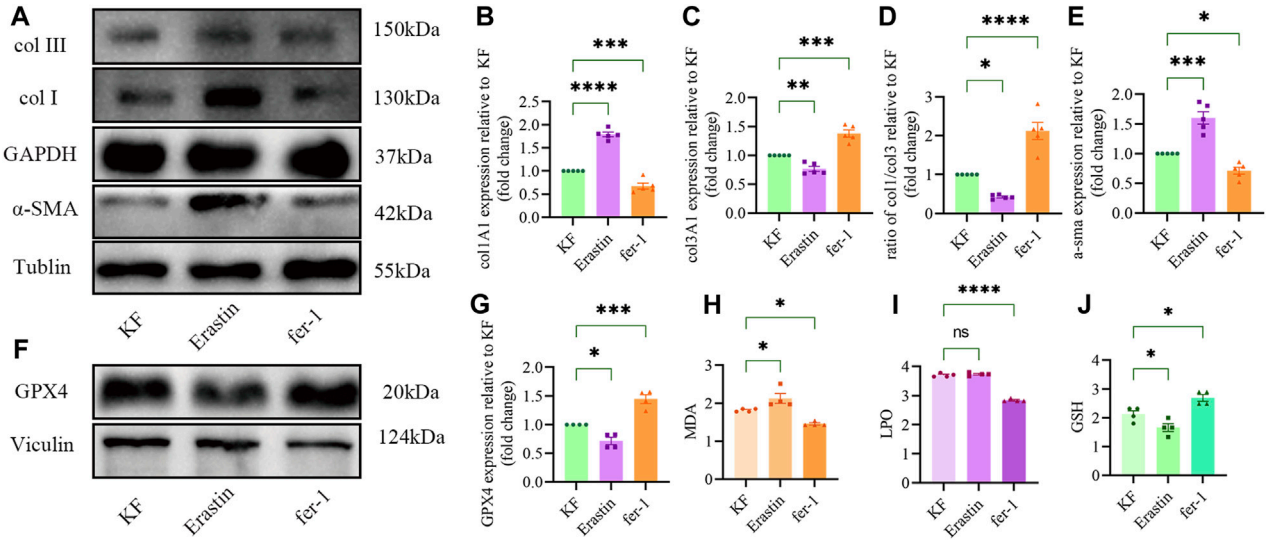

FIGURE 1
Effects of Erastin and fer-1 on fibrosis and ferroptosis in KF. **(A)** Western blot images of keloid fibrosis affected by Erastin (2.5 μm/mL) and fer-1 (1.7 μm/mL). **(B)** Quantification of the expression of col1A1, **(C)** col3A1, and **(D)** the ratio of col1A1 to col3A1, **(E)** α-SMA relative to keloid fibroblast (KF) (n = 5). **(F)** Western blot images of GPX4 in KF treated with Erastin and fer-1. **(G)** Quantification of GPX4 and **(H)** MDA levels. **(I)** LPO and **(J)** GSH relative to KF. GPX4, glutathione peroxidase 4; MDA, malondialdehyde; LPO, liposomal peroxidation; GSH, glutathione. Four repetitions were carried out for each experiment. The error bars represent the standard deviation. One-way ANOVA followed by Tukey's multiple comparison test was carried out for comparisons. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$, **** represents $p < 0.0001$, ns represents no significance.

cells were then moved to a medium supplemented with serum-free DMEM for 48 h at 37°C in a 5% CO_2 atmosphere. The media underwent a series of centrifugation procedures after the incubation time. The entire centrifugation process was performed at 4°C, and the initial centrifugation was performed at 300 × g for 10 min. Afterward, the supernatant was centrifuged for 10 min at 1,000 × g and 30 min at 10,000 × g. The supernatant was centrifuged at an ultrahigh pressure for 70 min at 100,000 × g. The precipitate was then placed in PBS to resuspend the pellet made up of ADSC-Exos and stored at −80°C refrigerated for later use after the final ultracentrifugation (100,000 × g for 70 min).

For the identification of ADSC-Exos, 1.0×10^9 vesicles were used. Using a transmission electron microscope, the ultrastructure of ADSC-Exos was examined. The particle dispersion size was analyzed by nanoparticle tracking analysis (NTA) and Nanosight LM10 (Malvern et al., United Kingdom). The expression of the common marker proteins for exosomes CD63 (Abmart, M051014, CHINA), TSG101 (Abmart, T55985, CHINA), and CD81 (Abmart, T557425, CHINA) were examined by Western blot.

2.5 Exosome uptake assay

To verify that ADSC-Exos could be internalized by KF, they were tagged with a PKH67 fluorescent cell linker kit (Sigma–Aldrich, MIDI67-1KT) according to the manufacturer's instructions. After the nuclei were stained with DAPI (Solarbio, C0065), the labeled ADSC-Exos were cocultured with P3 KF for 24 h, and images were obtained at 0 and 24 h with an Olympus IX81 fluorescence microscope.

2.6 Analysis of ferroptosis and fibrosis in keloids

After treatment with Erastin and fer-1, P3 KF were cultured in three groups: the first with a predetermined volume of PBS (control group), the second with Erastin (Erastin group), and the third with fer-1 (fer-1 group). After 24 h, each plate was subjected to Western blot analysis.

2.7 Analysis of ferroptosis and fibrosis in keloids cocultured with ADSC-Exos

After coculture with ADSC-Exos, P3 KF were cultured in three groups: the first with a predetermined volume of PBS (control group), the second with ADSC-Exos (ADSC-Exo group), and the third with ADSC-Exos + Erastin (ADSC-Exo + Erastin group). After 24 h, each plate was subjected to Western blot analyses.

2.8 Iron metabolism level determination in tissue and cells

Ferroptosis is characterized by free ferrous iron overload and lipid peroxide accumulation. The lipid peroxide (LPO, E-BC-K176-M, Elabscience), reduced glutathione (GSH, A006-1-1, Nanjing, China), and malondialdehyde (MDA E-BC-K027-M, E-BC-K025-M, Elabscience) values of each sample were calculated according to the formula. ROS levels were measured in a medium supplemented with the fluorescent probe DCFH-DA (Solarbio, Shanghai, China) for 20 min at 37°C. A Nikon confocal microscope was used to capture the images.

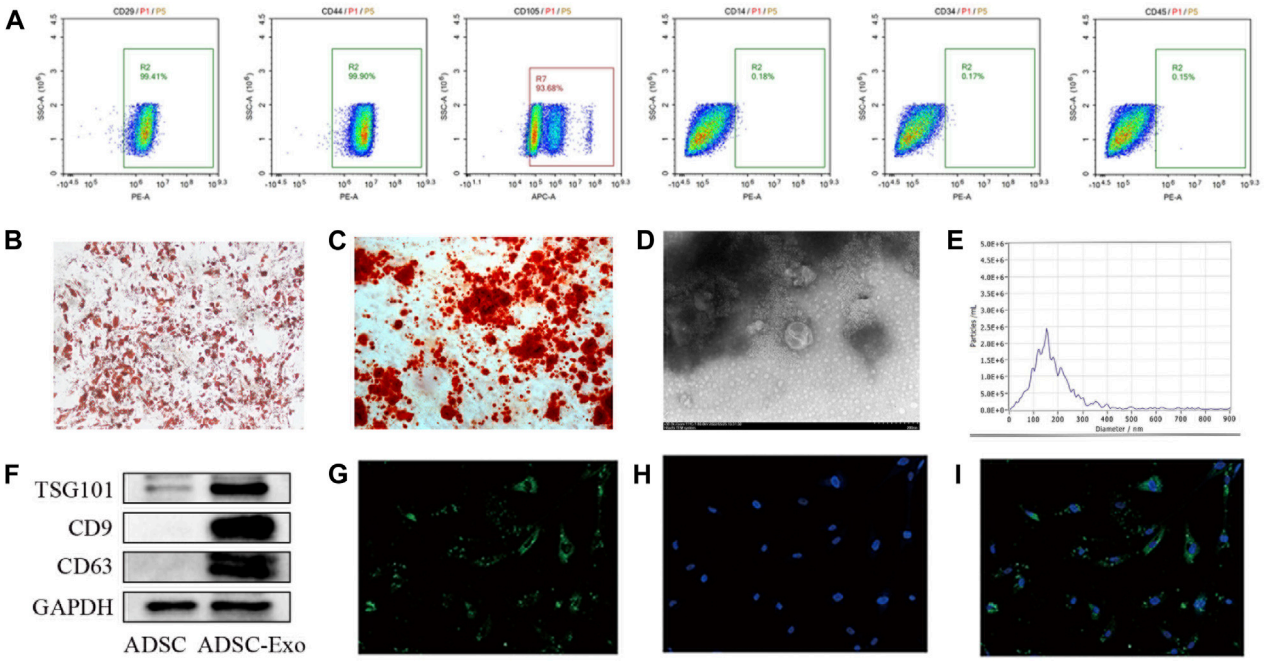

FIGURE 2
Characteristics of adipose-derived stem cells (ADSCs). **(A)** Results of flow cytometry analysis of ADSCs showing the expression of mesenchymal stem cell surface markers. CD29, CD44, and CD105 were positive; CD14, CD34 and CD45 were negative. **(B)** Adipogenic and **(C)** osteogenic differentiation measured by Oil Red O staining and Alizarin Red S staining; scale bars = 100 μm. **(D)** The morphology of ADSC-Exos was analyzed by TEM; scale bar = 200 nm. **(E)** The particle size of ADSC-Exos was measured by NTA. **(F)** Western blot images of the exosome markers CD9, CD81, and TSC101. **(G–I)** Confocal microscopy was used to observe the internalization of ADSC-Exos labeled with PKH-67 into **(G)** KF, **(H)** DAPI, and **(I)** merged images.

2.9 Western blot analysis

Total protein was extracted in RIPA (Bryotime, P0013B) lysis buffer with a loading buffer (Solarbio, Beijing, China) containing a 1% protease inhibitor cocktail (Solarbio, Beijing, China) and a 1% protein phosphatase inhibitor combination (Solarbio, Beijing, China). The protein concentration was measured with an Instant BCA assay kit (Beyotime, Beijing, China). Twenty micrograms of protein samples were separated by 12.5% and 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. At 25°C, the membranes were blocked in 5% nonfat milk in TBST solution for 60–80 min. COLIA1 (A1352, 1:500, ABclonal, United States), COLIIIA1 (ab184993, 1:1,000, Abcam, MA, United States), α-SMA (53-9760-82, 1:1,000, Thermo Fisher, MA, United States), GPX4 (ab125066, 1:1,000, Abcam, MA, United States), and SLC7A11 (ab175186, 1:1,000, Abcam, MA, United States) were added to the membranes. The protein bands were visualized using a BeyoECL Plus kit (Beyotime, 0018 M, China). Relative gene expression was determined using ImageJ software (http://rsb.info.nih.gov/ij/), with GAPDH (AF7021, 1:3,000, Affinity, United States), β-actin (AF70181:1,000, Affinity, United States), tubulin (M20005, 1:1,000, Abmart, China) or vinculin (T40106, 1:500, Abmart, China) used as the internal loading proteins for normalization.

2.10 qRT-PCR assay

TRIzol reagent (TaKaRa) was used to extract total RNA. cDNA was measured using a NanoDrop spectrophotometer (Thermo Fisher, MA, United States). Using a Roche Transcriptor cDNA Synth. Using a kit (Roche, GERMAN), 200 ng of RNA was reverse-transcribed into first-strand cDNA. The FastStart Universal SYBR Green Master Mix (Rox) (Roche GERMAN.) was then used on a Step One Plus Real-time PCR System (Applied Biosystems, Carlsbad, CA, United States). The internal loading of mRNA was performed with β-actin and GAPDH; the fold change in gene expression was computed using the $2^{-\Delta\Delta CT}$ method. A PCR array (wc-Mrna0271-H) was used to determine which mRNAs related to ferroptosis in KF were affected by ADSC-Exos.

2.11 A nude mouse model was established

Twenty-four 4-week-old nude mice (20 ± 5 g) purchased from Harbin Medical University were individually maintained in conventional animal rooms with free access to chow and water. After 3 days of adaptation, sliced fresh keloid tissue (1 cm^3) was embedded in nude mouse dorsalis, as previously reported. The mice were first injected intraperitoneally (IP) with 50 mg/kg pentobarbital and 10 mg/kg xylazine. The incision was sutured with suture-free glue. In subsequent

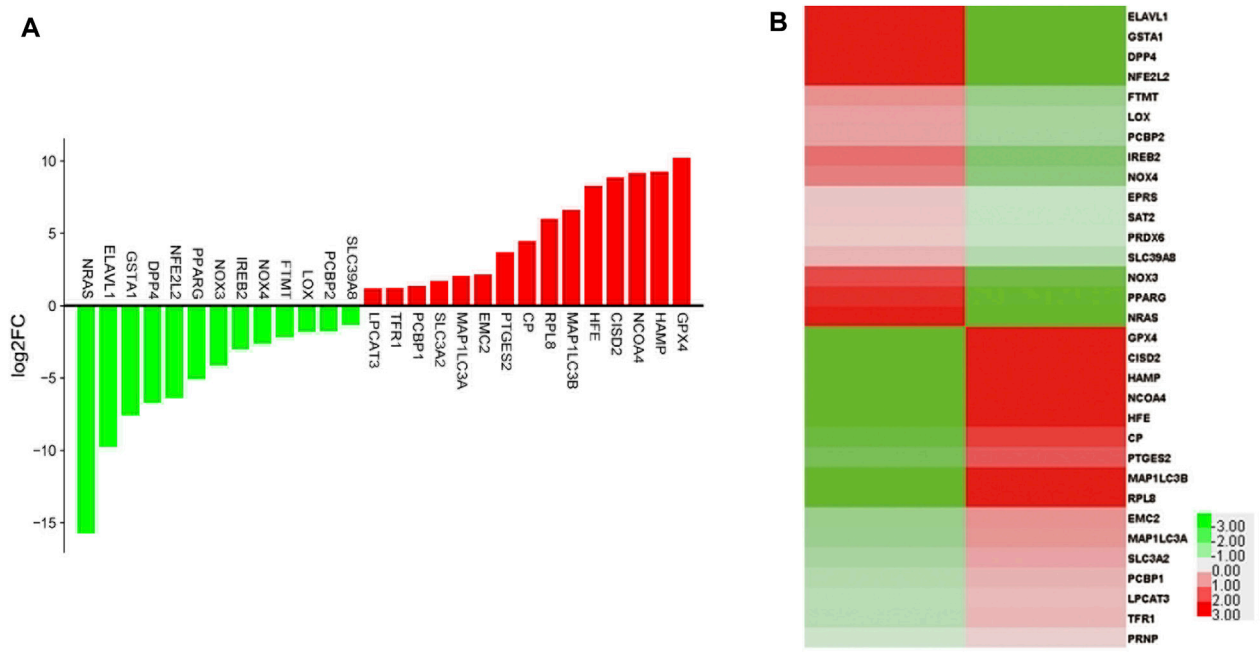

FIGURE 3
Twenty-eight DEGs were identified in KF cocultured with ADSC-Exos and in the control group by ferroptosis-related qPCR array. **(A)** Histogram and **(B)** heatmap of gene expression in KF cocultured with ADSC-Exos and control cells.

procedures, the mice were separated into three groups, with 8 mice in each group until 28 d, when the tissue was stable. Then, 200 μg of ADSC-Exos and ADSC-Exos + Erastin (1.25 mg) dissolved in PBS, as well as an equal volume of PBS solution, was injected into the interior of the keloids and injected radially into the backs of the nude mice; this process was repeated every 3 days.

Histological analysis of the mice was terminated 21 days after keloid implantation with an overdose of sodium pentobarbital (150 mg/kg, i.H.). Keloid tissue was fixed in 10% formalin at 4°C, and gradient dehydration was performed using ethanol. The tissue was embedded in paraffin wax and sectioned. HE and Masson's trichrome staining kits (Solarbio, Beijing, China) were used to assess tissue fibrosis. After staining, the sections were photographed with an attached digital camera and examined microscopically (Olympus, Japan). ImageJ software was used for quantitative analysis. LPO and MDA were used to calculate the levels of lipid peroxides and metabolites in keloid tissues from each group. The GSH content was a significant component for assessing the antioxidant capacity of each group.

After being washed with various ethanol concentrations, Keloid explant specimens have been deparaffinized and rehydrated with xylene. Antigen retrieval was conducted by microwaving the sections in an antigen retrieval solution for 10 min. Sections were cleaned and shaded, incubated in 3% hydrogen peroxide for 10 min at room temperature, followed by 10% goat serum for 10 min. Subsequently, antibodies against collagen I, collagen III, α-SMA, and GPX4 that segment were applied to the slides at a dilution of 1:50 with PBS. The slices were nuclear staining with DAPI (H-1200, Vector Laboratories, Burlingame, CA, United States) after being incubated for 1 h at room temperature with a secondary antibody solution. All slides were counterstained with hematoxylin (Cat. No.H8070, Solarbio, China) and imaged with a microscope.

2.12 siRNA transfection

SLC7A11-targeting siRNA and scrambled control siRNA were purchased from Gene Pharma. Lipofectamine 3000 (Invitrogen) was used to transfect the cells with siRNA according to the manufacturer's instructions. KF were seeded in 6 healthy plates, followed by transfection with 10 nM SLC7A11-targeting siRNA (si-SLC7A11) or nc-RNAi control (si-NC) for 24, 48, or 72 h. The transfection efficiency was determined by observing the position of liposomes under a fluorescence microscope. The transfection efficacy was assessed by examining the position of liposomes under a fluorescence microscope. The results are represented as a percentage of the absorbance ratio between treated and control cells during qRT-PCR verification of gene knockout status.

2.13 Statistics

GraphPad Prism 9 (GraphPad Inc., La Jolla, CA, United States) was used for data analysis. Data from three or more experiments were collected and are presented as the mean ± SEM. Student's *t*-test was used to determine the significance of the differences between the two groups. Three or more independent control and experimental samples were evaluated

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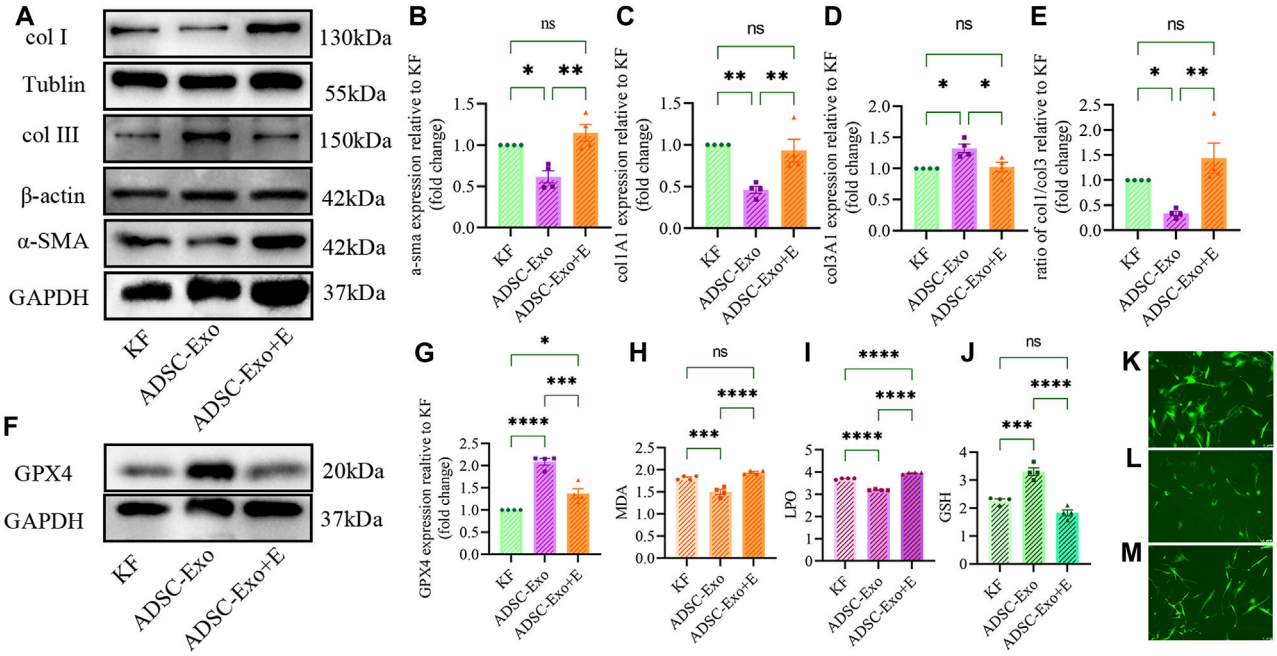

FIGURE 4
ADSC-Exos upregulated the GPX4-GSH axis and inhibited collagen synthesis and fibronectin *in vitro*. **(A)** Western blot images of fibrosis in KF cocultured with ADSC-Exos and ADSC-Exos + Erastin. **(B)** Quantified expression of α-SMA, **(C)** col1A1, **(D)** col3A1, **(E)** the ratio of col1A1 to col3A1; **(F)** Western blot images of GPX4 in KF cocultured with ADSC-Exos and ADSC-Exos + Erastin. **(G)** Quantification of GPX4 and **(H)** MDA levels. **(I)** LPO and **(J)** GSH relative to KF (n = 4). **(K–M)** Representative images of the detection of ROS. **(K)** KF, **(J)** KF cocultured with ADSC-Exos. **(M)** KF cocultured with ADSC-Exos + Erastin.The data are shown as the mean ± SEM. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$, **** represents $p < 0.0001$, ns represents no significance.

using one-way ANOVA. A p-value < 0.05 indicated statistical significance.

3 Results

3.1 Keloid fibrosis was induced by ferroptosis

Transmission electron microscopy revealed characteristic changes in ferroptosis in keloid tissues. In this study, we compared the expression of ferroptosis genes in KF and normal fibroblasts. The gene and protein expression levels of GPX4 and GSH in keloids are lower than those in normal skin. Our results showed that lipid peroxide and malondialdehyde levels in tissues or cells from keloids were greater than those in tissues or cells from normal skin. The expression of oxidative stress-related target genes related to ferroptosis, such as PTGS2 (COX2), NRF2, and nuclear factor E2-related factor 2 (Nrf2), was detected (Supplementary Material S1).

Erastin can activate the fibrogenic ability of KF. Increased the expression of type I collagen and α-SMA but decreased that of type III collagen, promoting the ratio of type I collagen to type III collagen in keloid centers, facilitating the formation of fibrosis in keloids (Figures 1A–E).

By activating ferroptosis, Erastin can increase the levels of liposomal peroxidation (LPO) metabolites, increase malondialdehyde (MDA) levels, decrease GSH levels, and decrease the expression of the GPX4 protein. However, fer-1 had the opposite effect on Erastin (Figures 1F–J).

3.2 Characterization of ADSCs and ADSC-Exos

First, the acquired ADSCs were negative for CD45, CD14, and CD45 but positive for the MSC surface indicators CD29, CD44, and CD105. These findings suggested that the ADSCs were suitable for further application (Figure 2A). The capacity of the ADSCs to differentiate into osteoblasts and idioblasts was further validated by alizarin red staining and Oil red O staining, respectively (Figures 2B, C). The particles that were removed from the ADSCs were also identified. The particles were observed to have a characteristic oval shape via TEM (Figure 2D). The NTA results revealed that the sizes of the isolated particles were primarily in the 100–170 nm range (Figure 2E). TSG101, CD9, and CD63 are recognized exosome markers that were further analyzed by Western blot analysis (Figure 2F). ADSC-Exos were tagged with PKH67 and cocultured with KF. After 24 h, ADSC-Exos were removed and delivered to the cytoplasm of KF (Figures 2G–I). These results indicate that ADSC-Exos were effectively separated and transferred to KF.

3.3 ADSC-Exos alleviated KF fibrosis by inhibiting ferroptosis

The differential gene expression between the ADSC-Exos and control groups was compared by ferroptosis PCR array. Compared with that in the untreated group, the expression of GPX4, the core gene involved in ferroptosis, was greater (Figure 3). Surprisingly, we

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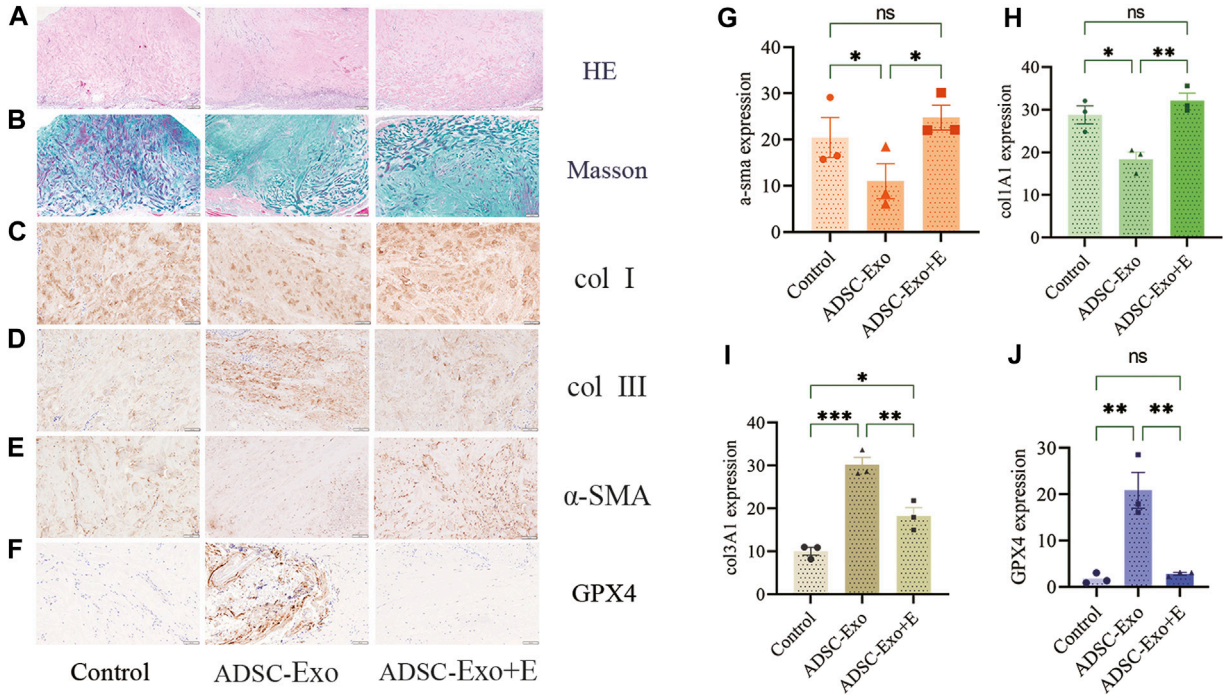

FIGURE 5
ADSC-Exos upregulated the GPX4 axis and inhibited collagen synthesis and fibronectin *in vivo*. The tissues on the backs of the mice treated with PBS, ADSC-Exos, or ADSC-Exos + Erastin were collected on day 21 postintervention. Typical histological images of keloid tissues stained with **(A)** H&E and **(B)** Masson's trichrome. Immunohistochemical images of **(C)** col1A1, **(D)** col3A1, **(E)** α-SMA, and **(F)** GPX4. Quantification of the expression of **(G)** col1A1, **(H)** col3A1, **(I)** α-SMA, and **(J)** GPX4 (n = 3). The data are shown as the mean ± SEM. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$.

discovered that GPX4 was significantly differentially expressed between the keloid and normal skin groups, indicating that ADSC-Exos could be a novel but crucial target for preventing the occurrence and development of KF fibrosis.

ADSC-Exos, which resembles fer-1, can restrain the fibrogenic process of KF, decreasing the expression of type I collagen and α-SMA while decreasing the expression of type III collagen, reducing the ratio of type I collagen to type III collagen in keloids (Figures 4A–E). In contrast, GPX4 protein expression was increased, and KF fibrosis was alleviated by ADSC-Exos. Erastin prevents this therapeutic effect. The accumulation of MDA and LPO, on the other hand, was reduced by ADSC-Exos, increasing the expression of GSH in the KF (Figures 4F–J). The intracellular ROS level was decreased by ADSC-Exos. Erastin prevents all the therapeutic effects of ADSC-Exos by activating ferroptosis (Figures 4K–M).

3.4 ADSC-Exos alleviated pathological keloid injury *in vivo*

Using a nude mouse keloid model, we investigated the therapeutic efficacy of ADSC-Exos. The therapeutic impact of ADSC-Exos on keloid pathology was observed using H&E and Masson staining. Figures 5A, B depicts the usual histological alterations of keloids in each model category. After constructing the keloid model, we examined the expression of collagen I, collagen III, α-SMA, and GPX4 after 21 days. Immunohistochemical labeling (Figures 5C–J) confirmed the findings of Western blot analysis (Figure 6): the expression of collagen I and α-SMA and the ratio of collagen I to collagen III were significantly lower in the keloid region treated with ADSC-Exos than in the control group or the ADSC-Exo + Erastin group. Figures 6F–J shows the increase in the expression of GPX4 and GSH and the decrease in the accumulation of LPO and MDA induced by ADSC-Exos *in vivo*. However, Erastin impeded the effect of ADSC-Exos.

3.5 ADSC-Exos inhibits fibrosis in keloids by promoting SLC7A11-GPX4 *in vitro*

To further explore the role of ADSC-Exos in keloid fibrosis, we investigated how ADSC-Exos protects against keloid iron sagging. Here, more attention has been given to the regulation of the SLC7A11-GPX4 pathway. ADSC-Exos were cocultured with SLC7A11-silenced KF. Compared with those of the controls, ADSC-Exos and ADSC-Exos cocultured with si-NC KF inhibited the expression of fibrosis-related genes in keloids and increased the protein expression of SLC7A11 and GPX4. At the same time, SLC7A11 silenced ADSC-Exos and antagonized this effect (Figure 7).

4 Discussion

Keloids are pathological scars with a high incidence rate. It can not only cause pain and itching but also affect the patient's mental

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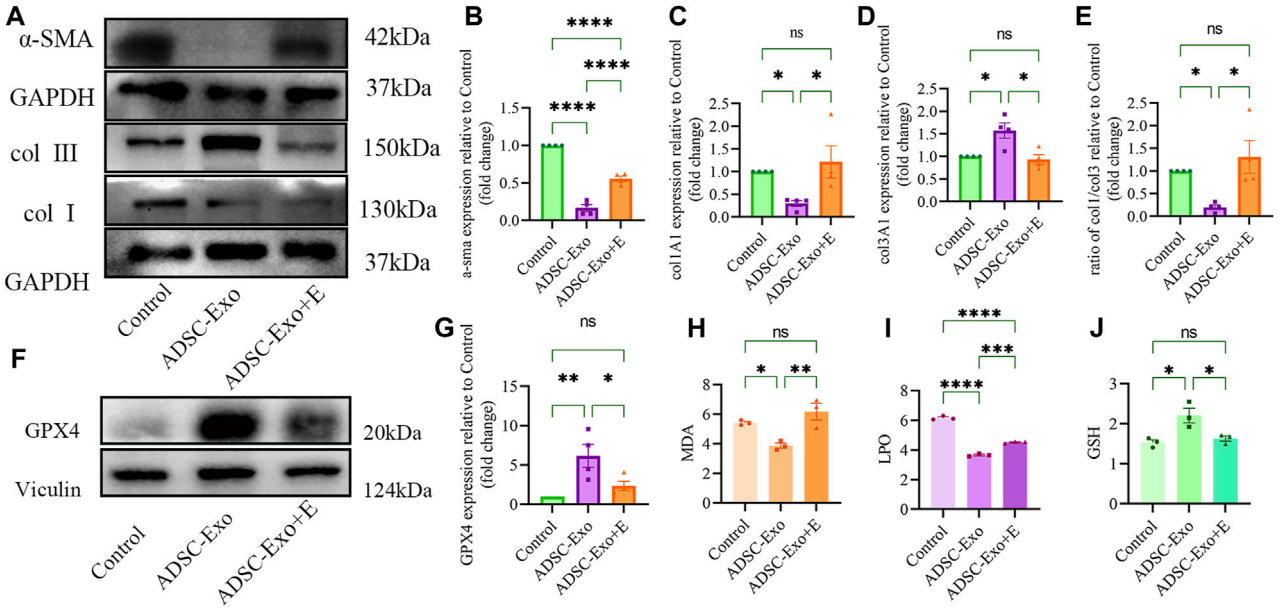

FIGURE 6
ADSC-Exos upregulated the GPX4 axis and inhibited collagen synthesis and fibronectin *in vivo*. **(A)** Western blot images of the control group, ADSC-Exo group and ADSC-Exos + Erastin group. **(B)** Quantified expression of α-SMA, **(C)** col1A1, **(D)** col3A1, **(E)** the ratio of col1A1 to col3A1 (n = 4); **(F)** Western blot images of GPX4 of the control group, ADSC-Exo group and ADSC-Exos + Erastin group. **(G)** Quantification of GPX4 (n = 4) and **(H)** MDA levels. **(I)** LPO and **(J)** GSH relative to KF (n = 3).

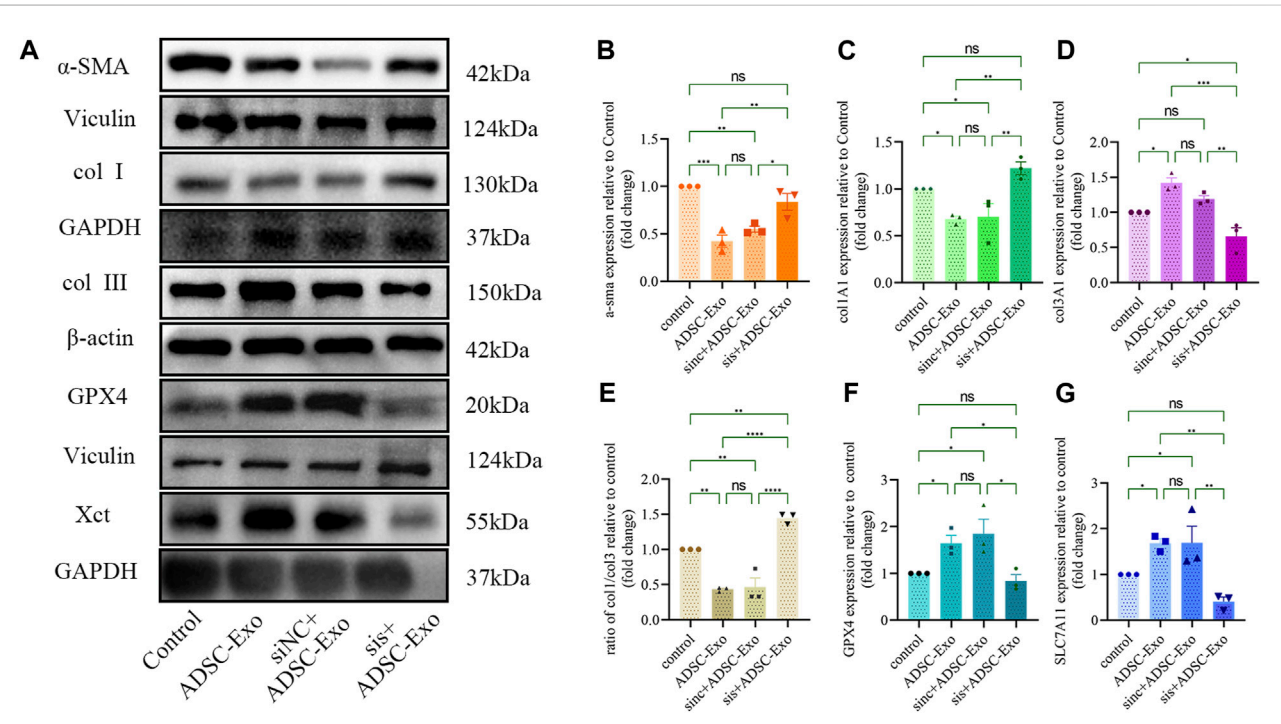

FIGURE 7
ADSC-Exos upregulated the SLC7A11-GPX4-GSH axis and inhibited collagen synthesis, fibronectin, and ferroptosis-related genes *in vitro*. **(A)** Western blot images of the control group, ADSC-Exo group, sinc + ADSC-Exo group, and sis + ADSC-Exo group. **(B)** Quantification of the expression of **(B)** α-SMA, **(C)** col1A1, **(D)** col3A1, **(E)** the ratio of col1A1 to col3A1, **(F)** GPX4, and **(G)** SLC7A11 relative to the control (n = 3). The data are shown as the mean ± SEM. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$, **** represents $p < 0.0001$, ns represents no significance.

state and quality of life. In severe cases, it can even affect affected limb function (Fu et al., 2024). Therefore, studying the mechanism underlying the formation and prevention of pathological scars is a hot topic in the medical field (Xu et al., 2022). Additionally, exploring a treatment method that can target the pathogenesis of keloids is the key to solving this problem.

Emerging research suggests that ferroptosis can modulate fibrosis (Qiu et al., 2024). Several genes influence keloid development. Although there are differences in gene expression between keloid and normal skin fibroblasts, the exact etiology of iron deficiency remains unexplained. In this study, we compared the expression of ferroptosis genes and metabolic products of iron in keloid and normal skin. The results showed that keloid fibrosis was associated with a reduction in GPX4 and GSH, which could not prevent the accumulation of lipid metabolite products during ferroptosis progression in keloids. Our research provides some evidence confirming the relationship between ferroptosis and the potential mechanism of keloid formation.

Exosomes from human adipose-derived mesenchymal stem cells can lower the activation of the fibrosis signaling system by preventing myofibroblast formation and increasing the level of transforming growth factor. Furthermore, by activating the ERK/MAPK pathway, ADSC-Exos increases the expression of matrix metalloproteinase-3 (MMP3) in dermal fibroblasts, resulting in a high ratio of MMP3 to tissue inhibitor of matrix metalloproteinase-1 (TIMP1), which is conducive to extracellular matrix (ECM) remodeling (Wang et al., 2017). The number of myofibroblasts increases during keloid repair. α-SMA is a myofibroblast differentiation marker that promotes myofibroblast release and wound healing. In our study, the exosomes of adipose-derived stem cells decreased the levels of α-SMA and collagen I and the ratio of type I to III collagen. Keloid is a dermal fibroproliferative tumor that can be recognized by excessive ECM accumulation. The ratio of Col1/Col3 is believed to improve in the later stage of ECM reshaping (Peeters et al., 2014). The ratio of type I to III collagen in fibroblasts in keloid tissue was greater than that in normal skin ($p < 0.05$) (Zhang et al., 2009). Collagen I is a stiff fibrillar protein that provides tensile strength (You et al., 2023), whereas collagen III forms an elastic network and stores elastic rebound kinetic energy (Wang et al., 2022). Our findings suggest that ADSC-Exos can transform thick and stiff collagen fibers into slender and elastic fibers in the dermis, which is likely to promote the development of keloids into normal skin. However, more research needs to be conducted on this topic.

Like fer-1, ADSC-Exos decreased fibrosis via ferroptosis in KF, decreased lipid peroxidation, and increased GPX4 and GSH expression. Erastin can promote ferroptosis in keloids and decrease the functionality of ADSC-Exos, accompanied by excessive fibrosis. These results suggest a new possible mechanism by which ADSC-Exos inhibits the myofibroblast differentiation of KF by decreasing ferroptosis in keloids.

The activity of GPX4 is dependent on the activation of the cystine transporter SLC7A11 (Bayir et al., 2023; Wang H. et al., 2023). We hypothesized that the increase in GPX4 signaling in keloids may be mediated by exosomes that increase the expression of SLC7A11 in keloids. By knocking out SLC7A11 in KF, the anti-ferroptosis or anti-fibrosis effects of ADSC-Exos were antagonized. Our results indicate that ADSC-Exos are involved in inhibiting myofibroblast differentiation and collagen production in KF by activating the SLC7A11-GPX4 signaling pathway to reduce ferroptosis.

The limitation of our study is that although an increase in SLC7A11-GSH-GPX4 was observed in KF treated with ADSC-Exos, it was not easy to detect the exact type of lipid. In subsequent studies, we combined ferroptosis-related oxidative lipidomics and keloids and further explored lipid metabolism after treatment with ADSC-Exos, accelerating the clinical transformation of ADSC-Exos.

In conclusion, iron metabolism disorder-induced ferroptosis is involved in the pathogenesis and persistent activation of myofibroblasts in KF. ADSC-Exos regulates GPX4 in KF and suppresses keloid fibrosis *in vitro* via SLC7A11-GPX4. By suppressing keloids, ADSC-Exos, and ferroptosis can become viable therapeutic targets.

5 Conclusion

Restraining ferroptosis can enhance the anti-fibrotic effect of keloid cells. ADSC-Exos can significantly reduce the degree of fibrosis in keloids by inhibiting ferroptosis. By regulating the occurrence of the SLC7A11-GPX4 signaling pathway, it can inhibit ferroptosis in keloid cells, thereby reducing fibrosis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University, China. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal studies were approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University, China. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YT: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. ML: Writing–review and editing, Investigation, Methodology, Project administration. RC: Writing–review and editing, Conceptualization, Data curation, Formal Analysis. XC: Resources, Software, Writing–review and editing. ZX: Project administration, Resources, Validation, Writing–original draft. JY: Conceptualization, Data curation, Investigation, Resources, Writing–original draft. ZD: Funding acquisition, Resources, Supervision, Writing–original draft, Writing–review and editing. LH: Conceptualization, Funding acquisition, Methodology, Project

administration, Validation, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1431846/full#supplementary-material

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Research progress of knee fibrosis after anterior cruciate ligament reconstruction

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Anterior cruciate ligament (ACL) injury is a common sports injury, and ACL reconstruction is an effective surgery for this trauma. Most cases gain good recovery after surgery, while some patients may experience knee stiffness, which is characterized by joint fibrosis, leading to reduced joint mobility, pain, and dysfunction. Currently, various research studies have been conducted to unveil the mechanisms underlying this condition, identifying pre-, intra-, and post-operative risk factors, and testify the efficacy of different therapeutic methods against it. In this review, we summarize the current progress regarding the advancements in knee fibrosis after ACL reconstruction. The risk factors associated with knee fibrosis are systematically delineated, accompanied by an evaluation of the efficacy of various treatment modalities for both the prevention and mitigation of fibrosis. Furthermore, recommendations for future research directions are proposed, offering a foundational basis for subsequent investigations.

KEYWORDS

anterior cruciate ligament injury, anterior cruciate ligament reconstruction, knee fibrosis, risk factors, treatment

1 Introduction

Anterior cruciate ligament (ACL) injury is a common sports-related knee injury among athletically active people (Chia et al., 2022). Arthroscopic reconstruction of the ACL is the prevalent therapy at present, with generally good recovery and a relatively low complication rate (Hanus and HudÁk, 2020). Still, knee fibrosis, intractable pain, hemarthrosis, fever, deep vein thrombosis, and infection may occur (Hanus and HudÁk, 2020). Knee fibrosis after ACL reconstruction poses a serious problem. According to the literature review, the prevalence of knee fibrosis after ACL reconstruction is 2.0%–35.0% (Eckenrode and Sennett, 2011). Knee fibrosis is characterized by an inflammatory and fibrotic response, which is manifested as a limited range of motion (ROM) and pain, affecting functional recovery (Millett et al., 2001). Knee arthrofibrosis is a joint disorder induced by an overactive inflammatory response. It is characterized by knee pain and decreased range of motion, resulting in impaired joint function. This not only causes great pain and a heavy medical burden for patients but also has a negative impact on the recovery process and long-term prognosis. To improve postoperative outcomes, it is essential to understand the mechanisms, risk factors, and treatment approaches associated with knee fibrosis following ACL reconstruction.

2 Pathophysiological mechanisms of knee fibrosis

Knee fibrosis is characterized by the uninhibited deposition of extracellular matrix proteins around the joint, resulting in symptomatic joint stiffness. Fibrosis is the final common pathway of many chronic inflammatory injuries and is a pathological feature of almost all organ diseases (Lee et al., 2022). This article discusses several possible pathological mechanisms, such as inflammatory response, activation and differentiation of fibroblasts, remodeling of the extracellular matrix, and abnormal proliferation of synovial cells in joints (Bayram et al., 2020). In addition, some articles have pointed out that the occurrence of connective tissue fibrosis is multifactorial, including immune cell infiltration caused by tissue damage and the involvement of a series of mediators, such as transforming growth factor-β (TGF-β), bone morphogenetic protein, connective tissue growth factor, and interleukin (Usher et al., 2019; Disser et al., 2023). TGF-β is the pivotal driver of fibrosis, resulting in the activation of fibroblasts and the migration of exogenous cells invading from outside of the tissue. It is a key factor in the regulation of fibroblast proliferation and collagen deposition (Usher et al., 2019). Many of these cells are defined as myofibroblasts, which can produce high levels of alpha-smooth muscle actin and lead to upregulation of collagen synthesis. The excessive activation of immune cells, signaling molecules, and myofibroblasts leads to unresolved post-injury inflammation, which in turn leads to the dysregulation of normal regenerative pathways and formation of fibrous scars (Bayram et al., 2020; Disser et al., 2023). A related report examines the molecular pathological features of human knee fibrosis using RNA sequencing (Jovic et al., 2022). In patients with knee fibrosis, members of the collagen family are commonly expressed as extracellular matrix-related genes, among which COL1A1, COL3A1, and COL6A1 are consistent with fibrosis characteristics (Disser et al., 2023; Morita et al., 2016; Theocharidis et al., 2016; Tao et al., 2018; Samokhin et al., 2018). In addition, integrins are another prominent family in the gene family associated with extracellular matrix organization, and the role of integrins in fibrosis has been confirmed (Disser et al., 2023; Kuivaniemi and Tromp, 2019). Moreover, LOX genes also play a potential role in fibrosis development (Disser et al., 2023; Schnittert et al., 2018). These findings provide new targets for diagnosis and drug therapy.

3 Risk factors for knee fibrosis

Knee fibrosis is a multifactorial disease, and its risk factors run through the preoperative, intraoperative, and postoperative periods. Understanding these risk factors can provide guidance for clinical intervention and improve recovery. Personalized treatment and rehabilitation programs are particularly important for patients with multiple risk factors.

3.1 Patient characteristics and preoperative risk factors

Studies have identified that factors such as female gender and older age are associated with an increased risk of revision operation after ACL reconstruction due to joint fibrosis. Female patients have a smaller femoral notch than male patients, indicating a structural difference in the joint that may predispose them to arthrofibrosis; older patients are also more prone to chronic injury, which, when combined with degenerative changes, may result in elevated inflammation (Hopper et al., 2024; Haley et al., 2023).

The timing of surgery after ACL injury is suspected to be relevant to the risk of joint stiffness and fibrosis (Freshman et al., 2023) since inflammatory mediators are present in the synovial fluid during the first week after ACL injury (Aman et al., 2024; Kingery et al., 2022; Haslauer et al., 2014). This belief is supported by the evidence that ACL reconstruction performed at least 6 weeks after injury can significantly reduce the risk of surgical intervention for subsequent knee fibrosis (Agarwal et al., 2023). However, this finding was not supported by recent evidence (von Essen et al., 2020). Given these controversial reports, Vermeijden et al. (2023) conducted a systematic review and identified that early surgery is not inferior to delayed surgery regarding knee fibrosis after isolated ACL reconstruction.

The application of anticoagulants is also related to joint fibrosis. Qin et al. found that, compared with patients who did not use thromboprophylaxis, those who took this medication were significantly associated with arthrofibrosis after subsequent surgery (Qin et al., 2021). Thromboprophylaxis results in increased rate of postoperative hematoma and, consequently, inflammatory cytokines within the joint, which may lead to fibrosis. Preoperative knee restriction is a well-established risk factor for arthrofibrosis (Mayr et al., 2004). Therefore, preoperative medication and the limited range of motion should be considered when making surgical plans to reduce the risk of joint fibrosis. In addition, other studies have found that preoperative depression has a negative impact on postoperative pain and functional recovery (García et al., 2024). Patients with preoperative depression have significantly higher pain interference scores and significantly lower physical function scores before and after surgery. At present, many scholars have found that there is a certain relationship between knee joint fibrosis and genetic factors (Skutek et al., 2004; Dagneaux et al., 2020). Comorbidities, including but not limited to type 2 diabetes mellitus, ankylosing spondylitis, and rheumatoid arthritis, are also found to increase the risk of knee fibrosis (Huang et al., 2013; Owen et al., 2021).

3.2 Intraoperative risk factors

At present, the autograft options for ACL reconstruction include bone–patellar tendon–bone (BTB), hamstring tendon, and quadriceps tendon. An analysis of 378 patients found that the incidence of knee joint fibrosis with BTB grafts was approximately 10.0%, compared to 1.9% with hamstring tendons and 6.3% with quadriceps tendons (Ouweleen et al., 2021). This phenomenon is suspected to be a consequence of higher collagen content in BTB grafts (Huleatt et al., 2018). Previous studies have suggested a link between graft type and knee fibrosis. Nwachukwu et al. (2011) found that using an autologous patellar tendon was a risk factor for arthrofibrosis after ACL reconstruction (Nwachukwu et al., 2011). Furthermore, Sanders et al. (2017) found that using

allografts lowered the likelihood of arthrofibrosis as compared to bone-patellar tendon-bone grafts. Other studies noticed that a femoral tunnel diameter less than 9.25 mm was associated with a reduced risk of joint fibrosis compared to its counterpart in male patients (Haley et al., 2023).

In relation to graft tension, some believe that increasing graft tension creates excessive constraints on the joint and results in loss of movement (Elias et al., 2009). However, studies have shown that although high graft pretension may cause graft wear in the femoral tunnel, it does not lead to complete loss of knee extension (Markolf et al., 1996). Conversely, inadequate graft tension may lead to anterior–posterior laxity, resulting in instability, poor graft healing, and failure (McDermott et al., 2024; Lee et al., 2018; Magit et al., 2007). Increasing the tension of the graft reduces the postoperative loss of tension and mobility due to viscoelasticity. This means that by increasing the tension of the graft, postoperative knee laxity can be reduced. Therefore, there is a relationship between graft tension and knee stiffness, yet there is no clear answer as to whether increasing or decreasing graft tension leads to loss of motion.

In addition, the effect of bone tunnel position and graft placement on fibrosis during ligament reconstruction is important. Placing ACL grafts in anatomical positions can reduce the risk of joint stiffness, while placing ACL grafts in non-anatomical positions may lead to higher rates of fibrosis (Yaru et al., 1992; Tanksley et al., 2017; Romano et al., 1993; Śmigielski et al., 2016; Vignos et al., 2020; Markolf et al., 2002). Multiple studies have found that ACL reconstruction combined with meniscus repair surgery increases the risk of knee fibrosis (Hopper et al., 2024; Haley et al., 2023; Huleatt et al., 2018). Meniscal repair often requires fixation to the joint capsule, which may limit the range of motion of the knee, thus increasing the risk of fibrosis. Moreover, the increase in intra-articular blood loss is also linked to a higher rate of joint fibrosis (Karaaslan et al., 2015).

3.3 Postoperative risk factors

Non-standard or excessive postoperative rehabilitation training and postoperative infection may lead to further injury in the joints and increase the risk of fibrosis. Some studies have found that different postoperative weight-bearing protocols (delayed weight-bearing, progressive weight-bearing, and immediate weight-bearing) have different complication rates, among which the delayed weight-bearing protocol has the highest risk of developing stiffness (Morris et al., 2021). Furthermore, reports have pointed out that patients who undergo progressive rehabilitation training after ACL reconstruction surgery have knee function, range of motion, and muscle strength (Grindem et al., 2015; Noyes et al., 2000). The application of a brace can also contribute to the prevention of knee stiffness following ACL reconstruction (Skalsky and McDonald, 2012), while a brace in the hyperextension position for at least 3 weeks was more effective in preserving extension function (Melegati et al., 2003).

4 Treatments

Treatments are mainly non-surgical and surgical (Figure 1). Non-surgical treatment includes physical therapy and medication. In severe cases of fibrosis, arthroscopic surgery is required to restore joint mobility. Additionally, postoperative rehabilitation after secondary surgical release is still needed to avoid recurrence.

4.1 Non-surgical treatment

Low-level laser therapy (LLLT) and continuous passive motion (CPM) are commonly used physical therapies. Studies have shown that LLLT after ACL reconstruction can reduce the formation of joint contractures by inhibiting inflammation and fibrosis (Kaneguchi et al., 2019). LLLT has anti-inflammatory and anti-fibrotic effects and causes fewer adverse reactions (Kaneguchi et al., 2019; Zhang et al., 2022; Wickenheisser et al., 2019). Moreover, it is a low-cost treatment and is widely used for a wide range of inflammatory and fibrotic diseases (Zhang et al., 2022; Khansa et al., 2016; Soleimanpour et al., 2014). Similarly, CPM treatment can reduce the incidence of knee fibrosis after various knee surgeries (Bram et al., 2019; Haller et al., 2015; Harvey et al., 2010). A recent study using an animal model of ACL rupture showed that immediate CPM therapy has a chondroprotective effect against post-traumatic osteoarthritis (Chang et al., 2017). On the contrary, in two recently published systematic reviews regarding CPM on knee ROM after ACL reconstruction, no evidence is noticed to support the application of this method in the index knee after ACL surgery (Thrush et al., 2018; D'Amore et al., 2021). Therefore, further research is required to evaluate the potential utility of CPM in the long run.

Regarding medications, the main anti-inflammatory drugs used to treat knee fibrosis can be categorized into glucocorticoids and non-steroidal anti-inflammatory drugs (Usher et al., 2019; Liu et al., 2017). The most commonly used non-steroidal drug is aspirin. Aspirin inhibits the development of fibrosis through a variety of mechanisms (Xu et al., 2022; Peng et al., 2023). Aspirin inhibits NF-κB synthesis via IKK receptors and promotes the formation of stable and powerful specialized pro-resolving lipid mediators (SPMs) (Liu et al., 2017). It is possible that aspirin lowers the incidence of fibrosis by decreasing PI3K/AKT/mTOR (phosphorylated phosphatidylinositol 3 kinase, protein kinase B, and mechanistic target of rapamycin) and increasing autophagy (Peng et al., 2023). These mechanisms make aspirin the primary drug currently prescribed for the treatment of fibrosis. Both oral and intra-articular glucocorticoids have advantages and disadvantages in the treatment of joint fibrosis (Barel et al., 2010; Melgert et al., 2001). Oral glucocorticoids can reduce joint inflammation and pain through systemic circulation, but multiple doses are required to maintain the therapeutic effectiveness, which can cause systemic side effects. On the other hand, intra-articular injection can act directly on the inflammatory and fibrotic tissue, improving treatment efficacy and reducing systemic side effects.

By managing the pro-inflammatory and pro-fibrogenic pathways, bio-agents against fibrotic disorders have attracted increasing attention in recent years. Montelukast and Pranlukast are two cytoplasmic leukotriene receptor antagonists mainly used to treat respiratory diseases such as asthma and allergic rhinitis (Wenzel, 1998; Huang and Handel, 2010; Menkü Özdemir et al., 2022; Lynch et al., 1999). In the treatment of arthrofibrosis, these two drugs show therapeutic potential in reducing the postoperative

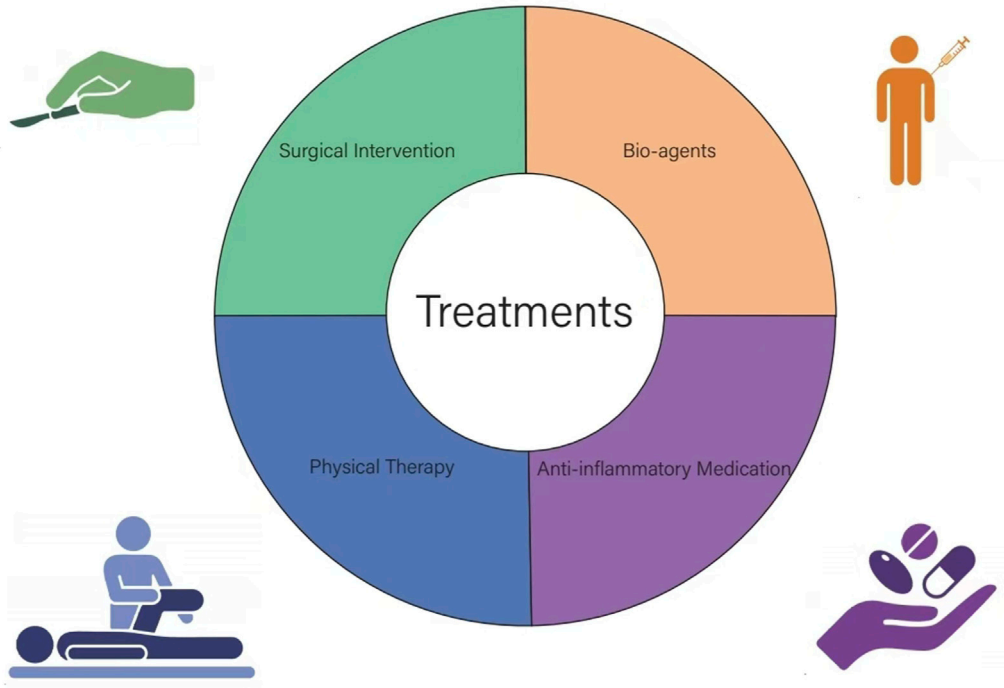

FIGURE 1
Treatments of knee fibrosis after ACL reconstruction.

inflammatory response after joint surgery (Chen et al., 2024). Relaxin-2 (RLX-2) is an endogenous anti-fibrotic peptide that is capable of alleviating TGF-β-induced myofibroblast differentiation (Wang et al., 2016; Samuel et al., 2016; Shabanpoor et al., 2012; Sassoli et al., 2013), and thus is used as an anti-fibrotic agent in knee contracture after ACL reconstruction. However, a major obstacle to the clinical translation of RLX is its short half-life (Metra et al., 2019; Khanna et al., 2009; Weiss et al., 2016), which requires further investigations regarding effective delivery modalities. Botulinum toxin type A is currently used as an anti-fibrotic agent for adhesive capsulitis (Blessing et al., 2021; Khenioui et al., 2016; Chen et al., 2011) and is observed to reduce scar formation in animal models of knee fibrosis (Namazi and Torabi, 2007; Gao et al., 2017). Platelet-rich plasma also has potential against joint fibrosis (Araya et al., 2020; Lin et al., 2023). Intra-articular delivery of hyaluronic acid is also a good method for treating knee fibrosis in animal models, while there are few clinical trials testing the efficacy of knee stiffness after ACL reconstruction (Kanazawa et al., 2015; Qu et al., 2023). In addition, vitamin D and angiotensin II receptor antagonists have also been successfully used under different fibrosis conditions and are becoming ideal candidates for joint fibrosis (Jagodzinski and Traut, 2022).

4.2 Surgical treatment

Surgical intervention for fibrosis mainly includes manual release under anesthesia (MUA) and arthroscopic lysis of adhesions (LOA). Patients who did not reach a full extension by 3 months postoperatively, defined as lacking 10°, and had a symptomatic difference in the range of motion relative to the unaffected knee were eligible for MUA or LOA. If MUA did not provide a sufficient range of motion, arthroscopy with LOA was recommended instead (Crabtree et al., 2023). MUA is also commonly used to treat knee fibrosis, either alone or in combination with arthroscopy (Crabtree et al., 2023; Baghdadi et al., 2022). For severe fibrosis, soft tissue release via LOA is still the recommended option. By removing the excessive extracellular matrix, LOA can not only relieve the joint movement restriction but also dilute the concentration of intra-articular pro-fibrotic mediators, thus blocking the vicious cycle formed by the ECM (Sanders et al., 2017; Lamba et al., 2023).

Arthroscopic LOA and MUA are safe and effective treatments for the postoperative fibrosis of the knee (Fackler et al., 2022). However, both techniques have complications. These surgical procedures may lead to neurological and vascular disorders, fractures, ligament relaxation, etc (Pivec et al., 2013; Egol et al., 2005; Laskin and Beksac, 2004; Fisher and Shelbourne, 1993). Therefore, careful pre-operative planning is necessary in facilitating knee function after the operation.

In current clinical practice, the prevention of fibrosis development is still challenging. If a physician or surgeon identifies a trend toward knee stiffness, interventions such as physiotherapy regimens and antifibrotic or anti-inflammatory medication can be considered. However, given the lack of evidence-based decision-making, the establishment of a sequential prevention method is still in progress.

Currently, there are studies on the treatment of arthrofibrosis, but reports are still in the basic research stage. In the future, one can consider exploring the mechanism of occurrence and development from the perspectives of molecular biology and genetics, while also searching for new biomarkers and therapeutic targets to facilitate early diagnosis and intervention. In addition, personalized

rehabilitation programs and prevention strategies based on specific patient characteristics can be developed to improve efficacy.

5 Conclusion

Knee fibrosis after anterior cruciate ligament reconstruction is a complex complication involving multiple risk factors. Early identification and intervention are essential in preventing or treating this condition. Conservative treatment may be useful in the early stages of joint fibrosis, while secondary surgery should be considered in the advanced stage. Determining the appropriate treatment plan requires assessment and decision-making by the physician based on the patient's specific situation. Future research is still required to explore the biological mechanisms and establish risk models to predict the occurrence of this condition, thereby improving the prognosis of patients after ACL reconstruction.

Author contributions

YL: writing–original draft. QZ: writing–review and editing. YF: writing–original draft and writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Decoding tumor-fibrosis interplay: mechanisms, impact on progression, and innovative therapeutic strategies

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Malignant tumors are a category of diseases that possess invasive and metastatic capabilities, with global incidence and mortality rates remaining high. In recent years, the pivotal role of fibrosis in tumor progression, drug resistance, and immune evasion has increasingly been acknowledged. Fibrosis enhances the proliferation, migration, and invasion of tumor cells by modifying the composition and structure of the extracellular matrix, thereby offering protection for immune evasion by tumor cells. The activation of cancer-associated fibroblasts (CAFs) plays a significant role in this process, as they further exacerbate the malignant traits of tumors by secreting a variety of cytokines and growth factors. Anti-fibrotic tumor treatment strategies, including the use of anti-fibrotic drugs and inhibition of fibrosis-related signaling pathways such as Transforming Growth Factor-β (TGF-β), have demonstrated potential in delaying tumor progression and improving the effectiveness of chemotherapy, targeted therapy, and immunotherapy. In the future, by developing novel drugs that target the fibrotic microenvironment, new therapeutic options may be available for patients with various refractory tumors.

KEYWORDS

fibrosis, tumor, CAFs, TGF-β, EMT

1 Introduction

Malignant tumors are a category of abnormal cellular proliferation diseases characterized by invasiveness and metastatic potential. In 2022, it was estimated that there were 20 million new cases and 9.7 million deaths worldwide (Bray et al., 2024). Although cancer treatment methods, such as surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, have continuously advanced, the complexity and heterogeneity of the disease make it challenging to cure, posing a significant global public health problem (Hirsch et al., 2017). Hanahan stated that the progression of tumors involves more than just an increase in tumor cell numbers and must be understood within the framework of the "tumor microenvironment (TME)." (Hanahan and Weinberg, 2011) TME is a complex system composed of tumor cells, stromal cells, immune cells, blood vessels, and the extracellular matrix (ECM). A growing body of research indicates that the TME is vital in the growth, invasion, metastasis, and treatment resistance of multiple tumors. Additionally, tumor cells can secrete cytokines and growth

factors, inducing stromal cell reprogramming to regulate the TME, providing new perspectives for clinical treatment (Ding et al., 2024; Mao et al., 2024; Nie et al., 2024).

Fibrosis is defined as the excessive accumulation of ECM components, like collagen, resulting in abnormal alterations in tissue structure and function. It is a chronic and progressive process, typically linked to prolonged inflammation and damage (Rimal et al., 2022). Additionally, fibrosis, as an essential part of the TME, is mainly manifested by the excessive deposition of ECM and the abnormal activation of stromal cells, including tumor-associated fibroblasts (CAFs) and myofibroblasts (Rimal et al., 2022). With deeper research, the intricate interactions between fibrosis and tumors are increasingly being clarified. Fibrosis facilitates tumor cell proliferation, invasion, and immune evasion (Thomas and Radhakrishnan, 2019; Metcalf et al., 2022). Additionally, tumor cells further aggravate fibrosis by secreting pro-fibrotic factors and inducing chronic inflammatory responses (Wu et al., 2020; Giarratana et al., 2024). Anti-fibrotic treatments, including anti-TGF-β therapy and targeting CAFs, have demonstrated important potential in the treatment of malignant tumors (Mohapatra et al., 2022; Li J. et al., 2023). Thus, comprehending the interaction mechanisms between fibrosis and different tumors is crucial for further research, developing novel therapeutic strategies, and enhancing cancer treatment efficacy.

2 Mechanisms that promote fibrosis in malignant tumors

2.1 The function of CAFs

2.1.1 Pro-fibrotic factors induce the activation of CAFs

Pro-fibrotic factors are vital in the activation and transformation of CAFs. Malignant tumor cells secrete pro-fibrotic factors (like TGF-β and PDGF), which can directly induce the transformation of fibroblasts into CAFs. CAFs represent one of the main cell types within the TME. The high expression of α-smooth muscle actin (α-SMA) and the biological characteristics of secreting multiple cytokines by CAFs play a vital role in tumor fibrosis (Geng et al., 2021). In renal clear cell carcinoma (RCC), cancer cells secrete TGF-β, which induces the transformation of normal fibroblasts into CAFs through the TGF-β-Smad2/3 pathway (Wang Y. et al., 2024). circ_0020256 is highly expressed in cholangiocarcinoma (CCA) and enhances CCA cells' secretion of TGF-β1, which subsequently activates CAFs via Smad2/3 phosphorylation. Mechanistically, circ_0020256 stabilizes KLF4 mRNA by recruiting EIF4A3 protein and increasing its expression. KLF4 then binds to the TGF-β1 promoter, enhancing its transcription in CCA cells (Li Z. et al., 2023). Hepatocellular carcinoma (HCC) cells secrete exosomes containing miRNA-21, directly targeting the PTEN gene and activating the PDK1/AKT signaling pathway, which promotes the transformation of normal hepatic stellate cells (HSCs) into CAFs with pro-cancer characteristics (Zhou et al., 2018). In oral squamous cell carcinoma (OSCC), PDGF secreted by cancer cells binds to PDGFR-β, activating lncRNA LURAP1L-AS1, which subsequently regulates the IKK/NF-κB signaling pathway, facilitating the activation and transformation of fibroblasts (Ren et al., 2021).

2.1.2 ECM remodeling

CAFs contribute to malignant tumor fibrosis by enhancing the synthesis of collagen, fibronectin, and other ECM components, resulting in excessive ECM accumulation in tissues. CAFs produce and secrete substantial quantities of type I and III collagen, the primary components of the ECM. The over-deposition of these collagens results in tissue stiffening and densification (Xu et al., 2024). Gastric cancer cells induce the abnormal expression and secretion of collagen by activating the FAK/AKT pathway in CAFs, driving malignant transformation and fibrosis (Zhang J. et al., 2024). CAFs secrete small extracellular vesicles (sEVs) that associate with the ECM; these sEVs are enriched with active lysyl oxidase (LOX). LOX interacts with collagen I under the action of sEVs, facilitating collagen cross-linking. Moreover, integrin α2β1 in sEVs mediates their binding to collagen, further strengthening the cross-linking process (Liu Y. et al., 2023). In lung cancer models, increased lipid droplet (LD) content in CAFs promotes their pro-tumor phenotype, characterized by high expression of α-SMA and collagen α-2 chain (COL1A2) (Zhang et al., 2022).

Moreover, CAFs secrete matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which control the degradation and remodeling of the ECM. In the process of fibrosis, CAFs aggravate fibrosis by adjusting the balance between MMPs and TIMPs, inhibiting normal ECM degradation, and facilitating ECM accumulation and stabilization (Najafi et al., 2019). For instance, when co-cultured with gastric cancer cells, CAFs significantly upregulate IL-17a expression and enhance the expression of MMP2 and MMP9, while downregulating their inhibitors TIMP1 and TIMP2 (Zhang J. et al., 2020).

2.2 Inflammation

Tumor cells promote fibrosis by persistently releasing inflammatory factors like IL-1, TGF-β1, TNF, and IL-6, which activate the NF-κB and JAK/STAT signaling pathways, inducing fibroblast differentiation into a pro-inflammatory phenotype or myofibroblasts. These fibroblasts further drive fibrosis (Anderson-Crannage et al., 2023). In a lung cancer mouse model, tumor cells induce an inflammatory response in the kidneys by secreting nephrotoxic proteins, which increase the expression of IL-6 and monocyte chemoattractant protein-1 (MCP-1), resulting in glomerular capillary collapse and tumor antigen deposition. Concurrently, the TGF-β signaling pathway is activated, triggering renal fibrosis (Hung et al., 2020). In pancreatic ductal adenocarcinoma (PDAC), tumor cells induce an inflammatory response in pancreatic stellate cells (PSCs) by absorbing lipids, which subsequently promotes PSC activation and triggers fibrosis (Hata et al., 2017). In pancreatic neuroendocrine tumors, cancer cells secrete interleukin-1 (IL-1), which induces CAFs to secrete stromal cell-derived factor 1 (SDF1), aggravating the extent of tumor fibrosis (Lai et al., 2024).

2.3 Signaling pathway

2.3.1 TGF-β/Smad signal transduction pathway

The TGF-β/Smad pathway serves as a key regulator in the fibrosis of malignant tumors. This pathway drives fibrosis formation and progression by modulating fibroblast proliferation, differentiation, activation, and the synthesis and deposition of ECM. TGF-β binds to the type II TGF-β receptor (TGF-βRII) on the cell surface, which then activates the kinase activity of TGF-βRI and triggers the phosphorylation of downstream Smad proteins. The phosphorylated Smad2 and Smad3 associate with the co-transcription factor Smad4, forming an active complex that moves into the nucleus to regulate the transcription of fibrosis-related genes (Lee and Massagué, 2022; Li J. et al., 2024).

Activation of the TGF-β-Smad2/3 pathway induces the expression of fibrotic factors, which leads to fibroblast activation, promoting their proliferation and differentiation into myofibroblasts. Myofibroblasts display increased α-SMA expression and an enhanced ability to synthesize ECM, thereby intensifying fibrosis within tumors and promoting tumor growth and progression (Su et al., 2020). The activation of the upstream Notch signaling pathway triggers the TGF-β/Smad pathway, which promotes the migration of mesenchymal stem cells to the stroma and their differentiation into fibroblasts (Peng et al., 2014). In breast cancer, a lack of glutamine can trigger the activation of TGF-β signaling, leading to the activation of associated fibroblasts and subsequent fibrosis. The activity of histone deacetylase 1 and the inhibition of mTORC1 are required for TGF-β signaling activation and the conversion of CAFs into a myofibroblast state (Mezawa et al., 2023). During cachexia, inflammation within tumors drives fibrosis. This process might be driven by TGF-β-induced differentiation of fibroblasts into myofibroblasts, resulting in imbalanced inflammatory cytokine expression, enhanced angiogenesis, and increased ECM components (Lima et al., 2019).

The TGF-β/Smad signaling pathway is essential in fibrosis, tumor progression, and metastasis by enhancing ECM synthesis and deposition. The epigenetic regulators UBR7 and histone methyltransferase EZH2 regulate TGF-β/Smad signaling. With the activation of the TGF-β/Smad pathway, collagen content and lysyl oxidase activity rise, directly impacting ECM stiffness (Adhikari et al., 2024). In unilateral breast cancer-associated lymphedema, TGF-β1 intensifies the fibrosis process by increasing the stiffness of fibroblasts, lymphatic endothelial cells, and lymphatic smooth muscle cells, and by enhancing ECM deposition (Baik et al., 2022).

2.3.2 JAK/STAT signal transduction pathway

The JAK/STAT signaling pathway drives fibrosis formation and reshapes the tumor microenvironment by mediating cell proliferation, differentiation, immune regulation, and inflammatory responses. Cytokines (like IL-6, IFN, and IL-13) bind to receptors, leading to JAK activation, followed by STAT protein phosphorylation. Phosphorylated STAT proteins dimerize and move into the nucleus, where they bind to DNA sequences to regulate the transcription of fibrosis-related genes (Liu X. et al., 2023). Bioinformatics analysis has shown that hub genes are significantly enriched in the JAK/STAT pathway in expression profiles associated with liver fibrosis and liver cancer (Hamdy et al., 2023). With the marked activation of pSTAT5 and pSTAT3, levels of pro-inflammatory and pro-tumor mediators rise, resulting in higher liver tumor burden and significantly increased fibrosis in mice (Cabrera-Galván et al., 2023). In RCC, RCC-derived CXCL5 promotes fibrosis by activating the JAK/STAT3 pathway, facilitating the transformation of normal fibroblasts into CAFs (Liu Y. et al., 2023). Research indicates that reprogrammed mouse liver cells, driven by IL6/Jak/Stat3 signaling pathways, convert into LGR5-positive cells. When transplanted into syngeneic mice, these LGR5-positive cells develop into invasive and metastatic tumors with marked fibrosis, underscoring the significance of the JAK/STAT pathway in malignant tumor fibrosis (Chaker et al., 2024). Research by Grohmann et al. shows that inhibiting STAT-1 signaling prevents T cell recruitment and fibrosis but does not prevent hepatocellular carcinoma; whereas correcting STAT-3 signaling can prevent liver cancer without affecting fibrosis. This research provides a more detailed explanation of the role of the JAK/STAT signaling pathway in malignant tumor fibrosis (Grohmann et al., 2018).

2.3.3 Wnt/β-catenin signal transduction pathway

Wnt proteins bind to cell surface receptors, activating β-catenin, leading to its accumulation and translocation to the nucleus, where it regulates the expression of fibrosis-related genes. These genes generally pertain to ECM synthesis and fibroblast activation (Feng et al., 2018). In lung adenocarcinoma, smoking induces the downregulation of filamin A interacting protein 1-like (FILIP1L), which activates the Wnt/β-catenin signaling pathway, resulting in mucin secretion, inflammation, and fibrosis (Kwon et al., 2022). In oral submucous fibrosis and OSCC tissues, hypermethylation of dickkopf-1 may lead to its downregulation, causing abnormal activation of the Wnt/β-catenin signaling pathway, potentially playing a crucial role in the pathogenesis of oral submucous fibrosis (He et al., 2020). Additionally, proteins associated with the Wnt/β-catenin pathway are highly expressed in pancreatic exocrine tissues, with significant alterations in their cellular and subcellular expression patterns, correlating with increased fibrosis (Bläuer et al., 2019). Stearoyl-CoA desaturase (SCD) in liver tumor-initiating stem-like cells (TIC) is regulated by Wnt/β-catenin signaling. The monounsaturated fatty acids produced by SCD stabilize LRP5/6 mRNA, forming a positive feedback loop that amplifies Wnt signaling, which in turn promotes liver fibrosis and tumor growth (Lai et al., 2017).

2.3.4 Notch signal transduction pathway

The Notch signaling pathway is activated by the interaction between Notch receptors and ligands. After receptor activation, proteolytic cleavage releases the Notch intracellular domain (NICD), which then translocates to the nucleus to regulate the transcription of specific genes. Suppressing Notch signaling can inhibit the activation of the classical TGF-β1 pathway and reduce the peritumoral desmoplastic reaction in cholangiocarcinoma (Mancarella et al., 2022). In liver cancer cells chronically exposed to low concentrations of cadmium, the activation of Notch and AKT/mTOR signaling pathways can induce the expression of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and its downstream target TNF-α-

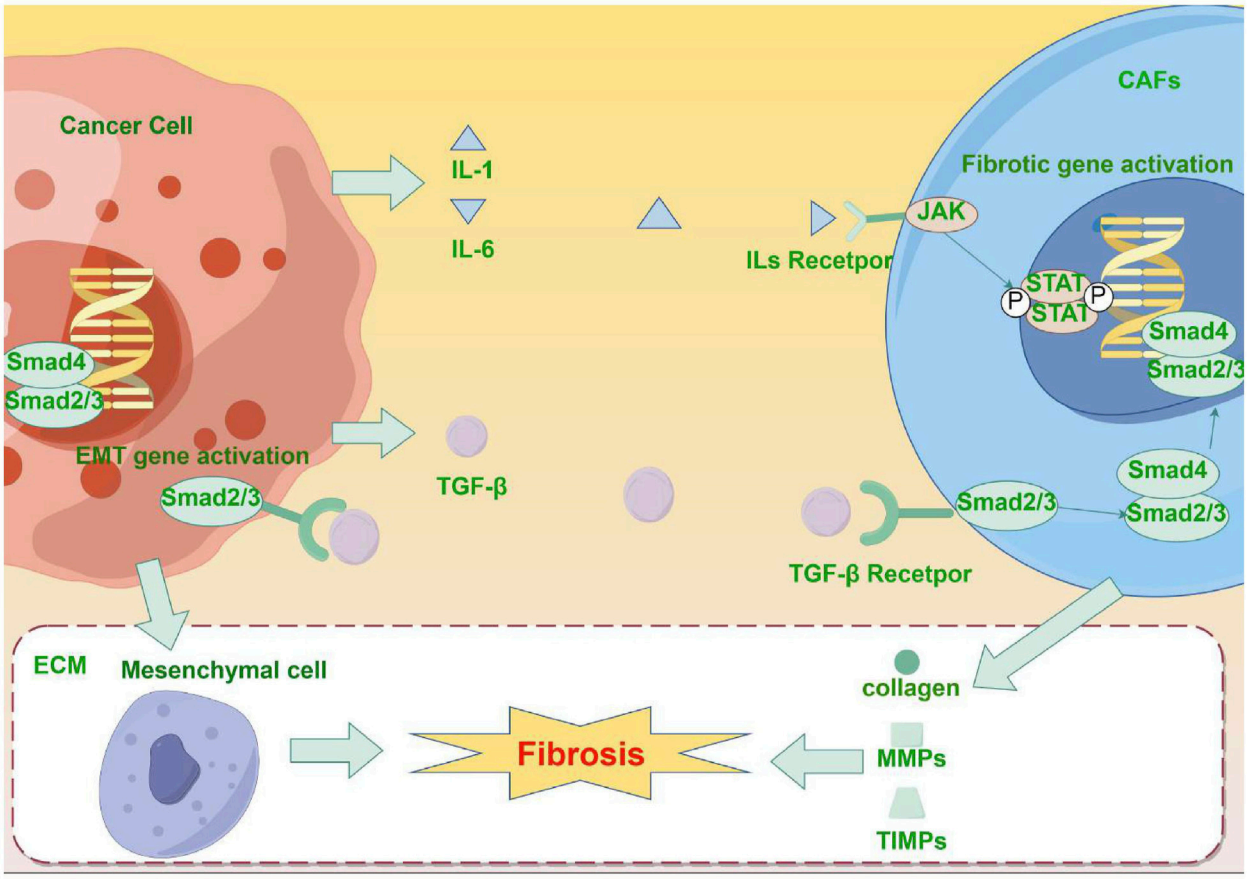

FIGURE 1
(By Figdraw, ID: RASTR41933) Mechanism of fibrosis promotion by malignant tumors: Tumor cells secrete pro-fibrotic factor TGF-β and activate CAFs through the TGF-β/Smad signaling pathway. Furthermore, tumor cells and CAFs secrete inflammatory factors (such as IL-1 and IL-6), which can further induce the production of additional pro-fibrotic factors, thus further activating CAFs. The activated CAFs promote the synthesis of collagen, fibronectin, and other ECM components, and affect ECM degradation and remodeling by regulating the secretion of MMPs and TIMPs. Additionally, the activation of the TGF-β/Smad signaling pathway is linked to the occurrence of EMT in malignant tumors, and the interaction between EMT and ECM remodeling further accelerates the fibrosis process.

induced protein 8 (TNFAIP8), thus regulating fibrosis and oncogenic signaling in liver cancer cells (Niture et al., 2023). Refer to Figure 1 for the mechanisms by which malignant tumors promote fibrosis.

2.3.5 The cross-talk effects of signaling pathways

It is worth noting that during the fibrosis process in malignant tumors, multiple signaling pathways do not function independently but often co-regulate fibrosis and tumor progression through complex interaction mechanisms. The interactions between different signaling pathways form a highly integrated network, which has a profound impact on the tumor microenvironment, cell proliferation, invasion, and treatment resistance. For instance, TGF-β1-induced activation of activating transcription factor 4 (ATF4) is dependent on the activation of the classical TGF-β1/Smad3 signaling and mTORC1-4E-BP1. ATF4 then promotes the *de novo* synthesis of enzymes from the serine-glycine biosynthesis pathway and transcription of the GLUT1 gene. This process meets the biosynthetic demands required for enhanced ECM synthesis (Selvarajah et al., 2019).

3 The effect of fibrosis on tumor progression

3.1 Enhances tumor proliferation and survival

Fibrosis results in the abnormal accumulation of extracellular matrix (ECM), especially the increase in collagen, fibronectin, and hyaluronic acid. These ECM components not only offer structural support for tumor cells but also interact with cell surface receptors, activating signaling pathways that promote proliferation and survival. In liver cancer, Sema3C supports tumor fibrosis by promoting the proliferation of hepatic stellate cells (HSCs). Moreover, Sema3C interacts with NRP1 and ITGB1 receptors, activating the AKT/Gli1/c-Myc signaling pathway, promoting the self-renewal and proliferation of HCC cells (Peng et al., 2024). The increased tissue stiffness due to fibrosis further promotes tumor cell proliferation and survival via mechanotransduction pathways, such as the YAP pathway (Schrader et al., 2011; Deng et al., 2022).

Fibroblasts and CAFs within the fibrotic microenvironment secrete numerous growth factors, such as TGF-β and EGF. These

factors facilitate tumor cell proliferation and survival by activating downstream signaling pathways. In prostate cancer, TGF-β1 is recognized as a highly secreted growth factor in CAFs, significantly enhancing tumor cell growth and proliferation in both *in vivo* and *in vitro* settings (Dy et al., 2019). In PDAC, CAF-derived thrombospondin 1 (TSP1) activates TGF-β signaling, leading to the loss of Smad4 expression in cancer cells and accelerating their proliferation and migration (Matsumura et al., 2022). In cholangiocarcinoma, CAF-secreted TSP-4 binds to integrin α2 on cancer cells, activating HSF1 and Akt signaling pathways. Activated HSF1 further enhances TGF-β1 expression and secretion, inducing the transformation of fibroblasts into CAFs and creating a positive feedback loop that promotes cell proliferation and advances cholangiocarcinoma progression (Shi et al., 2021).

3.2 Enhances angiogenesis

3.2.1 Secretion of angiogenesis-promoting factors

In malignant tumors, fibrosis promotes angiogenesis through various mechanisms, supplying the necessary nutrients and oxygen for tumor growth and expansion. During the fibrosis process in malignant tumors, fibroblasts and CAFs are activated, leading to the secretion of significant amounts of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF). These factors interact with receptors on vascular endothelial cells, activating signaling pathways that promote the formation of new blood vessels (Sobierajska et al., 2020).

In several malignant tumors, such as head and neck squamous cell carcinoma, RCC, and cholangiocarcinoma, CAFs can directly secrete VEGF to promote angiogenesis (Sun et al., 2022; Zhou et al., 2022; Liu J. et al., 2023). In colorectal cancer patients, exosomes released by CAFs enhance endothelial cell proliferation, migration, and angiogenesis by increasing the expression and secretion of VEGF. Specifically, circ_0084043 is highly expressed in CAF-derived exosomes and regulates HIF-1α and VEGFA by sponging miR-140-3p, suggesting that the circ_0084043/miR-140-3p/VEGF signaling pathway plays a critical role in CAF exosome-induced angiogenesis (Payervand et al., 2024). miR-210 secreted by lung cancer cells enhances angiogenesis by increasing VEGF via the activation of the JAK2/STAT3 signaling pathway in CAFs (Fan et al., 2020). Similarly, research by Dai et al. (2022) showed that CAF-derived extracellular vesicles promote angiogenesis in colorectal adenocarcinoma cells via the miR-135b-5p/FOXO1 axis, indicating the crucial role of non-coding RNAs in enhancing the secretion of angiogenic factors by CAFs.

Moreover, several signaling pathways are also crucial in regulating VEGF secretion. When CAFs are co-cultured with glioma C6 cells, the expression levels of VEGF-A and EGF proteins are significantly elevated, thereby enhancing glioma cell invasiveness, proliferation, and angiogenesis (Zhang S. et al., 2023). In triple-negative breast cancer (TNBC), particularly in patients with BRCA1 mutations, iCAFs have been found to be enriched and promote angiogenesis by interacting with tumor endothelial cells (TECs) via VEGF signaling. iCAFs activate angiogenesis-related genes (such as FLT1 and KDR) in TECs through the VEGF signaling pathway, promoting endothelial cell migration and sprouting angiogenesis (Lee et al., 2024). In breast cancer, the upregulation of VEGF-A and IL-8, along with their upstream effectors mTOR and HIF-1α, can enhance the pro-angiogenic potential of CAFs (Al-Kharashi et al., 2022). In melanoma, CD38-positive CAFs promote tumor cell migration and invasion, as well as endothelial cell tube formation, by secreting factors like VEGF-A, FGF-2, and CXCL-12 through paracrine signaling *in vitro* (Ben Baruch et al., 2020).

PDGF and other angiogenesis-promoting factors likewise play a crucial role in driving angiogenesis facilitated by CAFs. Chu et al. (2022) discovered that VEGF, angiopoietin, bFGF, and other factors secreted by CAFs are crucial in the angiogenesis of precancerous and malignant lesions in laryngeal cancer. In OSCC, reprogramming of glucose metabolism results in increased secretion of angiogenesis-promoting factors (VEGF-A, PDGF-C, and MMP9) by CAFs, which enhances the angiogenic phenotype (Li X. et al., 2022). In cholangiocarcinoma, CAFs secrete stem cell factor (SCF), which recruits mast cells and stimulates them to release hyaluronic acid (HA) via the MRGPRX2-Gαq signaling pathway. These bile-induced MCs subsequently release PDGF-B, which further enhances angiogenesis in cholangiocarcinoma (Shi et al., 2024).

3.2.2 ECM remodeling

Fibrosis caused by malignant tumors results in excessive extracellular matrix (ECM) deposition, offering a physical scaffold for new blood vessel formation and supporting vascular expansion within the dense matrix. In cholangiocarcinoma, the overexpression of PI3Kδ is closely related to stromal remodeling, manifesting as a thick ECM at the basement membrane and significant angiogenesis and lymphangiogenesis. The mechanism involves PI3Kδ promoting ECM remodeling via the TGFβ/Src/Notch signaling pathway, which in turn enhances angiogenesis (Bou Malham et al., 2023). In bladder cancer, the Sigma 1 receptor (Sig1R) can regulate crosstalk between the ECM and tumor cells, facilitating ECM-mediated cell proliferation and angiogenesis (Feng et al., 2023).

3.2.3 Hypoxia and the activation of HIF-1α

Fibrosis increases the density of tumor tissue, limiting oxygen diffusion and creating a hypoxic microenvironment. Hypoxia-inducible factors (HIFs) become stabilized and activated in hypoxic conditions, enhancing the expression of pro-angiogenic genes like VEGF, which drives the formation of new blood vessels (Yehia et al., 2015; De Marco et al., 2022). Pancreatic cancer features excessive desmoplastic reaction and a hypoxic microenvironment within the solid tumor mass. Hypoxia induces the production of HIF-1, which not only enhances the migration of pancreatic stellate cells (PSCs) and the expression of type I collagen but also increases VEGF secretion, promoting angiogenesis (Masamune et al., 2008; N et al., 2016).

3.3 Enhances immune evasion

The dense ECM structure created by fibrosis obstructs immune cell infiltration, diminishing their tumor-killing capacity and aiding tumor cells in evading immune surveillance. In the fibrotic tumor

microenvironment, tumor-associated macrophages (TAMs) initiate collagen synthesis via the TGF-β signaling pathway, causing tumor tissue stiffening and establishing a metabolic environment that impairs $CD8^+$ T cell function. Macrophages engaged in collagen synthesis deplete arginine in the environment and produce proline and secrete ornithine, which further suppresses the antitumor response of $CD8^+$ T cells. Therefore, fibrosis not only physically repels $CD8^+$ T cells but also weakens the immune response against cancer by altering the metabolic environment (Tharp et al., 2024). In non-small cell lung cancer (NSCLC), significant fibrosis corresponds with reduced T cell infiltration, resulting in impaired immune surveillance. Fibrosis not only accelerates tumor progression but also reduces the number and function of dendritic cells and alters macrophage phenotypes, further intensifying immune suppression (Herzog et al., 2023). Liver fibrosis enhances tumor immune evasion in hepatocellular carcinoma, resulting in decreased $CD8^+$ T cell infiltration and increased expression of the immune checkpoint molecule programmed death-ligand 1 (PD-L1). Specifically, Golgi membrane protein 1 (GOLM1) in fibrosis induces PD-L1 expression via the activation of the EGFR pathway, thereby suppressing antitumor immune responses (Ke et al., 2021). In the lung adenocarcinoma microenvironment, CAFs increase the expression of PD-L1 in tumor cells by secreting cytokines like CXCL2. High PD-L1 expression allows tumor cells to suppress $CD8^+$ T cell activity in the immune system, facilitating immune evasion (Inoue et al., 2019). Regulatory T cells (Tregs) and CAFs interact to collaboratively enhance fibrosis and immune suppression. Specifically, IL-33 enhances Treg cell activity through the IL1RL1 signaling pathway, and these Tregs interact with CAFs via the AREG/EGFR axis, inducing CAFs into a pro-fibrotic and immunosuppressive state (Sun et al., 2023). However, it is important to note that fibrosis in malignant tumors can facilitate tumor immune evasion while also constraining tumor size expansion (Li et al., 2021).

4 Fibrosis enhances treatment resistance

4.1 Chemotherapy resistance

Chemotherapy is a treatment approach that employs chemical agents to kill cancer cells or inhibit their growth and division, commonly used in the treatment of various malignant tumors. However, chemoresistance is a significant challenge in the treatment of malignant tumors, and it is often accompanied by fibrosis in affected patients. In a pancreatic ductal adenocarcinoma model, ectopic tumors showed more pronounced fibrosis, which led to increased resistance to FOLFIRINOX chemotherapy. Despite similar drug absorption in tumor tissues, fibrosis and microenvironmental differences significantly impacted the treatment response (Erstad et al., 2018). In breast cancer, fibrosis-related signaling pathways are significantly upregulated in patients who do not achieve a complete response to neoadjuvant chemotherapy; patients with high fibrosis have lower complete response rates and shorter survival durations (Wang X. et al., 2024).

Fibrosis is frequently accompanied by epithelial-mesenchymal transition (EMT), which converts tumor cells from an epithelial phenotype to a mesenchymal phenotype. EMT provides tumor cells with enhanced migratory ability and resistance to apoptosis, thereby increasing their resistance to chemotherapy. In 5-Fu-resistant (5-FU) breast cancer cell lines, tumor cells induce normal dermal fibroblasts to convert into a CAF phenotype via TGF-β1 paracrine signaling, promoting fibrosis, reducing E-cadherin expression, and facilitating EMT (Chandra Jena et al., 2021). CAFs can transfer exosomes to colorectal cancer cells, promoting stemness and EMT in CRC cells, which in turn enhances resistance to 5-FU/oxaliplatin (L-OHP) chemotherapy. Mechanistically, exosomes induce miR-92a-3p production, activating the Wnt/β-catenin pathway, inhibiting FBXW7 and MOAP1 expression, and suppressing mitochondrial apoptosis, thereby enhancing stemness and chemoresistance (Hu et al., 2019). In ovarian cancer, CAFs may activate the Wnt/β-catenin pathway via the CXCL12/CXCR4 axis, promoting cisplatin resistance by inducing EMT (Zhang F. et al., 2020). IL-6 derived from CAFs plays a crucial role in maintaining the paracrine loop between CAFs and NSCLC cells by enhancing EMT in NSCLC cells. This paracrine loop enhances intercellular communication, which subsequently leads to the development of chemoresistance (Shintani et al., 2016).

In addition to EMT, fibrosis can promote chemoresistance by activating tumor stem cell properties and anti-apoptotic signaling pathways. In PDAC, proliferating resident macrophages (proliferating rMφs) significantly increase tumor resistance to chemotherapy by promoting fibrosis and immune suppression. Multi-omics analysis found that these macrophages promote cancer cell survival during chemotherapy by producing more deoxycytidine (dC) and less dC kinase (dCK), reducing the absorption of gemcitabine (Zhang J. et al., 2023). Additionally, CAFs promote tumor fibrosis via the IL1β-IRAK4 signaling pathway, which enhances tumor cell survival and proliferation, resulting in gemcitabine resistance (Zhang et al., 2018). In lung adenocarcinoma, cancer stem cells (CSCs) secrete the acute-phase protein serum amyloid A (SAA), remodeling the tumor microenvironment, promoting fibrosis, and enhancing cisplatin (DDP) chemoresistance (Wang et al., 2023). In ovarian cancer, high expression of CHI3L1 (a secretory glycoprotein) is closely linked to fibrosis. CHI3L1 activates the Akt and Erk signaling pathways, enhancing the expression of β-catenin and SOX2, promoting stem-like characteristics in ovarian cancer cells, such as resistance to apoptosis, thereby increasing paclitaxel chemoresistance (Lin et al., 2019). In CAF-derived exosomes, the significantly upregulated circBIRC6 promotes the SUMOylation of XRCC4, enhancing its interaction with SUMO1 at lysine 115, facilitating XRCC4 chromatin localization, and increasing pancreatic cancer cell resistance to oxaliplatin (Zheng et al., 2023). In conclusion, fibrosis can enhance chemotherapy resistance through multiple mechanisms, including EMT, CSC, and anti-apoptotic pathways.

4.2 Resistance to immunotherapy

Presently, immunotherapy is an emerging cancer treatment method that fights cancer by enhancing or regulating the immune system. Immune checkpoint inhibitors are widely used immunotherapy strategies, among which PD-1/PD-L1 inhibitors

(like pembrolizumab and nivolumab) and CTLA-4 inhibitors (such as ipilimumab) have shown significant therapeutic potential in tumor immunotherapy (Li W. et al., 2022). In tumors that respond to immunotherapy, the TME shows enrichment of immune cells and CAFs, along with pro-inflammatory signaling and ECM remodeling, which aligns with proliferative fibrosis and immune-mediated tumor regression. However, tumor heterogeneity may result in immune-deficient regions, promoting immune evasion and early recurrence via HCC-CAF interactions and the expression of cancer stem cell markers. This indicates that fibrosis may contribute to immunotherapy resistance in certain cases, heightening treatment challenges (Zhang M. et al., 2023). In breast cancer, fibrosis facilitates immunotherapy resistance by increasing TAMs, EMT, fibroblast proliferation, ECM enhancement, and Wnt pathway activation. These alterations together create an immune-tolerant microenvironment, diminishing the effectiveness of PD-1 inhibitors (Yuan et al., 2022). Further research by Song et al. (2024) revealed a link between anti-PD-L1 therapy and fibrosis: During liver fibrosis, pathogenic Th17 cells (pTh17) significantly increase, and anti-PD-L1 therapy promotes pTh17 cell infiltration and activation in the liver. These pTh17 cells secrete IL-17A, which increases PD-L1 expression on the surface of hepatocellular carcinoma cells, further worsening liver cirrhosis and leading to resistance to anti-PD-L1 therapy (Song et al., 2024).

4.3 CAFs enhance resistance to targeted therapy

Targeted therapy is a form of cancer treatment that specifically targets certain molecules or signaling pathways in cancer cells. Unlike traditional chemotherapy, targeted therapy precisely identifies and inhibits abnormal proteins or genetic mutations in cancer cells, preventing tumor growth and spread while minimizing harm to normal cells.

4.3.1 Resistance to tyrosine kinase inhibitors

In the fibrotic microenvironment, CAFs play a key role in promoting resistance to tyrosine kinase inhibitors (TKIs). For example, in RCC, CAFs facilitate resistance to VEGFR-TKIs (Ambrosetti et al., 2022). In HCC, bioinformatics analysis identified SPP1 secreted by CAFs as a candidate molecule for resistance to sorafenib and lenvatinib. CAF-secreted SPP1 activates the RAF/MAPK and PI3K/AKT/mTOR pathways via the integrin-PKCα signaling pathway and promotes EMT, resulting in TKI resistance (Eun et al., 2023). CAFs enhance the secretion of HGF and IGF-1, activating the c-met and IGF-1R receptors, leading to increased ANXA2 expression and phosphorylation, inducing EMT and resulting in resistance to EGFR-TKIs (e.g., gefitinib) in NSCLC (Yi et al., 2018). Similarly, in NSCLC, CAFs derived from osimertinib-resistant cells secrete higher levels of IL-6, IL-8, and hepatocyte growth factor (HGF), express stronger CAF markers such as α-SMA, FAP, and PDGFR, and increase stemness and osimertinib resistance in NSCLC cells (Huang W. et al., 2021). In EGFR-TKI-resistant tumors, part of the CAF-derived tumor stroma is composed of EMT-derived tumor cells that express resistance markers, such as epithelial membrane protein-1. CAFs secrete paracrine factors that reduce the inhibitory effects of TKIs on pEGFR and pMAPK, thereby promoting tumor cell survival and drug resistance (Sr et al., 2010). In gastric cancer, cancer cells secrete lactate, inducing CAFs to produce BDNF, activating the TrkB-Nrf2 signaling pathway, inhibiting anlotinib-induced apoptosis and reactive oxygen species (ROS) generation, thus reducing drug efficacy (Jin et al., 2021). In RCC, CAFs increase sunitinib resistance by secreting CXCL3, which activates the CXCR2-ERK1/2 signaling pathway in tumor cells, promoting EMT and stemness (Wang Y. et al., 2024).

4.3.2 Resistance to monoclonal antibodies

Monoclonal antibodies (mAbs) are a crucial class of drugs in targeted therapy, specifically targeting certain antigens or receptors on cancer cell surfaces. They kill specific cancer cells by directly blocking signal transduction, activating ADCC, or inducing complement-dependent cytotoxicity (CDC). Common monoclonal antibodies include trastuzumab, bevacizumab, and cetuximab.

Trastuzumab can target the HER2 receptor and is used for treating HER2-positive breast cancer and gastric cancer. CAFs are enriched in trastuzumab-resistant HER2-positive breast cancer cases. These CAFs secrete immunosuppressive factors IDO1 and TDO2, inhibiting NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC), thereby causing resistance to trastuzumab (Du et al., 2023). CAF-derived Neuregulin 1 (NRG1) also mediates trastuzumab resistance in breast cancer by activating the HER3/AKT signaling pathway. However, pertuzumab may reverse resistance by targeting this pathway (Guardia et al., 2021). Mao et al. (2015)'s research shows that CAFs can induce trastuzumab resistance by expanding cancer stem cells and activating multiple pathways including NF-κB, JAK/STAT3, and PI3K/AKT.

Bevacizumab targets VEGF to inhibit angiogenesis and is used in the treatment of colorectal cancer, non-small cell lung cancer, renal cell carcinoma, and more. In OSCC, CAFs play a key role in angiogenesis by secreting sEVs. CAF-derived sEVs bind to VEGF and activate the VEGFR2 signaling pathway in human umbilical vein endothelial cells (HUVECs). Even after Bevacizumab treatment, VEGF bound to sEVs can continue to activate VEGFR2. This indicates that sEVs secreted by CAFs can bind VEGF via heparan sulfate proteoglycans on their surface, making them resistant to Bevacizumab (Li et al., 2020).

Cetuximab targets EGFR and is used for targeted therapy in colorectal cancer and head and neck squamous cell carcinoma (HNSCC). In CRC, CAFs significantly increase CRC cell resistance to Cetuximab by regulating the expression of the EMT key factor SNAI1 and remodeling the ECM (Galindo-Pumariño et al., 2022). In HNSCC, TGF-β-activated CAFs limit Cetuximab efficacy by upregulating the TGF-β signaling pathway, thereby enhancing drug resistance in the tumor microenvironment (Yegodayev et al., 2020). Further studies indicate that CAF-derived MMP-1 expression increases in both tumor cells and CAFs, promoting resistance to Cetuximab (Johansson et al., 2012). Refer to Figure 2 for fibrosis-promoted malignant tumor progression and treatment resistance. Refer to Table 1 for details on how fibrosis promotes treatment resistance.

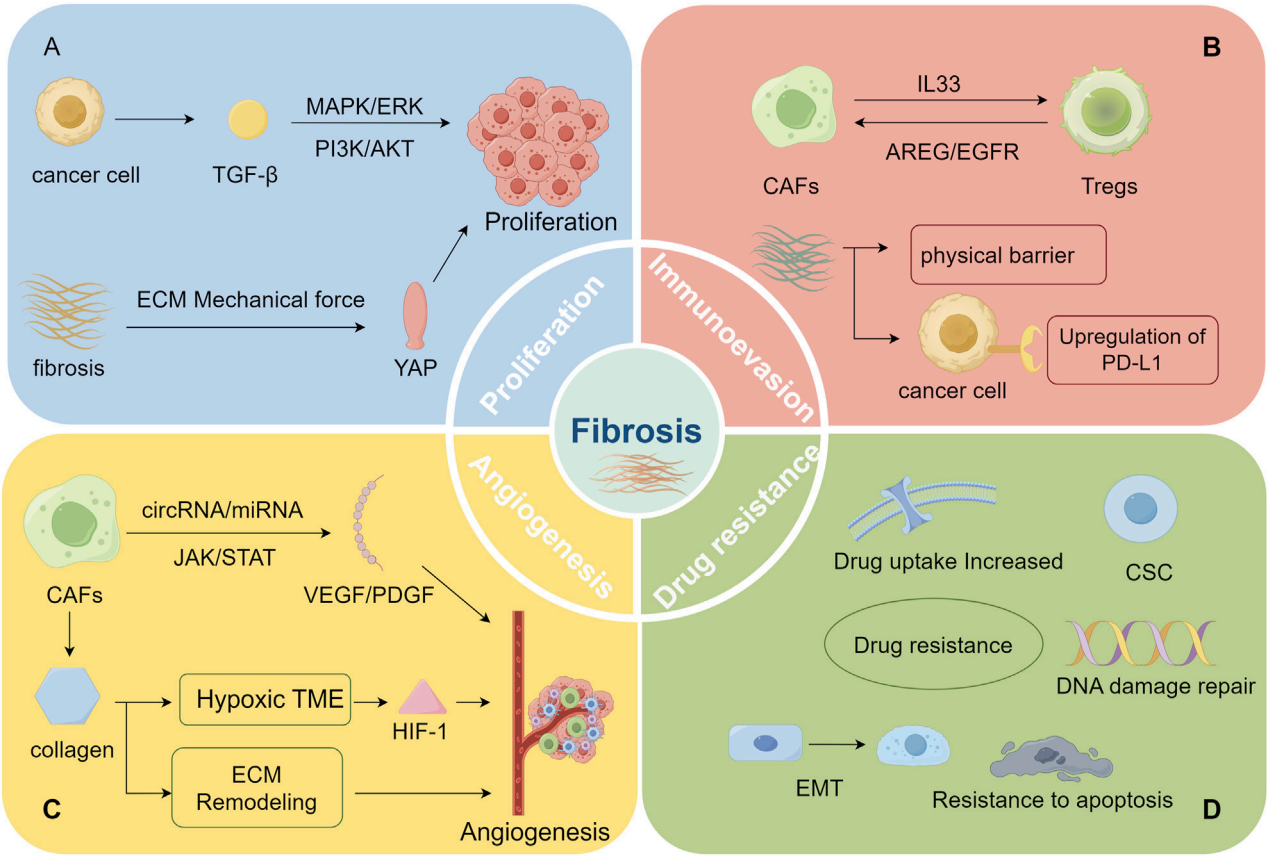

FIGURE 2
(By Figdraw, ID: RYWAR2b2b6) Fibrosis facilitates malignant tumor progression: **(A)** enhances tumor proliferation, **(B)** promotes immune evasion, **(C)** drives angiogenesis, **(D)** increases treatment resistance **(A)** Malignant tumors enhance tumor cell proliferation by secreting TGF-β, which activates pathways such as PI3K-AKT and MAPK/ERK. Furthermore, the increased tissue stiffness from fibrosis promotes tumor cell proliferation via mechanotransduction pathways (like the YAP pathway). **(B)** The interaction between Treg cells and CAFs not only promotes fibrosis but also intensifies immunosuppression. Moreover, the dense ECM structure resulting from fibrosis obstructs immune cell infiltration and aids tumor cells in evading immune surveillance by upregulating PD-L1 expression. **(C)** CAFs enhance the secretion of pro-angiogenic factors like VEGF and PDGF via the regulation of non-coding RNAs and signaling pathways such as JAK, thereby promoting tumor angiogenesis. The excessive deposition of ECM provides a physical scaffold for new blood vessel formation, supporting vascular expansion within the dense matrix. Additionally, excessive ECM accumulation restricts oxygen, activating HIF-1 and further promoting angiogenesis. **(D)** Fibrosis facilitates treatment resistance in malignant tumors through multiple mechanisms, including CSC activation, EMT induction, apoptosis inhibition, enhanced DNA damage repair, and reduced drug uptake, thereby weakening the efficacy of chemotherapy, targeted therapy, and immunotherapy.

5 Tumor therapeutic strategies targeting fibrosis

5.1 Nintedanib

5.1.1 Clinical trials

Nintedanib is a small-molecule TKI with antifibrotic and anti-inflammatory properties, mainly used in the treatment of idiopathic pulmonary fibrosis. The clinical use of antifibrotic drugs such as Nintedanib can significantly improve the survival time of patients with certain malignant tumors. In refractory metastatic CRC, Nintedanib combined with capecitabine is well tolerated and clinically more effective than regorafenib or trifluridine/tipiracil monotherapy. In a study of 36 patients, the median progression-free survival (PFS) was 3.4 months, and the median overall survival (OS) was 8.9 months after 18 weeks (Boland et al., 2024). Nintedanib combined with chemotherapy significantly improved PFS in NSCLC patients, though it had no significant impact on OS. A meta-analysis of three randomized controlled trials involving 2,270 patients showed that PFS in the Nintedanib group was significantly better than in the placebo group (HR = 0.79; 95% CI 0.71–0.88, $p < 0.0001$) (Alhadeethi et al., 2024). Additionally, a multicenter retrospective study indicated that Nintedanib combined with docetaxel had some efficacy in advanced NSCLC patients following the failure of immune checkpoint inhibitors (ICI) and/or chemotherapy. In 96 patients, the objective response rate (ORR) was 18.8%, the disease control rate (DCR) was 57.3%, the median PFS was 3.0 months, and the median OS was 8.0 months. Particularly in patients treated with Nintedanib and docetaxel after first-line ChT-ICI therapy, the ORR was 29.2%, the DCR was 66.7%, and the median PFS was 4.0 months (Ljubicic et al., 2023). These studies indicate that Nintedanib can effectively improve survival time in patients with certain malignant tumors.

However, there are ongoing debates regarding the response rate and safety of Nintedanib. In a double-blind, randomized, phase 2 trial adding Nintedanib to neoadjuvant chemotherapy for muscle-

TABLE 1 Summarize the mechanisms and associated signaling pathways through which fibrosis promotes resistance to chemotherapy, immunotherapy, and targeted therapy.

Type of drug	Drug	Disease	Mechanism of resistance	Signaling pathway	Reference
Chemotherapy	5-Fu/L-OHP	CRC	EMT, CSC	miR-92a-3p/Wnt/β-catenin	Hu et al. (2019)
	5-Fu	Breast cancer	EMT	--	Chandra Jena et al. (2021)
	Gemcitabine	PDAC	Drug uptake Increased	--	Zhang J. et al. (2023)
	Gemcitabine	PDAC	Resistance to apoptosis	IL1β-IRAK4	Zhang et al. (2018)
	DDP	Ovarian cancer	EMT	CXCL12/CXCR4-Wnt/β-catenin	Zhang F. et al. (2020)
	DDP	Lung adenocarcinoma	CSC	--	Wang et al. (2023)
	Paclitaxel	Ovarian cancer	CSC	CHI3L1/Akt/Erk-β- catenin	Lin et al. (2019)
	L-OHP	PDAC	DNA damage repair	circBIRC6-XRCC4	Zheng et al. (2023)
ICI	PD-1 mAb	Breast cancer	EMT, TAM increase	Wnt signaling pathway	Yuan et al. (2022)
TKI	Sorafenib/ lenvatinib	HCC	EMT\	RAF/MAPK、PI3K/AKT/ mTOR	Eun et al. (2023)
	Gefitinib	NSCLC	EMT	HGF/IGF-1/c-met、IGF-1R-ANXA2	Yi et al. (2018)
	Axitinib	NSCLC	CSC	--	Huang W. et al. (2021)
	Sunitinib	RCC	CSC, EMT	CXCR2-ERK1/2	Wang Y. et al. (2024)
	Anlotinib	Gastric cancer	Resistance to apoptosis and ROS	BDNF-TrkB-Nrf2	Jin et al. (2021)
mAbs	Trastuzumab	Gastric cancer, Breast cancer	Resistance to ADCC	--	Du et al. (2023)
	Trastuzumab	Breast cancer	Resistance to apoptosis	NRG1/HER3/AKT	Guardia et al. (2021)
	Trastuzumab	Breast cancer	CSC	NF-κB, PI3K/AKT and JAK/STAT3	Mao et al. (2015)
	Bevacizumab	OSCC	Continuous activation of VEGF	--	Li et al. (2020)
	Cetuximab	HNSCC, CRC	EMT, ECM Remodeling	--	Galindo-Pumariño et al. (2022)
	Cetuximab	HNSCC	Upregulation of the TGF-β signaling pathway	TGF-β signaling pathway	Johansson et al. (2012)

5-Fu, 5-Fluorouracil; L-OHP, Oxaliplatin; CRC, Colorectal cancer; EMT, Epithelial–mesenchymal transition; CSC, Cancer stem cell; PDAC, Pancreatic Ductal Adenocarcinoma; DDP, Cisplatin; TAM, Tumor-associated macrophages; HCC, Hepatocellular carcinoma; NSCLC, Non-small cell lung cancer; RCC, Renal cell carcinoma; ADCC, Antibody dependent cell-mediated cytotoxicity; OSCC, Oral squamous cell carcinoma; HNSCC, Head and neck squamous cell carcinoma.

invasive bladder cancer, the pathological complete response rate (pCR) was similar between the Nintedanib and placebo groups (37% vs. 32%). However, the Nintedanib group showed a higher incidence of grade 3 or higher toxic events (93% vs. 79%), with the most common serious adverse events being thromboembolic events (30% vs. 21%) and neutropenia (39% vs. 11%) (Hussain et al., 2022). In ovarian cancer, the Nintedanib treatment group showed worse PFS and OS compared to the placebo group, along with higher toxicity (92% vs. 69% for grade 3/4 adverse events), primarily consisting of hematologic and gastrointestinal side effects (Ferron et al., 2023). Refer to Table 2 for the clinical trial results of Nintedanib.

5.1.2 Sensitization to chemotherapy and immunotherapy

Combining Nintedanib with immunotherapy or chemotherapy drugs can significantly improve treatment outcomes and promote tumor cell death. Nintedanib significantly inhibits tumor growth in mouse models. When combined with anti-PD-1 antibodies, Nintedanib enhances antitumor efficacy primarily by reducing the number of TAMs and polarizing them into the antitumor M1 phenotype. The combination therapy also restores macrophage phagocytic function, enhancing treatment effectiveness (Tada et al., 2023). In malignant tumors, combining Nintedanib with PD-L1 enhances immune cell infiltration and activation within the tumor, boosts interferon-γ response, and activates MHC class I-mediated antigen presentation. It also promotes PD-L1 expression and STAT3 phosphorylation, thereby improving the effectiveness of immunotherapy (Tu et al., 2022). In PDACs, Nintedanib inhibits CAF secretion of IL-6 by blocking the PDGFRβ signaling pathway. Moreover, MSLN-targeted chimeric antigen receptor-NK cells combined with Nintedanib significantly enhanced tumor-killing ability in xenograft models, triggering robust NK cell infiltration (Lee et al., 2023).

TABLE 2 Efficacy and adverse reactions of Nintedanib in different malignant tumors.

Disease	Trial-registration	Phase	Case	OS (month)	PFS (month)	PFS HR (95% CI)	Serious treatment-related adverse events (Grade 3–4)	Reference
RAIR DTC	NCT01788982	II	56	--	3.7	0.65 (0.42–0.99)	50%	Leboulleux et al. (2024)
MTC	NCT01788982	II	20	--	7.0	0.49 (0.16–1.53)	59.1%	Leboulleux et al. (2024)
NSCLC	NCT02299141	--	20	11.3	4.3	--	35%	Auberle et al. (2024)
CRC	NCT02393755	I/II	42	8.9	3.4	--	44%	Boland et al. (2024)
SCLC	jRCTs031190119	II	33	13.4	4.2	--	81.8%	Ikeda et al. (2024)
NSCLC	--	--	27	15.8	5.4	--	44.4%	Makiguchi et al. (2023)
Ovarian Cancer	NCT01583322	II	188	37.7	14.4	1.50	96%	Ferron et al. (2023)
NSCLC	jRCTs071180049	III	243	15.3	6.2	0.68 (0.50–0.92)	72.5%	Otsubo et al. (2022)
NSCLC	--	II	59	6.9	2.7	--	53.7%	Auliac et al. (2021)
CRC	NCT01362361	II	53	17.1	8.1	0.65 (0.32–1.30)	73.1%	Ettrich et al. (2021)

PFS, Progression-Free-Survival; OS, Overall survival; HR, Hazard Ratio; RAIR DTC, Radioiodine-refractory differentiated thyroid cancer; MTC, Medullary thyroid cancer.

In a xenograft model derived from gastric adenocarcinoma cells, Nintedanib inhibited tumor cell proliferation, reduced tumor angiogenesis, and increased tumor cell death. Notably, when combined with docetaxel and irinotecan, it significantly extended the animals' survival (Awasthi et al., 2023).

5.2 Pirfenidone (PFD)

5.2.1 PFD suppresses tumor invasion capability

Clinically, PFD is an approved drug used to treat idiopathic pulmonary fibrosis. It alleviates fibrotic responses by inhibiting TGF-β and other profibrotic factors and can significantly reduce tumor invasiveness by inhibiting EMT, regulating immune responses in the tumor microenvironment, and remodeling the ECM. For instance, PFD can inhibit the growth of breast tumors in mice and alcohol-promoted metastasis (Li H. et al., 2024). In TNBC, PFD reduces the expression of EMT-related transcription factors and mesenchymal genes by inhibiting the TGF-β/Smad signaling pathway, thereby inhibiting the proliferation, migration, and invasion of breast cancer cells while promoting apoptosis (Luo et al., 2023). PFD promotes the downregulation of ZEB1 via miR-200 in NSCLC exosomes, slowing down migration, invasion, and EMT processes (Liu et al., 2022). In RCC, PFD significantly inhibits the progression of renal cancer by targeting the TGF-β signaling pathway. PFD decreases TGF-β expression and secretion, blocking TGF-β-induced EMT and thus reducing the proliferation, migration, and invasion of renal cancer cells. Additionally, PFD enhances the immunosuppressive tumor microenvironment by limiting the recruitment of tumor-infiltrating myeloid-derived suppressor cells (MDSCs) (Wang et al., 2022). PFD targets CAFs, inhibiting EMT and stemness features in breast cancer cells. In breast cancer samples with a high stromal index, CAFs promote cancer cell spheroid formation and induce the expression of YAP1, VIM, and CD44. PFD treatment significantly reduces cancer cell migration and the protein expression levels of these genes (Es et al., 2021). PFD inhibits the expression of CAFs, hyaluronic acid, and collagen I, reducing tumor stromal pressure, eliminating the immunosuppressive microenvironment, and increasing cytotoxic T lymphocyte infiltration, thereby remodeling the desmoplastic tumor microenvironment. Moreover, PFD, in combination with therapies targeting the mitochondrial ROS-PYK2 pathway, significantly inhibits the growth and metastasis of malignant breast cancer (Zuo et al., 2021). PFD effectively eliminates the ethanol-mediated promotion of the TGF-β/RUNX3/Snail axis in CRC metastasis by specifically blocking the TGF-β signaling pathway (Zheng et al., 2019).

5.2.2 Sensitization to chemotherapy and immunotherapy

In chemotherapy, PFD can significantly enhance tumor cell death. PFD can reprogram several biological pathways, inhibiting tumor cell secretion of PDGF by downregulating the TGM2/NF-kB/PDGFB pathway, thus exerting antifibrotic effects. This leads to a reduction in collagen X and fibronectin secretion by CAFs, and in a mouse pancreatic tumor orthotopic model, PFD showed the potential to enhance gemcitabine sensitivity (Lei et al., 2024). PFD's use in NSCLC primarily focuses on its antitumor and chemosensitizing effects. PFD exerts anticancer effects by inhibiting the TGF-β1 signaling pathway, reducing lactate and

ATP production, and thus inhibiting glycolysis. When combined with cisplatin, PFD enhances the targeted inhibition of TGF-β1, improving chemotherapy sensitivity in A549 and H1299 cells (Zhang S. et al., 2024). In TNBC, PFD inhibits the TGF-β/Smad signaling pathway, reducing the expression of EMT-related transcription factors and mesenchymal genes, inhibiting breast cancer cell proliferation, migration, and invasion, and promoting apoptosis. Additionally, although PFD has a relatively mild standalone antitumor effect *in vivo*, its combination with nab-paclitaxel (nab-PTX) significantly enhances the anticancer effect in TNBC (Luo et al., 2023).

PFD demonstrates significant potential when combined with immunotherapy. When combined with PD-L1 inhibitors, PFD significantly delays tumor growth, improves survival rates, enhances both innate and adaptive immune responses, increases immune cell infiltration, and optimizes T cell localization. This combination therapy also effectively alleviates lung fibrosis and reduces tumor growth (Qin et al., 2020). In bladder cancer, the combination of PD-L1 inhibitors and PFD can significantly inhibit bladder cancer progression, potentially by modulating the tumor immune microenvironment and inhibiting tumor cell epithelial-mesenchymal transition (Chen et al., 2024).

5.2.3 Targeted drug delivery increases therapeutic efficacy

In pancreatic cancer, PFD combined with miR-138-5p, delivered through targeted engineered exosomes, successfully reprogrammed CAFs, inhibiting their pro-tumor effects. The combination inhibited the TGF-β signaling pathway and collagen synthesis, significantly improving the TME, reducing tumor pressure, enhancing the penetration of the chemotherapeutic drug gemcitabine, and increasing the sensitivity of cancer cells to chemotherapy (Zhou et al., 2024). In Jia et al. (2024)'s study, cell membrane-fused liposomes were used for targeted delivery of PFD and doxorubicin to inhibit CAF activity and remodel the TME, thereby significantly enhancing chemotherapy efficacy in TNBC. Furthermore, the optimized delivery strategy amplified the effects of anti-PD-L1 immunotherapy (Jia et al., 2024). Targeted drug delivery provides new insights for precision medicine in clinical practice.

5.3 Galunisertib

5.3.1 Clinical trials

Galunisertib is a selective inhibitor of TGF-β receptor type I (ALK5), capable of blocking TGF-β signaling, inhibiting tumor growth and metastasis, and demonstrating potential in the treatment of malignant tumors. In a trial evaluating Galunisertib combined with nivolumab for NSCLC treatment, patients received Galunisertib (150 mg, 14 days on/14 days off) along with nivolumab (3 mg/kg IV every 2 weeks). 24% of patients showed confirmed partial responses, and 16% of patients exhibited stable disease. The median progression-free survival was 5.26 months, and the median overall survival was 11.99 months. The response rate for locally advanced NSCLC is generally low, about 10%–20%, suggesting that this drug may partially increase patient survival rates (Nadal et al., 2023). In another study, patients with locally advanced rectal cancer received Galunisertib-containing neoadjuvant chemoradiotherapy, resulting in an increase in the complete response rate to 32% with good tolerability, markedly increases the complete response rate compared to the previous treatment regimen (Yamazaki et al., 2022). Galunisertib has demonstrated potential in increasing complete response rates clinically, and its efficacy deserves further evaluation in randomized trials.

5.3.2 Sensitization to chemotherapy and immunotherapy

Fibrosis forms a physical barrier and also creates an immunosuppressive microenvironment by secreting multiple cytokines. Anti-TGF-β drugs reduce fibrosis and can partially relieve this immunosuppression, promoting the infiltration of T cells and other immune effector cells into the tumor area. In OSCC, Galunisertib downregulates TGF-β signaling, enhances $CD8^+$ T cell activity, and improves the efficacy of anti-PD-1 immunotherapy (Tao et al., 2024). In aggressive B-cell non-Hodgkin lymphoma (B-NHL), Galunisertib promotes immune system activation, reduces detrimental Treg cells, and prevents $CD8^+$ T cell exhaustion (Rej et al., 2023). In PDAC, Galunisertib combined with dual immune checkpoint inhibitors (anti-PD-L1 and CTLA-4) significantly inhibits tumor growth and induces the infiltration of antitumor M1 macrophages. Additionally, it can enhance the immune system's tumor-attacking ability by reducing the number of tumor-associated immunosuppressive cells (Rana et al., 2022). Galunisertib combined with IL-15-activated dendritic cells significantly enhances immunotherapy efficacy in highly invasive and metastatic mouse lymphoma. This combination therapy improves prognosis by inhibiting Treg cells in tumor-draining lymph nodes and spleen and through the inactivation of p-SMAD2 and Neuropilin-1 (Hira et al., 2020).

Galunisertib, when combined with chemotherapy drugs, enhances therapeutic efficacy. In B-NHL, Galunisertib enhances the antiproliferative and pro-apoptotic effects of doxorubicin and further inhibits tumor growth by upregulating p-P38 MAPK and inhibiting the TGF-β/Smad2/3 and PI3K/AKT signaling pathways (Rej et al., 2023).

5.4 Tranilast

Tranilast is an anti-allergic medication originally used to treat allergic conditions such as bronchial asthma, allergic rhinitis, and eczema. However, as research has advanced, Tranilast has demonstrated potential in the treatment of fibrosis-related diseases and certain cancers by inhibiting fibroblast activation, reducing malignant tumor resistance, and decreasing tumor proliferation. For instance, in CRC, Tranilast inhibits tumor growth by reducing tumor size, fibrosis, and angiogenesis. When combined with 5-FU, Tranilast further enhances the antitumor effect, leading to increased ROS production, decreased collagen deposition, and enhanced tumor necrosis (Hashemzehi et al., 2021).

5.4.1 Tranilast impacts CAF function

Tranilast inhibits the migration of M2 macrophages by suppressing CXCL12 secretion by CAFs, while also inhibiting tumor growth, fibrosis, and the infiltration of M2 macrophages and mast cells. Additionally, it significantly promotes $CD8^+$

lymphocyte infiltration into the tumor, thereby inducing cancer cell apoptosis via immune response (Nakamura et al., 2022). In NSCLC, Tranilast inhibits IL-6 secretion by CAFs, blocks CAF-induced upregulation of the STAT3 signaling pathway, reduces EMT, and reverses CAF-mediated resistance of NSCLC to osimertinib/selumetinib (Ochi et al., 2022). Furthermore, Tranilast, by inhibiting CAF activity, prevents them from promoting the survival and radioresistance of nasopharyngeal carcinoma cells via the IL-8/NF-κB pathway following radiotherapy (Huang W.-C. et al., 2021).

5.4.2 Tranilast suppresses the TGF-β signaling pathway

In lung cancer, Tranilast inhibits TGF-β1-induced EMT and cell invasion by suppressing Smad4 expression, leading to reduced pleural dissemination of cancer cells (Takahashi et al., 2020). In breast cancer, Tranilast modulates the TGF-β signaling pathway by increasing AKT1 phosphorylation and reducing ERK1/2 phosphorylation, causing cell cycle arrest after the G1/S phase. Additionally, Tranilast upregulates p53, induces PARP cleavage, promotes tumor cell apoptosis, and modulates cell migration and invasion by inhibiting TGF-β (Subramaniam et al., 2010).

5.4.3 Tranilast enhances the TME

Tranilast combined with Doxil treatment normalizes the TNBC TME by significantly reducing ECM components, increasing tumor blood vessel diameter and pericyte coverage, and improving tumor perfusion and oxygenation. These changes enhanced the antitumor immune response and improved therapeutic efficacy. Additionally, Tranilast restored T cell infiltration and reduced the migration of T cells away from immunosuppressive CAFs. The combination of Tranilast and Doxil also significantly increased the levels of immunostimulatory M1 macrophages in tumor tissue, enhancing the efficacy of immune checkpoint inhibitors (such as anti-PD-1/anti-CTLA-4) (Panagi et al., 2020).

6 Conclusion and outlook

In conclusion, as research deepens, the interaction between fibrosis and malignant tumors has received increasing attention. Fibrosis is not merely a consequence of tumor development but plays a crucial role in tumor progression, resistance to therapy, and immune evasion. Fibrosis facilitates tumor cell proliferation, migration, and invasion by altering the composition and structure of the extracellular matrix, while also offering a protective niche for tumor cells to evade immune surveillance. Additionally, fibrosis is closely linked to the activation of tumor-associated fibroblasts, which secrete various cytokines and growth factors, further exacerbating the malignancy of tumors.

Strategies targeting fibrosis in tumor treatment exhibit broad prospects, as fibrosis plays a key role in tumor progression and drug resistance. Inhibiting fibrosis-related signaling pathways, such as TGF-β, can not only suppress tumor cell proliferation and metastasis but also enhance the effects of chemotherapy, targeted therapy, and immunotherapy. For instance, TGF-β inhibitors can decrease fibrosis, improve drug permeability, and increase treatment effectiveness. Moreover, drugs targeting CAFs in the tumor microenvironment have demonstrated potential in preclinical research (Conte, 2022).

In the future, anti-fibrosis therapies are likely to become a crucial part of cancer treatment, especially when combined with current therapies, providing new options for patients with difficult-to-treat tumors. Through ongoing research, anti-fibrosis strategies will offer crucial support in enhancing treatment outcomes and improving patients' quality of life.

Author contributions

HC: Conceptualization, Writing–original draft. XX: Conceptualization, Writing–review and editing. JL: Writing–original draft. YX: Writing–review and editing. XnL: Writing–review and editing. KZ: Writing–review and editing. HJ: Writing–review and editing. XaL: Writing–review and editing. ML: Writing–review and editing, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The impact of diabetes mellitus on tendon pathology: a review

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Diabetes is one of the most common metabolic diseases worldwide, leading to complications, mortality, and significant healthcare expenditures, which impose a substantial social and financial burden globally. A diabetic environment can induce metabolic changes, negatively affecting tendon homeostasis, leading to alterations in biomechanical properties and histopathology. Numerous studies have investigated the mechanisms through which diabetes exerts pathological effects on tendons, including increased free radical production, oxidative stress, inflammatory responses, deposition of advanced glycation end products (AGEs), and microvascular changes. These metabolic changes damages tendon structure, biomechanics, and tendon repair processes. The proliferation of tendon stem cells decreases, apoptosis increases, and abnormal differentiation, along with abnormal expression of myofibroblasts, ultimately lead to insufficient tendon repair, fibrosis, and remodeling. Although researches unveiling the effects of diabetes on tendinopathy, fibrosis or contracture, and tendon injury healing are growing, systematic understanding is still lacking. Therefore, this review summarizes the current research status and provides a comprehensive overview, offering theoretical guidance for future in-depth exploration of the impact of diabetes on tendons and the development of treatments for diabetes-related tendon diseases.

KEYWORDS

diabetes, tendon, tendinopathy, pathology, fibrosis

1 Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia, primarily caused by insufficient insulin secretion (type 1 diabetes, T1DM) or insulin resistance (type 2 diabetes, T2DM) (DiMeglio et al., 2018; Chatterjee et al., 2017). The latter accounts for approximately 90% of all diabetes cases and is one of the most prevalent metabolic diseases worldwide (Chatterjee et al., 2017; Giha et al., 2022). By 2045, the prevalence is projected to rise to 12.2% of the population worldwide (Sun et al., 2022). The high prevalence of DM has significant social, economic, and developmental implications (Chatterjee et al., 2017; Vasiljević et al., 2022). Complications, mortality, and healthcare costs associated with DM impose a considerable social and financial burden (Cho et al., 2018; Nichols et al., 2019).

DM induces metabolic changes in microenvironment, such as increased free radical production, oxidative stress, abnormal expression of inflammatory factors (Vasiljević et al., 2022), copper metabolism abnormalities (Jia et al., 2024a), and the deposition of advanced

glycation end products (AGEs) (Lee and Veres, 2019; Haus et al., 2007) These diabetes-related microenvironmental changes lead to numerous clinical complications, such microvascular diseases (Puri et al., 2022) and macrovascular diseases (Puri et al., 2022; Kato et al., 2024). In addition, musculoskeletal abnormalities, including tendon dysfunction, are also common complications of diabetes (Giha et al., 2022; Shalit et al., 2024).

Tendons connect muscles and bones, effectively transmitting muscle forces during musculoskeletal movements (Lu et al., 2020; Sharma and Maffulli, 2005; Singh et al., 2022). While there are extensive research on the impact of DM on musculoskeletal disorders, including arthritis (Wang et al., 2024a; Banu and Köseoğlu, 2023), osteoporosis (Li et al., 2024a), skeletal muscle atrophy (Atala et al., 2021; Cruz-Jentoft et al., 2019), and fibrosis (Singh et al., 2022; Wu et al., 2024), recent years witnesses an increasing number of investigations on the effects of DM on tendon homeostasis, providing knowledgeable foundation for further studies.

2 Impact of DM on normal tendons

The primary components of tendons are dense fibrous connective tissue and collagen, connecting muscles to bones and efficiently transmits forces during movements (Lu et al., 2020; Sharma and Maffulli, 2005). T2DM leads to impaired cellular glucose uptake and chronic hyperglycemia, exposing tissues to abnormally high glucose concentrations (Chatterjee et al., 2017). In both basic and clinical studies, the impact of T2DM on tendon homeostasis is generally overlooked, possibly due to a lack of recognition of the chronic pathological changes in tendon structure caused by T2DM (Nichols et al., 2019; Kim et al., 2022; Filgueiras et al., 2022).

DM alters muscle microcirculation and metabolic responses. In diabetic patients with a high risk of peripheral arterial or neurological disease, microcirculation deterioration is present in muscles and tendons, and tendon homeostasis may be affected by hyperglycemia (Kim et al., 2022; Kim et al., 2021; Panji Sananta et al., 2019), leading to structural changes and inflammation (Nopparat et al., 2023). Furthermore, T2DM is associated with increased oxidative stress (OS), which negatively affects tendon conditions (Vasiljević et al., 2022; Atala et al., 2021; Alabadi et al., 2023). Advanced glycation end products (AGEs) are compounds formed by aging and DM, which activate NADPH oxidase (NOX), increase reactive oxygen species (ROS) production and leads to OS (Kato et al., 2023; Shinohara et al., 2022a). AGEs also induces OS and triggers inflammatory responses (Shinohara et al., 2022a). The accumulation of AGEs, combined with other systemic and behavioral factors, further complicates tendon dysfunction (Singh et al., 2022; Zellers et al., 2021). AGEs, formed by non-enzymatic reactions, bind to membrane receptors to exacerbate inflammation and accelerate protein degradation (Puri et al., 2022; Cruz-Jentoft et al., 2019).

Structural changes in tendons of DM patients include collagen fiber disorder and micro-tears (Lo et al., 2013; Zaib et al., 2024; Chang et al., 2022). The metabolic changes in the microenvironment affect tendon stiffness, collagen composition, and physiology (Lee and Veres, 2019; Shi et al., 2021), which may be associated with AGEs (Fessel et al., 2014; Li et al., 2013). Research has shown that the crosslinking of AGEs in DM tendon inhibits the biomechanical plasticity and significantly disrupts tissue morphology (Lee and Veres, 2019; Indyk et al., 2021). The accumulated AGEs not only crosslinks adjacent collagen molecules to weaken biomechanics (Lee and Veres, 2019), but also induces inflammatory responses (Indyk et al., 2021). Moreover, pro-inflammatory chemokines, such as CCL-1, 2, 4, and 5, are highly expressed in the circulation of T2DM, further mediating inflammation (Mir et al., 2024).

Degenerative changes in tendons are common in DM patients (Abate et al., 2010). For example, histological studies confirm that hyperglycemia caused by DM is associated with degeneration of the rotator cuff or Achilles tendons (Kim et al., 2022; Kent and Bailey, 1985). Even asymptomatic DM patients may exhibit morphological abnormalities in the Achilles tendon (Afolabi et al., 2020), such as thickening, collagen disorder, or calcific changes at the tendon-bone junction (Harish et al., 2020; Vaidya et al., 2022; Xu et al., 2022). Specifically, Sneha et al. (Harish et al., 2020) evaluated the Achilles tendons of 61 healthy volunteers and 81 T2DM patients using ultrasound. Compared with healthy volunteers, the Achilles in T2DM patients was thickened and softened. DM can also lead to increased risk of Achilles tendon and plantar fascia contracture, impairing foot biomechanics and contributing to foot ulcers (Zellers et al., 2021; Harish et al., 2020; Ra and Hn, 2022). These changes reduce the extensibility of normal tendons and the strain energy of rupture (Lopez-Pedrosa et al., 2024; Su et al., 2024).

3 Impact of DM on tendinopathy

Tendinopathy is a common connective tissue disease, widely described as involving cellular proliferation, changes in extracellular matrix (ECM) turnover/synthesis, and inflammation associated with chronic tendon pathology (Sikes et al., 2021). The etiology is multifactorial and not yet fully understood (Giha et al., 2022; Xu et al., 2022). Tendinopathy is usually caused by overuse, metabolic disorders, and other metabolic factors related to micro-injuries in tendons. Tendinopathy is a challenging complication in diabetic patients (Shi et al., 2021; Cannata et al., 2020), often leading to chronic pain, restricted joint mobility, and even tendon rupture. DM, especially hyperglycemia, leads to elevated levels of acetylated p53, promoting cell apoptosis and OS, shifting the response of tenocytes from anabolic to pathogenic (Chang et al., 2022; Shinohara et al., 2022b), increasing the risk of developing tendinopathy (Panji Sananta et al., 2019; Harish et al., 2020). The potential pathogenic mechanisms by which DM leads to tendinopathy can generally be categorized into several aspects (Figure 1).

Chronic Inflammation: It is well known that diabetic patients are in a pro-inflammatory state, and the hyperglycemic environment in diabetes may lead to chronic inflammation in tendons, eventually progressing to tendinopathy (Kwan et al., 2020). Diabetic patients typically exhibit elevated levels of pro-inflammatory cytokines, such as prostaglandins, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and leukotriene B4, which are significantly elevated in the serum of diabetic patients (Vasiljević et al., 2022; Zaib et al., 2024). These elevated levels of pro-inflammatory cytokines and chemokines may contribute to the chronic development of

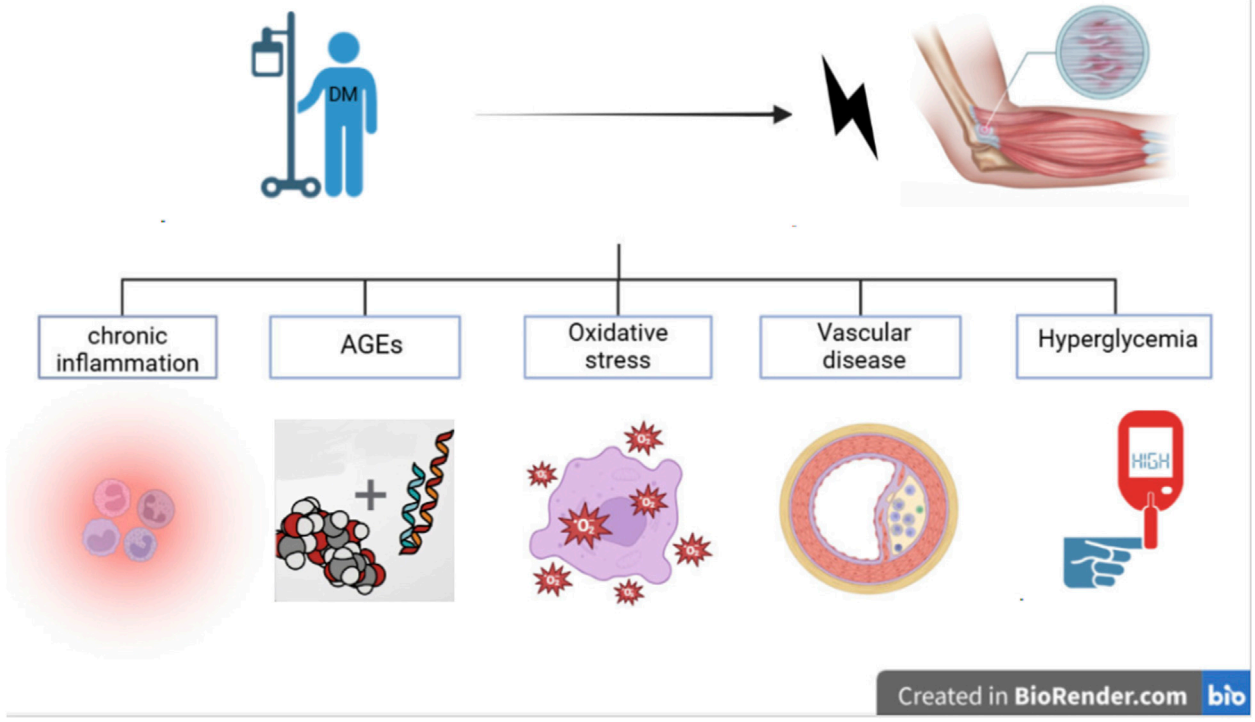

FIGURE 1
Schematic illustration of the potential mechanisms by whcich diabetes mellitus triggers tendinopathy.

tendinopathy (Indyk et al., 2021; Xu et al., 2022; Kwan et al., 2020). Evidence indicates that the chronic inflammation observed in tendinopathy may be due to the reduced proteolytic response of tendon-derived stem cells (TDSCs) in tendinopathy, where the hyperglycemic environment may stimulate chronic inflammation and reduced proteolytic response, leading to tendinopathy (Kwan et al., 2020). Studies on the role of T2DM in rotator cuff tendinopathy suggest that persistent hyperglycemia may impair the proliferation and autophagy of tenocytes, further leading to increased expression of pro-inflammatory and pro-fibrotic mediators (Song et al., 2022).

Excessive Production of AGEs: AGEs can alter collagen within tendons, increase collagen crosslinking, reduce tendon fiber sliding and viscoelasticity, inhibit the biomechanical plasticity of natural tendons, and disrupt tendon morphology (Lee and Veres, 2019; Indyk et al., 2021). TDSCs are involved in tendinopathy, and AGEs can alter the pathophysiology of tendons in diabetic patients by regulating the proliferation and differentiation of TSPCs (Lu et al., 2020). However, other studies suggest that the relationship between AGE content and tendon tensile mechanics may be obscured by collagen disorder (Zellers et al., 2021).

OS: Diabetic patients may experience impaired angiogenesis, promoting tissue hypoxia and the production of ROS, leading to OS and pathological damage (Abu Khadra et al., 2024). In addition, DM patients have lower levels of catalase (CAT) activity, with an imbalance between oxidants and antioxidants, which increases OS to induce cell death and trigger tendinopathy (Lu et al., 2020; Abu Khadra et al., 2024; Yoon et al., 2024).

Vascular Changes: Vascular disease is one of the most common long-term complications of poorly DM, leading to functional and structural changes in the macrovascular and microvascular systems of tendons (Panji Sananta et al., 2019). These biochemical and structural abnormalities are also observed in various organs and tissues, including nephropathy, retinopathy, peripheral neuropathy, atherosclerosis, etc. (Kato et al., 2024; Tavares et al., 2021; Zheng et al., 2021). Diabetes-induced endothelial cell damage reduces the synthesis and secretion of protective factors, resulting in vasoconstriction and inflammation (Sharma and Maffulli, 2005).

Circulating AGEs are associated with vascular complications (Kato et al., 2024). Impaired vascular supply may also reduce the nutrients and oxygen supply to connective tissues, leading to degenerative changes and hindering tendon healing, thus promoting tendinopathy (Kato et al., 2024; Indyk et al., 2021; Abu Khadra et al., 2024). Some studies suggest that dysregulated glucose and lipid metabolism exacerbate the aging of TDSCs and promote osteogenic differentiation (Chen et al., 2024).

Calcific tendinopathy of the Achilles tendon is common, but most patients are asymptomatic. The incidence of Achilles tendon insertional calcific tendinopathy increases with age and is significantly higher in diabetic patients (Giai Via et al., 2022). Research shows that the risk of developing calcific tendinopathy of the shoulder increases by 27% at 8 years following DM diagnosis (Su et al., 2021). On the other hand, the etiology and pathogenesis of calcific tendinopathy remain unclear. Riley et al. (1994). proposed a theory suggesting that ischemic injury and rotator cuff degeneration associated with metabolic diseases lead to further calcification, indicating that metabolic diseases may be related to calcific tendinopathy. Chen et al. (2024) demonstrated that dysregulated glucose and lipid metabolism can activate the CXCL13-CXCR5 axis in aged TDSCs, thereby promoting ectopic ossification.

Hyperglycemia, inflammatory responses, AGEs, OS, and diabetic vascular changes can all influence tendon cell behavior. However, the extent to which these specific changes lead to diabetic tendinopathy and impaired healing remains unclear (Vaidya et al., 2022). Antidiabetic drugs may have beneficial effects on diabetic tendinopathy. Pioglitazone improves TDSC dysfunction caused by AGEs through autophagy promotion, and pioglitazone has been identified as a potential pharmacological option for tendinopathy (Xu et al., 2020). Research on metformin suggests that it may affect gene expression of myogenesis and adipogenesis, while whether metformin benefits tendinopathy remain unclear (Chang et al., 2022). Further efforts are required to develop effective therapeutics.

4 Impact of DM on tendon fibrosis

DM is associated with several fibrotic conditions, such as frozen shoulder, Dupuytren's contracture, trigger finger, Achilles tendon contracture, and plantar fasciitis, which limit the range of motion of the affected joints, impairing function and the ability to perform daily activities (Abate et al., 2013; Al-Matubsi et al., 2011). Fibrosis is characterized by the accumulation of ECM, usually involving changes in ECM quality. The morphological and biochemical disruption of the ECM is directly related to the loss of target organ function (Primadhi and Herman, 2021; Ramirez et al., 2024). The excessive production of AGEs under hyperglycemic conditions can alter collagen within tendons, increase collagen crosslinking, reduce tendon fiber sliding and viscoelasticity, inhibit the biomechanical plasticity of natural tendons, and disrupt tendon morphology (Lee and Veres, 2019; Indyk et al., 2021; Gautieri and Silván, 2016). By stimulating transforming growth factor-beta (TGF-β) pathway, AGEs and ROS regulate the expression of various matrix proteins, forming fibrotic tissue (Primadhi and Herman, 2021; Li et al., 2024b; Noonin and Thongboonkerd, 2024). Myofibroblasts, the main producers and organizers of collagen/ECM during tissue healing, are also sensitive to DM related pathological changes, initiating hypertrophic scar formation and tissue fibrosis (Schuster et al., 2023). Given the aberrant fibrogenesis process, T2DM significantly impairs tendon healing by inducing scar formation (Zhao et al., 2017).

Tendon injuries can occur at the muscle-tendon junction (e.g., gastrocnemius, quadriceps), within the tendon itself (e.g., Achilles tendon), and at the tendon-bone interface (e.g., rotator cuff) (Sharma and Maffulli, 2005; Tavares et al., 2021; Takahashi et al., 2021; Yuan et al., 2024). Tendon healing occurs in three overlapping phases: the initial inflammatory phase, where erythrocytes and inflammatory cells, particularly neutrophils, infiltrate the injury site, with monocytes and macrophages predominating within the first 24 h, leading to the phagocytosis of necrotic material; a few days later, the proliferative phase begins and lasts for several weeks, during which the synthesis of type III collagen peaks; approximately 6 weeks later, the remodeling phase begins, characterized by a reduction in cell numbers, and decreased collagen and glycosaminoglycan synthesis. The remodeling phase can be divided into a consolidation phase, beginning around 6 weeks and lasting up to 10 weeks, and a maturation phase, starting 10 weeks after injury and continuing for up to a year, during which fibrous tissue gradually transforms into scar-like tendon tissue (Sharma and Maffulli, 2005; Farkas et al., 1973; Adawhhflf et al., 1983).

The increased risk of rotator cuff tears (RCTs) in diabetic patients may be related to impaired microcirculation (Yuan et al., 2024). Studies have shown that sodium-glucose cotransporter 2 inhibitors (SGLT2is) promote systemic anti-inflammatory effects by increasing fat utilization and regulating macrophage-mediated inflammatory pathways. SGLT2 inhibitors may prevent rotator cuff tears and subsequent repairs by reducing inflammation (Su et al., 2024). Diabetes leads to severe damage to the inflammatory, angiogenic, and proliferative processes, which may adversely affect tendon healing or remodeling after injury (Chbinou and Frenette, 2004).

Diabetic patients are at a higher risk of requiring tendon repair surgery (Cho et al., 2015), and diabetes can affect tendon healing post-operatively (Tavares et al., 2021; Takahashi et al., 2021; Griffith et al., 2022). Elevated hemoglobin A1c levels 3–6 months after rotator cuff repair surgery in diabetic patients are associated with an increased rate of re-tears (Kim et al., 2023). Nevertheless, for diabetic patients with perioperative glycemic control, the re-tear rate following rotator cuff repair is observed to be comparable to that of non-diabetic patients (Smith et al., 2021), underlying the importance of blood glucose control.

Tendon-bone healing is a challenging process in orthopedics and sports medicine (Wang et al., 2024b), while DM is a significant risk factor for poor tendon-to-bone healing. The hyperglycemic microenvironment inhibits TDSCs proliferation and inducing osteochondral differentiation, a potential mechanism by which diabetes impairs tendon-to-bone healing (Cao et al., 2022). Additionally, diabetes-induced hyperglycemia increases the expression of AGE and RAGE, resulting in significantly elevated mRNA expression levels of NOX1, NOX4, IL-6, RAGE, type III collagen, MMP2, TIMP1, and TIMP2 in the rotator cuff tendon, along with an increase in ROS-positive cells and apoptotic cells (Lee and Veres, 2019; Shinohara et al., 2022b; Yoshikawa et al., 2022). These inflammatory factors also induce a crosstalk between immune cells and tenocytes/TDSCs, while breaking this vicious cycle has therapeutic potential against this condition (Peng et al., 2024). Fibroblasts is closely correlated with collagen levels, and a hyperglycemic environment negatively impacts fibroblast quantity, adversely affecting tendon healing (Panji Sananta et al., 2019). AGEs-related increased expression of inflammatory factors can result in insufficient type I collagen synthesis of fibroblasts, delaying recovery process (Yoshikawa et al., 2022; Jia et al., 2024b).

5 Potential therapies under development

The ability to manage targets related to tendinopathy/tendon healing and strictly control diabetes may be effective in treating tendon pathology in diabetic patients (Yoon et al., 2024). However, the cellular and molecular components involved in various aspects of tendons disrupted by diabetes remain to be elucidated (Yoon et al., 2024). AGE inhibitors that prevent AGE formation could be a novel approach to treating diabetic tendon-to-bone healing (Jud and Sourij, 2019) (Menè and Pugliese, 2003). These therapeutic options include AGE crosslink breakers, AGE inhibitors, RAGE antagonists,

clinically approved drugs for various indications (e.g., antidiabetic and antihypertensive drugs, or statins), and dietary and herbal treatments (Jud and Sourij, 2019). Direct AGE inhibitors include pyridoxamine and aminoguanidine, which reduce AGE/RAGE by increasing activation of the detoxifying enzyme Glo-1 and inhibiting ROS derived from NOX, as well as by inhibiting the formation of reactive dicarbonyl compounds (such as methylglyoxal) (Sourris et al., 2020). Hyperglycemic conditions increase intracellular ROS levels, a major cause of OS, which may interfere with the repair capacity of damaged or degenerated tendons under hyperglycemic conditions (Yoon et al., 2024; Osonoi et al., 2020). Inhibiting OS and improving mitochondrial function is another manner to facilitate tissue repair (Li et al., 2024c; Quetglas-Llabrés et al., 2024). Dietary polyphenols is noticed to mitigate OS and mitochondrial dysfunction in the crosstalk between type 2 diabetes and tendinopathy (Wang et al., 2024c). Polyphenols, such as pomegranate peel extract, have also shown beneficial effects on inflammatory states and OS biomarkers in T2DM (Vasiljević et al., 2022).

The decline in regenerative function of adipose-derived stem cells is partly mediated by the OS and inflammatory environment induced by diabetes. The induction of antioxidant stress factors in adipose-derived stem cells may represent an adaptive mechanism to cope with the increased OS in the diabetic microenvironment (Ahmed et al., 2024). After applying adipose tissue-derived stromal vascular fraction (SVF) in diabetic rats, the number of tenocytes, capillaries, and collagen increased, improving Achilles tendon rupture healing (Panji Sananta et al., 2019). 3D-printed biological scaffolds have the potential to improve rotator cuff healing by enhancing osteogenesis, reducing inflammation, and promoting macrophage polarization (Wang et al., 2024b). Some studies also suggest that antidiabetic drugs may have beneficial effects on tendon healing. For example, pioglitazone can prevent the harmful effects of AGEs on Achilles tendon healing, improving the biomechanical properties of the Achilles tendon (Jia et al., 2024b). Pioglitazone is a peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonist widely used in clinical practice to treat T2DM. It can also reduce RAGE expression and block its downstream signaling pathways, thereby alleviating OS and inflammation in tissues (Xu et al., 2020; Yuan et al., 2011). Diabetes has adverse effects on the neurotrophic pathways in tendon regeneration. Therefore, new therapeutic strategies for regenerating tendons after injury in diabetic patients may include the modulation of neurotrophic pathway molecules, such as NGF and its receptors (Quaini et al., 2017).

6 Conclusion

In summary, DM alters the microcirculation and metabolic responses in tendons, leading to negative changes that affect the biomechanical properties and histopathology. Specifically, increased free radical production, OS, inflammatory responses, and the deposition of AGEs collectively damage tendon structure, biomechanics, and tendon fibrosis and repair. The decreased proliferation of tendon stem cells, increased apoptosis, and incorrect differentiation ultimately result in insufficient tendon repair, maintenance, and remodeling. Although current research has explored the impact of diabetes on tendons, tendinopathy, and tendon injury healing, detailed evidence on the underlying mechanisms remains to be revealed. Future researches are needed to delve deeper into the mechanisms DM-associated tendon pathology to provide references for developing treatment methods against this disorder.

Author contributions

JX: Writing–original draft, Writing–review and editing. JW: Writing–original draft, Writing–review and editing. YJ: Conceptualization, Writing–original draft. YL: Data curation, Investigation, Writing–original draft. JJ: Methodology, Writing–review and editing. YaW: Formal Analysis, Writing–original draft. XC: Methodology, Writing–review and editing. YuW: Funding acquisition, Writing–original draft. BG: Writing–review and editing. HY: Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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P4HA1: an important target for treating fibrosis related diseases and cancer

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Fibrosis is significantly associated with a wide variety of diseases and is involved in their progression. Fibrosis activated under the influence of different combinations of factors is considered a double-edged sword. Although there has been much research on organ fibrosis in recent years, a variety of organ fibrosis diseases and cancers are not well controlled in terms of prevention, treatment, and prognosis. Clinical studies still lack exploration and discovery of effective targets for the pathogenesis of organ fibrosis. Prolyl 4-hydroxylase subunit alpha 1 (P4HA1) is a protein kinase and the synthesis and secretion of collagen are related to the sustained activation of P4HA1. As further studies are being conducted, the potential role of P4HA1 in the development of fibrosis-associated diseases and cancer is becoming clear. Consequently, we conducted a systematic review and discussion on the role of P4HA1 in the pathogenesis of various fibrosis-related diseases and cancers. We reviewed the possible strategies of P4HA1 in the diagnosis and treatment of fibrosis-related diseases and cancers, and analyzed its potential relevance as a biomarker in the diagnosis and treatment of fibrosis-related diseases and cancer.

KEYWORDS

prolyl 4-hydroxylase subunit alpha 1, fibrosis, cancer, cardiovascular diseases, mechanism

1 Introduction

Fibrosis is the result of tissue repair responses following multiple organ injury. Several cell types, including epithelial cells, vascular endothelial cells, and cells of the innate or acquired immune system, participate in fibrosis by secreting factors that recruit and activate fibroblasts to produce extracellular matrix proteins. After tissue damage, local tissue fibroblasts are activated, and the proliferative capacity and extracellular matrix (ECM) synthesis of fibroblasts increase, providing structural support for tissue repair and resulting in repair effects (Henderson et al., 2020; Antar et al., 2023; Yasuma and Gabazza, 2024). Under chronic injury and persistent inflammatory stimuli, the fibrosis process is often uncontrollable, and uncontrolled fibrosis leads to the continued accumulation of ECM components, which may cause tissue structural damage, organ dysfunction, and ultimately organ failure (Henderson et al., 2020; Antar et al., 2023; Yasuma and Gabazza, 2024; Weiskirchen et al., 2019). At present, treatment for organ fibrosis is still in the stage of actively controlling the primary disease (Ngu et al., 2023; Naehrig et al., 2017). Therefore,

TABLE 1 Studies reporting P4HA1 in cancer.

Disease model	P4HA1 expression	Main function	References
Colorectal Cancer	↑	P4HA1 knockdown inhibits colon cancer cell proliferation and reduces stemness	Xu et al. (2019); Li et al. (2020); Chen et al. (2021)
Gliomas	↑	P4HA1 promotes GBM cell migration and invasion	Shin et al. (2023); Tanaka et al. (2020): Gawel et al. (2019)
Lung Cancer	↑	P4HA1 promotes lung adenocarcinoma cell invasion and metastasis	Yang et al. (2024)
Prostate Cancer	↑	P4HA1 promotes prostate cancer cell growth, tumor progression, and cancer cell stemness	Zhou et al. (2020); Yang et al. (2023)
Pancreatic Cancer	↑	P4HA1 promotes PDAC cell proliferation, drug resistance, and stemness	Zhang et al. (2023); Chakravarthi et al. (2014); Walenta et al. (2004)
Breast Cancer	↑	P4HA1 promotes breast cancer cell metastasis, invasiveness and stemness	Hu et al. (2020); Cao et al. (2019); Li et al. (2023)
Esophageal Cancer	↑	P4HA1 promotes esophageal cancer progression	Hollern et al. (2014)
Hepatocellular Carcinoma	↑	P4HA1 promotes the proliferation of liver cancer cells	Polley et al. (2021)
Ovarian Cancer	↑	P4HA1 promotes ovarian cancer cell migration and invasion	Li et al. (2022); Gou et al. (2023a)

↑: GDF11 expression increased.

there is an urgent need to explore the pathogenesis and regulatory network of fibrosis-related diseases, identify effective intervention targets, and develop drugs with targeted precision.

Several studies have shown that P4HA1, a key protein involved in collagen synthesis, is a promising therapeutic target for fibrosis-related diseases (Chen et al., 2018; Lou et al., 2017). P4HA1 is composed of two identical alpha subunits and two beta subunits (Zhu et al., 2021; Zou et al., 2017) and plays a central role in the formation and stability of collagen triple helix domains (Kivirikko and Pihlajaniemi, 1998). It plays important roles in various cancers (Table 1), liver diseases, and cardiovascular diseases. P4HA1 is widely distributed in various tissues. For example, P4HA1 mRNA is highly expressed in body parts such as the muscle tissue, kidney, liver, and female tissues, and P4HA1 protein is highly expressed in body parts such as the cerebral cortex, nasopharynx, and bronx (Figure 1). This phenomenon may be attributed to post-transcriptional modifications of RNA (Delaunay et al., 2024), including N6-methyladenosine (m6A) and N5-methylcytosine (m5C), as well as post-translational modifications of proteins (Lee et al., 2023), such as phosphorylation and ubiquitination. Splicing, capping, and tailing processes after transcription of RNA may affect the stability of mRNA, potentially leading to elevated transcription levels of mRNA and diminished protein expression (Hao et al., 2024; Gilbert and Nachtergaele, 2023). Post-translational modifications of proteins affect a number of key biological processes, including expression, localization, and enzyme activity (Wang et al., 2023). Consequently, an increase in protein stability and a reduction in the degradation rate may result in a reduction in mRNA transcription levels, while protein expression levels remain elevated. A deeper study on the role of P4HA1 in fibrosis will broaden the perspective of potential targets for treatment. In this article, we discuss the regulatory factors of P4HA1 expression and the signaling pathways involved in diseases caused by P4HA1.

2 P4HA1 and cancer

Cancer is driven by genetic changes that disrupt the survival, proliferation, and spread of cancer cells (Kiri and Ryba, 2024). In 2020, there were a total of 4,546,400 new cases of cancer and 2,992,600 deaths in China, accounting for 25.1% and 30.2% of global cases, respectively (He et al., 2024). The noncancerous components of tumor tissues (including fibroblasts, inflammatory cells, and ECM) play a crucial role in tumorigenesis and cancer progression. This provides a mutagenic environment that allows cancer cells to develop, facilitating their survival, expansion, and invasiveness (Landolt et al., 2022; Mallikarjuna et al., 2022; Nicolini et al., 2023). This presents serious difficulties in the treatment of cancers, such as the emergence of immunotherapy and medication resistance (Naik and Leask, 2023; Xiao and Yu, 2021). Collagen promotes the infiltration, invasion, migration, and angiogenesis of malignant tumors by reshaping the ECM and influencing the tumor microenvironment (Xu et al., 2019; Su and Karin, 2023; Necula et al., 2022). P4HA1 is responsible for producing 4-hydroxyproline at the Yaa position of the Gly Xaa Yaa repeat sequence in collagen, which is necessary for the formation of the collagen triple helix structure (Taga et al., 2014). Previous studies have shown that increased P4HA1 expression is associated with poor prognosis in some solid cancers, such as pancreatic cancer, colon cancer, high-grade glioma, breast cancer, prostate cancer, and lung cancer (Zhou et al., 2023; Zhao and Liu, 2021; Li et al., 2020; Chen et al., 2021).

2.1 Colon cancer

Colorectal cancer (CRC) is the third most common malignant tumor of new cancer cases worldwide (Ionescu et al., 2023; Aljama et al., 2023). The metastasis of CRC is significantly correlated with matrix deposition and remodeling (Shin et al., 2023), indicating that P4HA1 may also have carcinogenic effects in CRC. Tanaka et al.

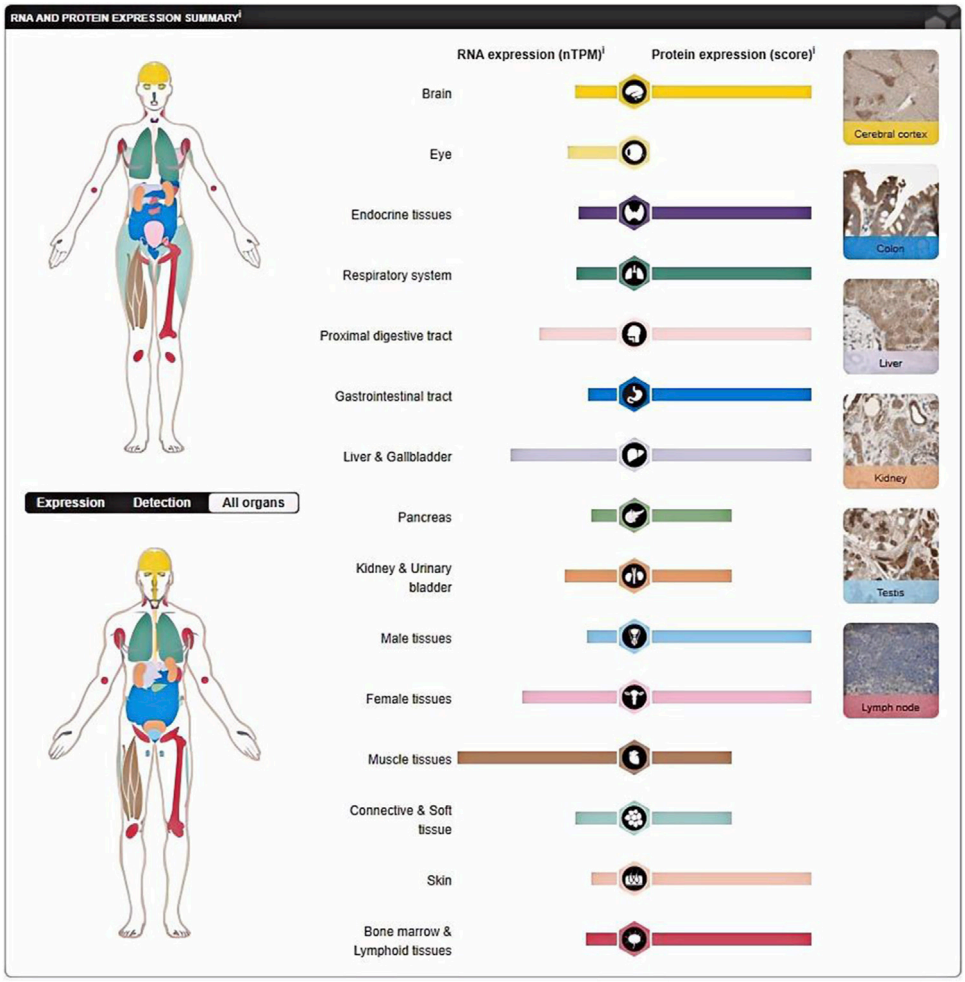

FIGURE 1
P4HA1 is widely distributed in different tissues. P4HA1 protein is highly expressed in cerebral cortex, nasopharynrynx, and broncius. The expression levels of P4HA1 mRNA are relatively high in muscle tissues, kidney tissues, liver tissues, and female tissues. (https://www.proteinatlas.org/ENSG00000122884-P4HA1/tissue).

(2020) found through tissue analysis of clinical cases of 599 patients with stage I or II CRC that P4HA1 is mainly expressed in the malignant epithelial components of CRC. In addition, Gawel et al. (2019) found that the combination of P4HA1 with tripartite motif-containing 28 (TRIM28), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (PLOD1) and carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5) proteins in the plasma of 80 newly diagnosed CRC patients and 80 healthy controls can serve as potential biomarkers for early diagnosis of colorectal cancer. This indicates that P4HA1 plays an important role in the occurrence, development, and diagnosis of CRC. However, the mechanism of action of P4HA1 in CRC is still unclear.

Zhang et al. (2021) found that P4HA1 expression can stabilize hypoxia inducible factor-1 alpha (HIF1α) and activate the Wnt signaling pathway, promoting the proliferation of CRC cells. Chen et al. found through gene expression profiling analysis using the Cancer Genome Atlas (TCGA) that the risk signal of P4HA1 related genes in CRC consists of 11 genes, including MIR210HG, solute carrier family 4 member 7 (SLC4A7), cell division cycle associated 2 (CDCA2), death associated protein kinase 1 (DAPK1), homeobox C6 (HOXC6), Troponin T 1 (TNNT1), UL16 binding protein 2 (ULBP2), serine protease inhibitor clade E member 1 (SERPINE1), WFDC21P, and forkhead box D1 (FOXD1) (Chen et al., 2021). In addition, Agarwal et al. found that P4HA1 is highly expressed in CRC tissues and promotes tumor cell proliferation, invasion, migration, and tumor growth. And diethyl pyhidc can inhibit the progression of invasive CRC by acting on P4HA1 (Agarwal et al., 2020) (Figure 2). The above research progress suggests that P4HA1 may serve as an early diagnostic biomarker and therapeutic target for CRC, but its pathogenic mechanism in CRC is still unknown.

2.2 Gliomas

Glioma is the most common malignant tumor of the central nervous system in adults and is divided into different subtypes. Among them, glioblastoma multiforme (GBM) has the highest number and the strongest lethality (Uddin et al., 2022). The ECM is significantly correlated with the stemness and invasion of

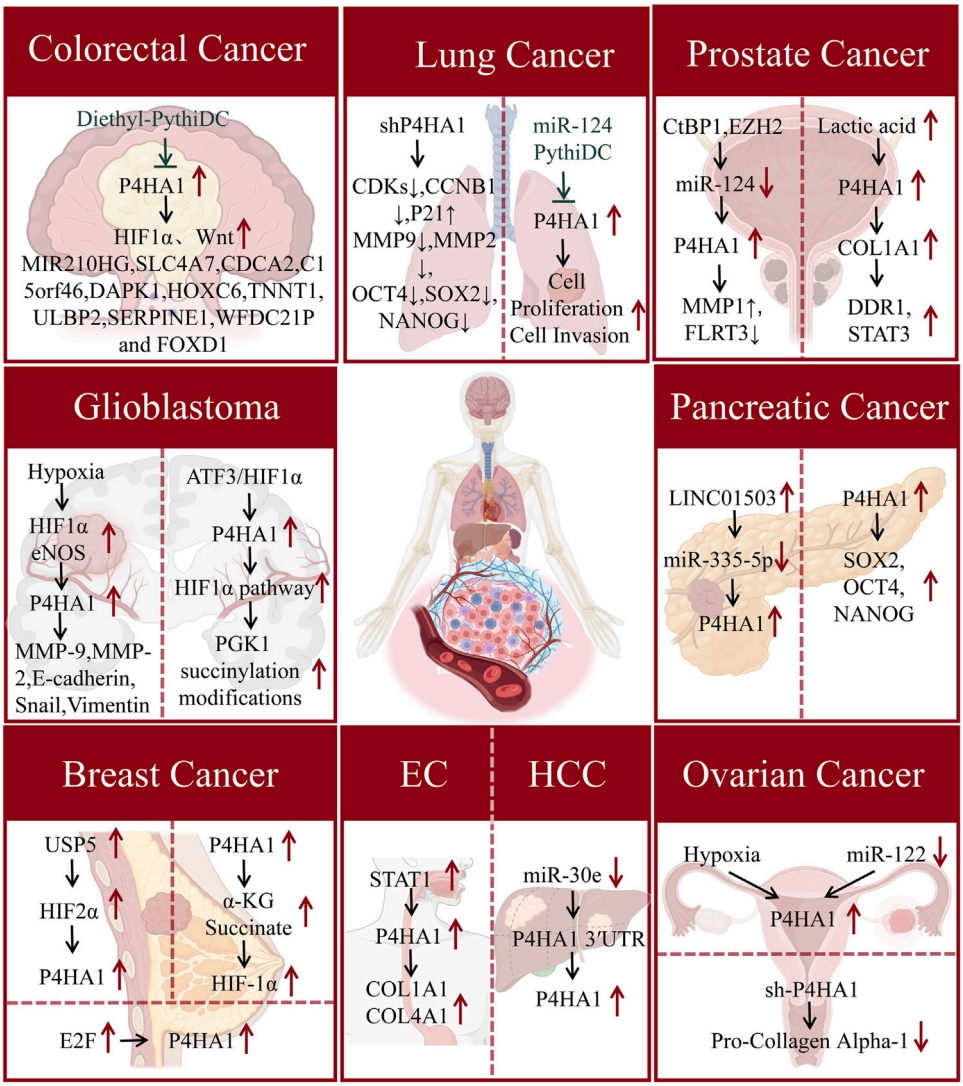

FIGURE 2
The mechanism of action of P4HA1 in various cancers. P4HA1 is involved in regulating the proliferation, migration, and invasion of various cancer cells. The figure summarizes the mechanism by which P4HA1 contributes to the occurrence and progression of cancers in the manuscript.

glioma cells. Cescon et al. (2023) found that collagen VI is involved in maintaining the stem cell-like properties of GBM cells and promoting invasive transcriptional programs for cancer cell proliferation and survival. P4HA1 is a key rate limiting protein in the process of collagen synthesis. Hu et al. found that P4HA1 expression is upregulated in gliomas. The high expression of P4HA1 is associated with the malignancy of glioma and can serve as a prognostic indicator for high-grade glioma patients (Hu et al., 2017). The hypoxic microenvironment affects the invasiveness of cancer cells., Hypoxia promotes cancer cell migration and invasion through the L-Arg/P4HA1 axis in GBM (Zhu et al., 2021). In addition, Yang et al. (2024) found that in GBM cells, P4HA1 enhances PK1 succinylation by affecting succinate concentration, and succinylation inhibits proteasomal degradation of phosphoglycerate kinase 1 (PGK1), significantly increasing aerobic glycolysis to produce lactate. Overexpression of activating transcription factor 3 (ATF3) inhibits the binding of HIF1α to the P4HA1 promoter region under hypoxic conditions, suppressing immune response and tumor growth (Figure 2). Chitosan gelatin microspheres loaded with P4HA1 siRNA can significantly inhibit the proliferation, metastasis, glial layer formation, and protein levels of stromal markers (N-cadherin, vimentin) and epithelial mesenchymal transition (EMT) transcription factors (Snail, Slug, Twist1) in glioma cells (Zhou et al., 2024). The above research suggests that P4HA1 correlates significantly with the expression of transcription factor HIF1α in GBM. Developing therapeutics targeting P4HA1 and HIF1α may be the way to go for treating GBM in the future.

2.3 Lung cancer

Lung cancer is the leading cause of cancer-related death worldwide. Histologically, lung cancer can be divided into small-cell lung cancer

(SCLC) and non-small cell lung cancer (NSCLC) (Jha et al., 2024). At present, the main treatment strategies for lung cancer include molecular targeted therapy, photothermal therapy, and immunotherapy (Feng and Zhang, 2023; Alduais et al., 2023; Lahiri et al., 2023). Research has shown that P4HA1 is essential for the growth and invasion of lung cancer cells, indicating that P4HA1 may be an effective therapeutic target for lung adenocarcinoma (Zhao and Liu, 2021). Zhou et al. (2020) found that the expression of P4HA1 was upregulated by 40% in tumor tissues compared to normal tissues of lung adenocarcinoma. In addition, both P4HA1 mRNA and protein are upregulated in NSCLC. Further research has found that P4HA1 promotes the invasion and metastasis of lung adenocarcinoma tumor cells by affecting EMT and matrix metalloproteinases (MMPs) expression (Ning et al., 2021). MicroRNAs (miRNAs) are a class of non-coding RNAs with a length of approximately 21 nucleotides, and studies have shown that the expression of some miRNAs is dysregulated in NSCLC (Yang et al., 2023; Lobera et al., 2023; Rajakumar et al., 2023). Robinson et al. found that overexpression of miR-124 can significantly inhibit the expression of P4HA1 protein in lung cancer cells, resulting in tumor-suppressive effects (Robinson et al., 2021).

The above studies indicate that P4HA1 plays an important role in the disease progression of lung cancer (Figure 2). Li et al. (2020) found through survival analysis that lung cancer patients with high P4HA1 have a poorer clinical prognosis. Targeting P4HA1 is a promising strategy for treating lung cancer. Therefore, there is an urgent need to develop small molecule inhibitors targeting lung cancer cell P4HA1. Robinson et al. discovered that the small molecule inhibitor PythiDC of P4HA1 can significantly inhibit the cell viability and invasion ability of lung cancer cells (Robinson et al., 2021). The above research indicates that P4HA1 plays a key role in the pathogenesis and prognosis of lung cancer. Furthermore, P4HA1 inhibitors have the potential to become a treatment for lung cancer. However, P4HA1 has not been reported in lung fibrosis, such as idiopathic pulmonary fibrosis. This suggests that research on P4HA1 in pulmonary fibrosis-related diseases is still lacking and that in-depth studies are highly valuable.

2.4 Prostate cancer

Prostate cancer (PCa) is a widespread cancer, which mainly affects men, with a high incidence rate and mortality. It is the second most common cancer in men, after lung cancer (Zhang et al., 2023). In general, there are no typical symptoms in the early stages of PCa, and most newly diagnosed PCa patients are often in the advanced stage. In addition, prostate biopsy is considered the gold standard for the diagnosis of PCa. Currently, there is a lack of relevant biomarkers for the diagnosis and prognosis of PCa. ECM is a major component of the tumor environment, promoting the establishment of pre-invasive behavior. A number of studies have shown that P4HA1 expression is associated with the progression of PCa. Chakravarthi et al. (2014) found that P4HA1 expression was significantly increased in metastatic prostate cancer tissues. Further mechanistic studies have shown that miR-124 regulates prostate cancer cell growth and tumor progression by acting on the expression of P4HA1 and MMP1. Lactic acid is one of the most abundant environmental metabolites in tumors, and its levels are significantly correlated with cancer metastasis in cancer patients (Walenta et al., 2004). Ippolito et al. (2024) found that lactate secreted by cancer-associated fibroblasts promotes an increase in alpha-ketoglutarate (α-KG) in prostate cancer cells, activating α-KG dependent P4HA1 to increase collagen hydroxylation, thereby inducing stemness and invasive features of prostate cancer cells. The above research progress indicates a significant correlation between P4HA1 and cancer metastasis in PCa.

2.5 Pancreatic cancer

Pancreatic cancer is the leading cause of cancer-related death worldwide. At present, clinical treatment for pancreatic cancer is mainly divided into surgery and chemotherapy (Kolbeinsson et al., 2023; Wood et al., 2022; Milella et al., 2022). However, there is still a lack of specific therapeutic targets and biomarkers for pancreatic cancer. Hu et al. (2020) analyzed tumor and normal samples in different datasets and showed that P4HA1 was significantly overexpressed in multiple pancreatic cancer datasets. Ductal adenocarcinoma of the pancreas (PDAC) is the main type of pancreatic cancer. After overexpression of P4HA1, KEGG pathway enrichment analysis showed a significant correlation with the HIF-1 signaling pathway. Research has found that P4HA1 enhances the stability of HIF1α, promotes glycolytic activity in PDAC cells, induces cancer cell proliferation, drug resistance, and stemness (Cao et al., 2019). Cao X. et al. (2023) found that ectopic expression of P4HA1 increased the levels of cancer stem cell-associated proteins [sex-determining region (SOX2), octamer-binding transcription factor 4 (OCT4), and nanog homeobox (NANOG)] in pancreatic ductal adenocarcinoma cells. However, the specific mechanism and key proteins of P4HA1 in the occurrence and malignant progression of pancreatic cancer are still unclear, which deserve further discussion. Hu et al. found that LINC01503/miR-335-5p is the most promising upstream regulation axis that affects P4HA1 in pancreatic cancer through correlation analysis (Hu et al., 2020). Previous studies have demonstrated that P4HA1 plays a significant role in the pathogenesis of pancreatic cancer. However, further investigation is required to elucidate the disease mechanisms and to develop targeted therapeutic agents.

2.6 Breast cancer

Breast cancer (BC) is a common malignant tumor in women globally. Collagen deposition is significantly related to the progress and metastasis of BC (Herrera-Quintana et al., 2024; Papanicolaou et al., 2022; Li et al., 2023). However, at present, the specific mechanism of BC is still unclear. Further clarification of new and more specific biomarkers for the diagnosis, prognosis, and risk prediction of BC is of great significance to achieve personalized treatment, improve treatment, and prevent overtreatment, undertreatment, and incorrect treatment. The regulation of P4HA1 has a significant impact on the prognosis of BC patients (Li et al., 2020; Murugesan and Premkumar, 2021). Hollern et al. (2014) found that E2F transcription factors promote the metastasis of breast cancer, while E2F downstream target genes include Vegfa, Bmp4, Cyr61, and P4HA1, suggesting that P4HA1 may regulate

collagen deposition and participates in the regulation of cancer metastasis and invasion. In addition, ubiquitin-specific peptidase 5 (USP5) is highly expressed in breast cancer. USP5 deubiquitination modifies HIF2α, and protects HIF2α from ubiquitin-proteasome degradation, thus promoting the transcription of HIF2α target genes, such as P4HA1, solute carrier family 2 member 1 (SLC2A1), PLOD2 and vascular endothelial growth factor A (VEGFA), providing a potential therapeutic target for BC (Huang et al., 2022) (Figure 2). Triple-negative breast cancer (TNBC) is the most aggressive and heterogeneous of all BC subtypes (Polley et al., 2021; Vagia et al., 2020; Rigiracciolo et al., 2020). The activation of the HIF-1 pathway in TNBC is at least partially regulated by P4HA1, promoting the stemness of cancer cells. In addition, elevated expression of P4HA1 is associated with poor prognosis and chemotherapy resistance in TNBC patients. The combination of P4Hi and chemotherapy drug doxorubicin can overcome TNBC chemotherapy resistance (Xiong et al., 2018).

2.7 Other cancers

Previous studies have elucidated the function and operational process of P4HA1 in colon cancer, gliomas, lung cancer, prostate cancer, and pancreatic cancer. What is the function of P4HA1 in other types of cancers? The ECM is the main component of the tumor microenvironment. Collagen can promote the invasion and migration of malignant tumors, and P4HA1 is a key enzyme of collagen. Li et al. (2022) inferred that P4HA1 may play an important role in the tumorigenesis of clear cell renal cell carcinoma (RCC) and may be a prognostic biomarker and therapeutic target for various malignancies, including RCC. Gou et al. (2023a) found that the expression of P4HA1 is related to the differentiation degree, location, lymph node metastasis, and tumor lymph node metastasis staging of esophageal squamous cell carcinoma. And it was discovered that P4HA1 is activated by STAT1 transcription, thereby promoting the progression of esophageal cancer (EC) (Gou et al., 2023b). Hepatocellular carcinoma (HCC) is the leading cause of cancer-related deaths around the world, particularly in populations in Asia and Africa. The expression level of miR-30e is reduced in liver cancer tissues. Further research has found that miR-30e can reduce the expression of P4HA1 at both mRNA and protein levels, inhibiting the proliferation of liver cancer cells (Feng et al., 2016).

Ovarian cancer is an invasive disease, and the deposition of collagen is significantly correlated with the invasion, prognosis, and metastasis of ovarian cancer (Akinjiyan et al., 2024; Lyu and Feng, 2021; Ho et al., 2021). Platinum-based chemotherapy is the cornerstone of ovarian cancer treatment, but the resistance of ovarian cancer cells to platinum-based chemotherapy seriously affects the prognosis and survival of ovarian cancer patients. Song et al. observed that hypoxia can significantly upregulate the mRNA and protein expression of P4HA1/2, while knocking down P4HA1/2 can significantly inhibit collagen secretion, migration, and metastasis of ovarian cancer cells (Song et al., 2023). In addition, miR-122 has tumor-suppressive effects on various cancers. Duan et al. (2018) found that miR-122 inhibited the migration, invasion, and EMT of ovarian cancer cells by downregulating P4HA1. MiR-122 and P4HA1 may be potential diagnostic markers and therapeutic targets in ovarian cancer.

Levofloxacin has broad-spectrum anticancer activity, and its combination with cisplatin further enhances the cytotoxicity of cancer cells by promoting apoptosis (He et al., 2022a). Levofloxacin prevents DNA replication in bacteria by inhibiting the activity of DNA helicase. He et al. (2022b) found that levofloxacin significantly inhibited cancer cell proliferation, colony formation, and xenograft tumor growth by blocking the G2/M cell cycle and promoting cell apoptosis. Additionally, P4HA1 is enriched in differentially downregulated genes. P4HA1 mediated high collagen deposition plays a crucial role in the tumor microenvironment and progression, and new therapeutic strategies or small-molecule inhibitors targeting collagen synthesis are being developed, which will be an important direction for future cancer research.

3 P4HA1 and cardiovascular diseases

Cardiovascular disease is the leading cause of morbidity and mortality worldwide. Fibrosis is a common feature of cardiovascular diseases. Cardiovascular fibrosis represents the activation of repair mechanisms for damaged organs. However, prolonged and uncontrolled activation of these repair mechanisms can result in excessive remodeling and hardening of the ECM, leading to impaired cardiac function and ultimately heart failure (Poe A et al., 2023; Ravassa Set al., 2023). The following section will further discuss the role and specific mechanisms of P4HA1 in the context of cardiovascular disease fibrosis.

3.1 Atherosclerosis

Atherosclerosis is the main cause of cardiovascular disease, which is characterized by the accumulation of lipids and fiber elements in the great arteries. Collagen synthesis by vascular smooth muscle cells (VSMCs) is very important in atherosclerosis because it affects plaque stability (Grootaert and Bennett, 2021; Miano et al., 2021; Zhai et al., 2022). miRNAs play an important role in cardiovascular diseases (Han et al., 2021; Bian et al., 2021; Gao et al., 2022). Chen et al. (2018) found a negative correlation between collagen and VSMC content in plaques and miR-124-3p levels. MiR-124-3p inhibits VSMC collagen synthesis by directly targeting P4HA1, which may reduce the stability of atherosclerotic plaques. Low shear stress and oscillatory shear stress can affect the size and phenotype of coronary atherosclerotic lesions. P4HA1 overexpression increases the fiber cap thickness and collagen content of carotid plaques induced by low shear stress and oscillatory shear stress, leading to a significant increase in the size of atherosclerotic plaques (Cao et al., 2016). Plaque rupture is the most common cause of coronary artery occlusion, which can lead to acute coronary syndrome. IL-6 significantly increased the phosphorylation of RAF, mitogen-activated protein kinase (MEK)1/2 and extracellular signal-regulated kinase (ERK) 1/2, and the transcription factor c-Jun mediated the reduction of P4HA1 transcription, downregulated the expression of P4HA1, thereby destroying the stability of mouse atherosclerotic plaques (Zhang et al., 2012). Melatonin is an endogenous neurohormone

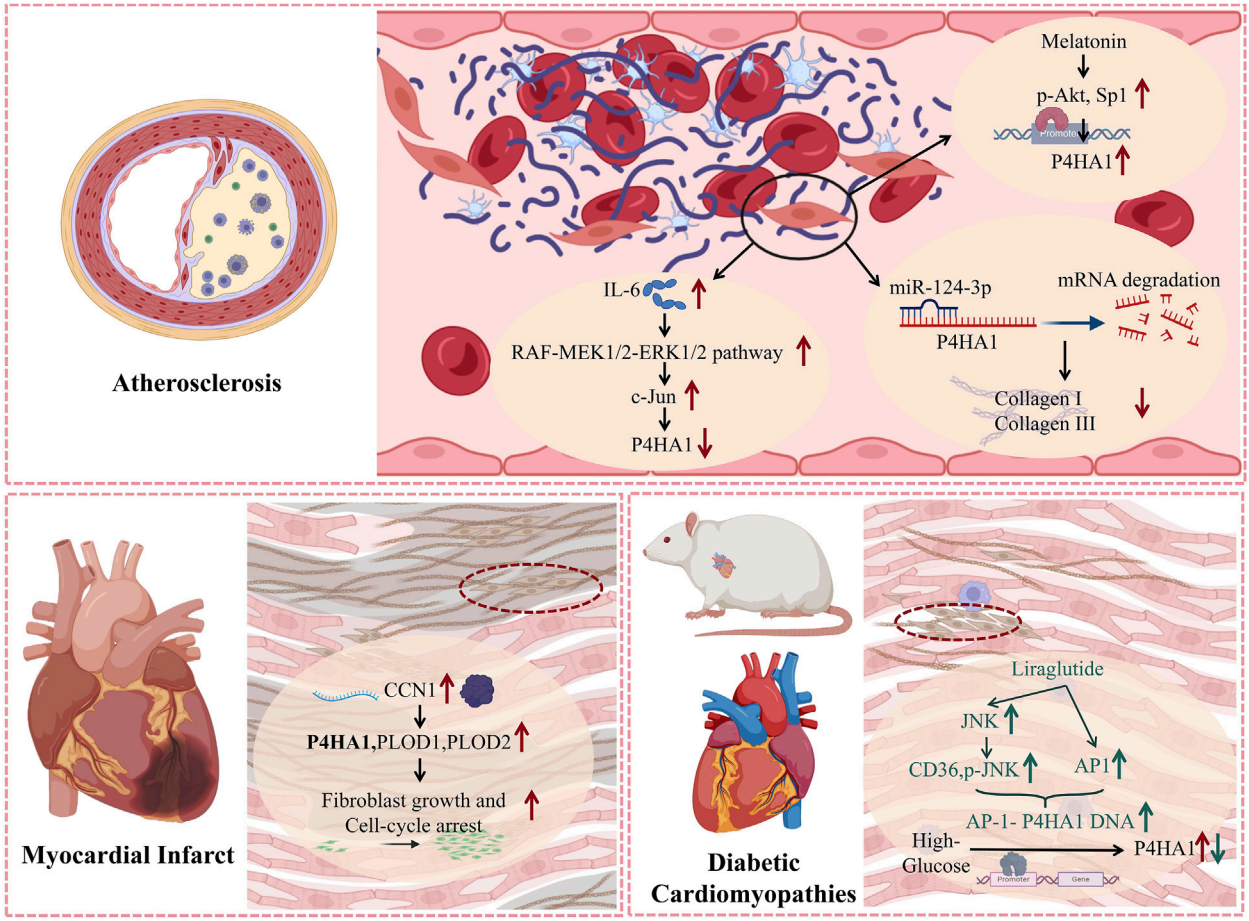

FIGURE 3
The mechanism of action of P4HA1 in various cardiovascular diseases. P4HA1 is involved in regulating the proliferation of fibroblasts and the synthesis of collagen in various cardiovascular diseases. The molecular processes of P4HA1 involvement in cardiovascular disease discussed in the text are summarized in this figure.

primarily secreted by the pineal gland, with multiple physiological functions. Li et al. (2019) found that melatonin increased Akt phosphorylation and transcription activation of specific protein 1 (Sp1), which binds to P4HA1 promoter, induces P4HA1 expression, and enhances the stability of atherosclerotic plaques in ApoE −/− mice.

3.2 Myocardial infarction

Myocardial infarction (MI) is the main cause of global incidence rate and mortality, and also the main cause of heart failure (HF) (Groenewegen et al., 2020; Frantz et al., 2022). The significant loss of myocardial cells and excessive deposition and arrangement of ECM after myocardial infarction leads to serious consequences such as cardiac fibrosis (Yin et al., 2023). Fischer et al. (2024) found that cellular communication network factor (CCN)1 plays a crucial role in scar formation after myocardial infarction, guiding the appropriate arrangement of extracellular matrix collagen components in mature scars - shaping the mechanical properties that support their structural stability. Further research has found that the absence of CCN1 reduces the expression of collagen processing and stabilizing enzymes (i.e., P4HA1, Procollagen-lysine 2-oxyglutarate 5-dioxygenase (PLOD)1, and PLOD2). CCN1 gene knockout mice showed higher ECM structural complexity in the scar area after myocardial infarction, including reduced local arrangement and increased curvature of collagen fibers, as well as a 90% decrease in tissue consistency, packaging, and size of collagen fibrils. The above studies indicate that P4HA1 plays an important role in the synthesis and arrangement of collagen during the fibrosis process after myocardial infarction.

3.3 Diabetic cardiomyopathy

Diabetic cardiomyopathy (DCM) is a serious complication of diabetes (Shao et al., 2022), leading to cardiac fibrosis, even heart failure and other serious consequences (Nakamura et al., 2022). Zhao et al. found that liraglutide can upregulate the expression levels of CD36 and p-JNK, enhance the DNA-binding activity of activator protein (AP)-1 to P4HA1, thereby downregulating P4HA1 expression and reducing myocardial fibrosis (Zhao et al., 2019). This provides a new therapeutic target for heart fibrosis caused by diabetic cardiomyopathy.

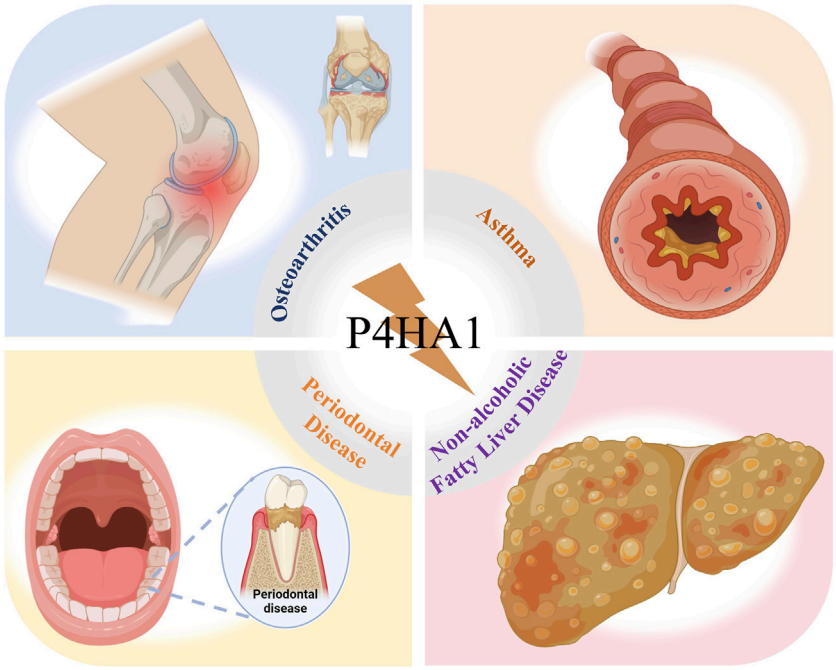

FIGURE 4
P4HA1 is significantly correlated with osteoporosis, asthma, periodontal disease, and NAFLD.

The above results indicate that P4HA1 mediates the synthesis and secretion of collagen, which influences the stability of atherosclerotic arterial plaques and the process of cardiac fibrosis in myocardial infarction and diabetic cardiomyopathy (Figure 3).

4 P4HA1 and other diseases

Non-alcoholic fatty liver disease (NAFLD) is currently the most common liver disease and a global disease that threatens human health. The progression of NAFLD may ultimately result in fibrosis and cirrhosis (Pouwels et al., 2022). In multiple studies, it has been found that P4HA1 is a hub gene in NAFLD, and its expression is downregulated by 95% in NAFLD (Jiang H. et al., 2023a). Cao J. et al. (2023) found a significant correlation between P4HA1 and neutrophils. The above research suggests that P4HA1 may participate in the disease progression of NAFLD by participating in cellular metabolism and inflammatory responses. In addition, P4HA1 is also involved in the process of liver fibrosis. Li et al. found that overexpression of miR-122 in hepatic stellate cells significantly reduced the expression of P4HA1 by targeting the binding site of P4HA1 mRNA 30-UTR, leading to decreased collagen maturation and ECM generation, and inhibited liver fibrosis (Li et al., 2013; Lou et al., 2017).

Periodontal disease is a multifactorial chronic disease. It is usually accompanied by a hypoxic environment, which affects metabolic activation and exacerbates pathological and physiological conditions (Gou et al., 2022). The extracellular matrix of periodontal connective tissue comprises a substantial proportion of type I collagen. Morimoto et al. found that hypoxia culture stimulates upregulation of P4HA1 expression in periodontal ligament cells, increasing collagen levels (Morimoto et al., 2021).

The airway remodeling in asthma airway inflammation is caused by the deposition of collagen on the airway wall. Chelidonium majus may alleviate airway remodeling induced by ovalbumin in asthmatic rats by affecting the expression of P4HA1 (Wang et al., 2024). The above research results indicate that P4HA1 could be used as one of the targets for developing therapeutic drugs for airway inflammation.

Osteoarthritis (OA) is the most common type of arthritis. In OA, the composition and viscoelasticity of the ECM produced by chondrocytes undergo alterations (Hodgkinson et al., 2022). According to reports, P4HA1 disrupts the structure of the vascular basement membrane by inhibiting collagen synthesis (Zhou et al., 2017). Jiang P. et al. (2023) found that miRNA-1 treatment led to a decrease in the expression levels of P4HA1 and aggrecan (ACAN), delaying articular cartilage degeneration (Figure 4).

5 Summary

According to existing research, the role of P4HA1 in pancreatic cancer, colon cancer, high-grade glioma, breast cancer, prostate cancer, lung cancer and other cancers has been preliminarily verified. However, the role of P4HA1 in cardiovascular diseases such as myocardial infarction, ischemia-reperfusion, and heart failure with preserved ejection fraction remains to be explored. Therefore, it is necessary to further expand the research scope and explore the specific roles and mechanisms of P4HA1 in different types of cardiovascular diseases. P4HA1 is expressed in

various organs. This explains its relationship with cancer and cardiovascular disease. Overall, research on the role of screening small-molecule drugs targeting P4HA1 in organ fibrosis diseases and cancer is limited. Therefore, based on current research results, more evidence is needed to apply strategies for treating organ fibrosis by inhibiting the expression of P4HA1 gene and protein. In addition, due to the limitations of research on the mechanism of P4HA1 fibrosis in cardiovascular diseases. Therefore, future research should explore the mechanism of action of P4HA1 through various methods such as cell experiments, animal models, clinical cases, and comprehensively analyze other related genes and signals to understand the role of P4HA1 in fibrosis in cardiovascular diseases. We hope that with the continuous advancement of technology and the continuous development of research, the potential of P4HA1 in treating cardiovascular and cerebrovascular diseases will gradually be discovered and realized.

Author contributions

XY: Writing–original draft. DZ: Writing–original draft. ML: Writing–review and editing. YS: Writing–review and editing. XZ: Writing–review and editing. YX: Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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New insights into SUMOylation and NEDDylation in fibrosis

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Fibrosis is the outcome of any abnormal tissue repair process that results in normal tissue replacement with scar tissue, leading to persistent tissue damage and cellular injury. During the process of fibrosis, many cytokines and chemokines are involved, and their activities are controlled by post-translational modifications, especially SUMOylation and NEDDylation. Both these modifications entail a three-step process of activation, conjugation, and ligation that involves three kinds of enzymes, namely, E1 activating, E2 conjugating, and E3 ligase enzymes. SUMOylation participates in organ fibrosis by modulating FXR, PML, TGF-β receptor I, Sirt3, HIF-1α, and Sirt1, while NEDDylation influences organ fibrosis by regulating cullin3, NIK, SRSF3, and UBE2M. Further investigations exhibit the therapeutic potentials of SUMOylation/NEDDylation activators and inhibitors against organ fibrosis, especially ginkgolic acid in SUMOylation and MLN4924 in NEDDylation. These results demonstrate the therapeutic effects of SUMOylation and NEDDylation against organ fibrosis and highlight their activators as well as inhibitors as potential candidates. In the future, deeper investigations of SUMOylation and NEDDylation are needed to identify novel substrates against organ fibrosis; moreover, clinical investigations are needed to determine the therapeutic effects of their activators and inhibitors that can benefit patients. This review highlights that SUMOylation and NEDDylation function as potential therapeutic targets for organ fibrosis.

KEYWORDS

SUMOylation, NEDDylation, fibrosis, ginkgolic acid, MLN4924

1 Introduction

Fibrosis is the outcome of abnormal tissue repair processes rather than diseases and has been known to cause persistent tissue damage and cellular injury (Antar et al., 2023; Chen et al., 2018; Taru et al., 2024). Fibrotic tissues are characterized by excessive extracellular matrix deposition and activated fibroblasts accompanied by chronic inflammation (Zhang et al., 2021). Wound healing is effective for repairing injured tissues when the damage is minor or non-repetitive, and only a transient increase in the extracellular matrix and a small amount of activated fibroblasts are observed. However, inflammatory and chronic wound-healing responses are aggravated when the damage is severe, and there is poor elimination of the induced profibrotic factors; in such instances, normal tissue is replaced by scar tissue and often results in organ failure (Henderson et al., 2020; Zhang et al., 2024b). The process of fibrosis begins from injury to the epithelial and/or endothelial cells that release

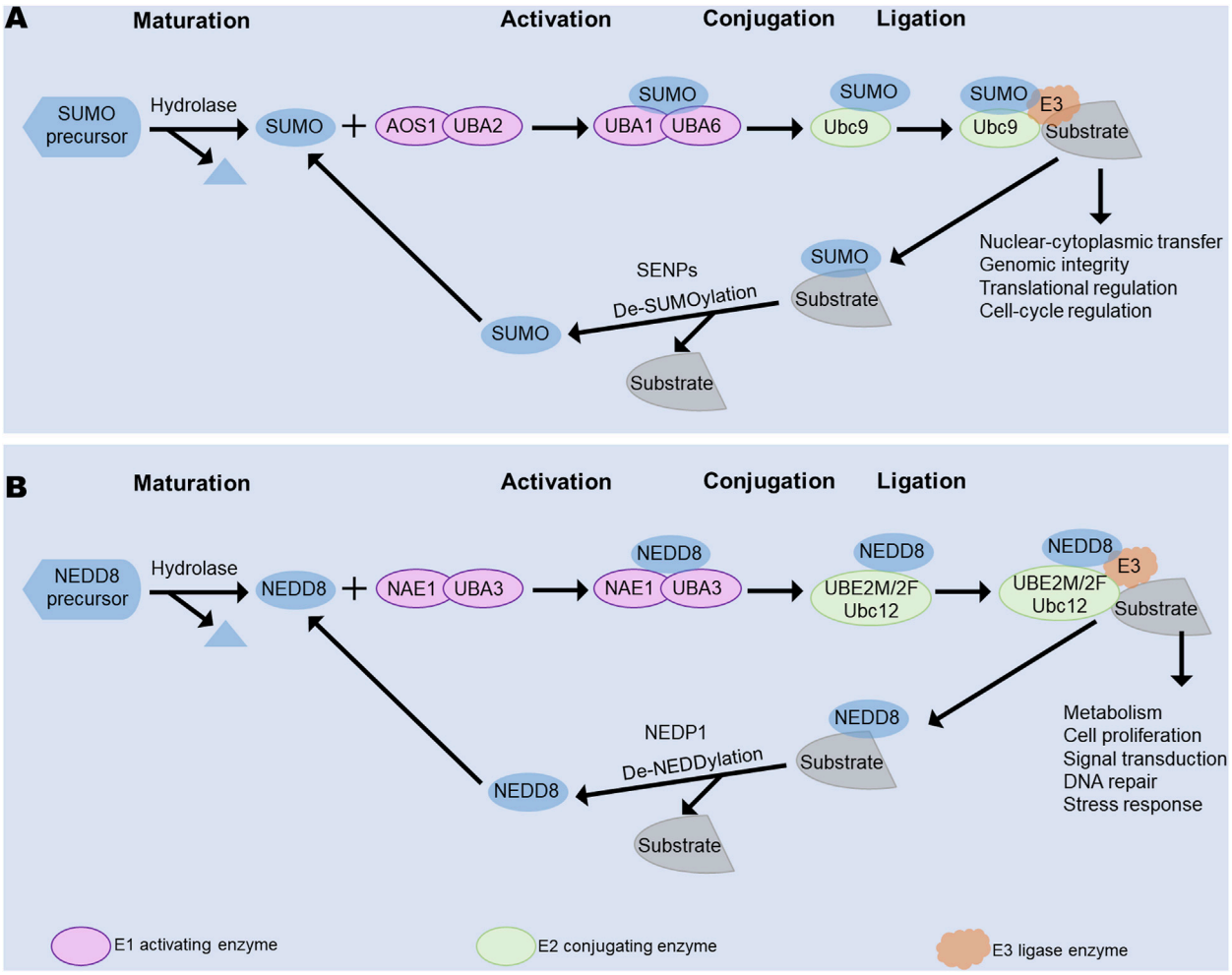

FIGURE 1
Main processes of SUMOylation and NEDDylation, including activation, conjugation, and ligation. **(A)** Main process of SUMOylation. **(B)** Main process of NEDDylation. Both SUMOylation and NEDDylation share these three processes that are mediated by different E1 activating, E2 conjugating, and E3 ligase enzymes. SENPs facilitate deSUMOylation, while NEDP1 controls deNEDDylation. NAE, NEDD8-activating enzyme; NEDP1, Nedd8 protease 1; SAE1, SUMO-activating enzyme subunit 1; SENPS, sentrin-specific proteases; UBA1, ubiquitin-like modifier activating enzyme 1; UBA2, ubiquitin-like modifier activating enzyme 2; UBA3, ubiquitin-like modifier activating enzyme 3; UBA6, ubiquitin-like modifier activating enzyme 6; UBE2M/2F, ubiquitin-conjugating enzyme E2 M/2F; Ubc9, ubiquitin-conjugating enzyme 9.

proinflammatory chemokines and profibrotic growth factors; then, macrophages and monocytes are recruited in the injured region and release massive amounts of cytokines and chemokines to induce fibroblast activation. The activated fibroblasts migrate to the injured region and transform into myofibroblasts. Excessive extracellular matrix is also accumulated in such instances, and some parenchymal cells are transformed into fibroblasts or myofibroblasts under stimulation by cytokines and chemokines.

During fibrosis formation, many cytokines and chemokines are involved, and their activities are mostly controlled by post-translational modifications (PTMs), including those involving transforming growth factor-β (TGF-β), promyelocytic leukemia (PML), and hypoxia-inducible factor (HIF)-1α (Dai et al., 2020; Lin et al., 2020; Peng et al., 2022). Ubiquitination, phosphorylation, acetylation, and methylation are some of the common PTMs, and numerous studies have confirmed the vital roles of PTMs in fibrosis (Chen et al., 2022; Liessi et al., 2020; Liu et al., 2023c). Notably, some novel PTMs like SUMOylation and NEDDylation are also known to affect fibrosis and have potential as new therapeutic targets against organ fibrosis. Both these modifications entail a three-step process of activation, conjugation, and ligation involving three kinds of enzymes (Figure 1), as will be described in detail in the next section.

In this review, we describe some important cellular and molecular mechanisms of SUMOylation and NEDDylation in organ fibrosis reported over the past 5 years, from their main regulatory enzymes to the processes themselves as well as introduce the roles of SUMOylation, NEDDylation, and their substrates in organ fibrosis. Then, we present the effects of SUMOylation/NEDDylation activators and inhibitors in organ fibrosis. We also discuss the benefits and limitations of SUMOylation/NEDDylation in the treatment of organ fibrosis with the goal of highlighting their therapeutic potentials and clinical treatment.

2 PTMs by ubiquitin-like (Ubl) proteins

2.1 Ubiquitination

Ubiquitination is a complex enzymatic cascade in which ubiquitin (Ub) units attach to specific residues of a protein, leading to protein degradation, transcriptional regulation, cell survival, protein–protein interactions, and intracellular trafficking. The process of ubiquitination is mediated by three types of enzymes, namely, E1 ubiquitin-activating, E2 ubiquitin-conjugating, and E3 ubiquitin ligase enzymes. First, the free Ub is activated by the E1 ubiquitin-activating enzyme through the participation of ATPs. Ubiquitin conjugates to substrates as a monomer (monoubiquitination) or at multiple sites (multi-monoubiquitination). Then, the activated Ub is transferred from E1 to cysteine at the active site of E2. Finally, the ubiquitinated protein is degraded by the proteasome into amino acids and small peptides or participates in biological processes (Gomarasca et al., 2022; Pellegrino et al., 2022). In this process, depending on E3, ubiquitin is transferred to the substrate via two mechanisms. The really interesting new gene (RING) E3 directly transfers ubiquitin to the substrate, while the homologous to E6AP carboxyl terminus (HECT) E3 or RING-between RING-RING (RBR) E3 transfers ubiquitin to itself and then to the substrate (French et al., 2021). The glycine residue of Ub covalently links to the lysine of the substrate, and Ub also forms Ub chains in this manner. Ub has seven lysine residues, namely, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63. Among these, Lys48-linked polyubiquitin chains are responsible for protein degradation, while Lys29-linked polyubiquitin chains control lysosomal degradation (French et al., 2021). Notably, ubiquitination is a reversible process that is mediated by deubiquitinating enzymes (DUBs) (Liu et al., 2023b). DUBs remove ubiquitin from substrates or ubiquitin chains by reversing the function of the E3 ligase enzyme.

2.2 SUMOylation

SUMOylation is an important and reversible PTM similar to ubiquitination; it participates in nuclear–cytoplasmic transfer, genomic integrity, translational regulation, and cell-cycle regulation (Qi et al., 2024; Sun et al., 2024). Small ubiquitin-like modifier (SUMO) proteins are the most well-known Ubls that share a similar three-dimensional structure with Ub. Five SUMO proteins (SUMO1–5) are expressed in mammals, where SUMO2 and SUMO3 are highly similar so as to be called SUMO2/3 (Wang and Matunis, 2023b). SUMO1–3 are widely expressed in tissues, while SUMO4 is mainly expressed in the spleen and kidneys and SUMO5 is mainly expressed in the blood and testes (Gomarasca et al., 2022). Similar to ubiquitination, SUMOylation relies on three classes of enzymes.

Before SUMOylation, the SUMO proteins are matured by removing several amino acids using the sentrin-specific protease (SENP) family of proteases. First, the E1 activating enzyme is a heterodimer comprising two SUMO-activating enzyme subunits (SAE1 and SAE2) that activates the SUMO protein at the C-terminal glycine residue to form a thioester bond with SAE2 at the cysteine residue. Then, SUMO is transferred to the only E2 enzyme, ubiquitin-conjugating enzyme 9 (Ubc9), through the formation of a thioester bond during SUMOylation (Zhu et al., 2024). Finally, SUMO is transferred to the substrate at the lysine residue to form a thioester bridge with the glycine residue at the C-terminus of SUMO (Wu and Huang, 2023) (Figure 2). Notably, a specific SUMO consensus motif ΨKXE is identified, where Ψ is a hydrophobic residue while K and E are the respective lysine acceptor and glutamic acid residues, and X is an amino acid. SUMOylation also includes a reversible process called deSUMOylation, in which a SUMO modification is cleaved from a substrate by the SENP family (Pei et al., 2024). The SENP family also mediates SUMO maturation (Brand et al., 2022; Chen et al., 2024a; Wen et al., 2024).

2.3 NEDDylation

NEDDylation is a type of PTM characterized by the covalent conjugation of neural-precursor-cell-expressed developmentally downregulated 8 (NEDD8) to a lysine residue in the substrate. NEDDylation plays important roles in metabolism, cell proliferation, signal transduction, DNA repair, and stress responses (Gonzalez-Rellan et al., 2023; He et al., 2023; Lu et al., 2023). NEDD8 is one of the Ubls sharing 80% homology with Ub. NEDDylation involves three enzymatic cascades with NEDD8-activating enzyme (NAE) E1, NEDD8-conjugating enzyme E2 (or ubiquitin-conjugating enzyme E2 M, UBE2M), and substrate-specific NEDD8-E3 ligase.

Before NEDDylation, the maturation of NEDD8 includes exposure to Gly76 through removal of the C-terminal amino acids from the NEDD8 precursor with a hydrolase such as UCHL3 or DEN1. First, a thioester bond is formed between NEDD8 and the UBA3 subunit of the E1 activating enzyme NAE along with participation of ATPs. Then, NEDD8 is transferred to an E2 conjugating enzyme by a trans-thiolation reaction. Lastly, a substrate-specific E3 ligase contributes to the bond between NEDD8 and the substrate through promotion of the bond between the E2 enzyme and substrate (Figure 3). The most identified NEDDylation substrates are cullins, which are subunits of the cullin-RING E3 ubiquitin ligases (CRLs) (Olaizola et al., 2022; Yu et al., 2022a). Notably, CRLs are common E3 ubiquitin ligases whose activities are facilitated by NEDDylation (Boh et al., 2011; He et al., 2023). In addition, PTEN, p53, and phosphoenolpyruvate carboxykinase 1 (PCK1) are substrates of NEDD8 (Gonzalez-Rellan et al., 2023; Liu et al., 2023a; Xie et al., 2021).

DeNEDDylation involves removal of NEDD8 from a substrate by a NEDD8 isopeptidase (Xiong et al., 2020). Nedd8 protease 1 (NEDP1) is a common NEDD8 isopeptidase that has two kinds of activities; NEDP1 belongs to a small ubiquitin-like-modifier-specific protease family that matures NEDD8 by exposure to the Gly76 residue and removal of the covalent binding between NEDD8 and the substrate (Bailly et al., 2019; Pellegrino et al., 2022).

3 Roles of SUMOylation and NEDDylation in fibrosis

3.1 Role of SUMOylation in fibrosis

Emerging evidence has revealed the important roles of SUMOylation and deSUMOylation in fibrosis; herein, we

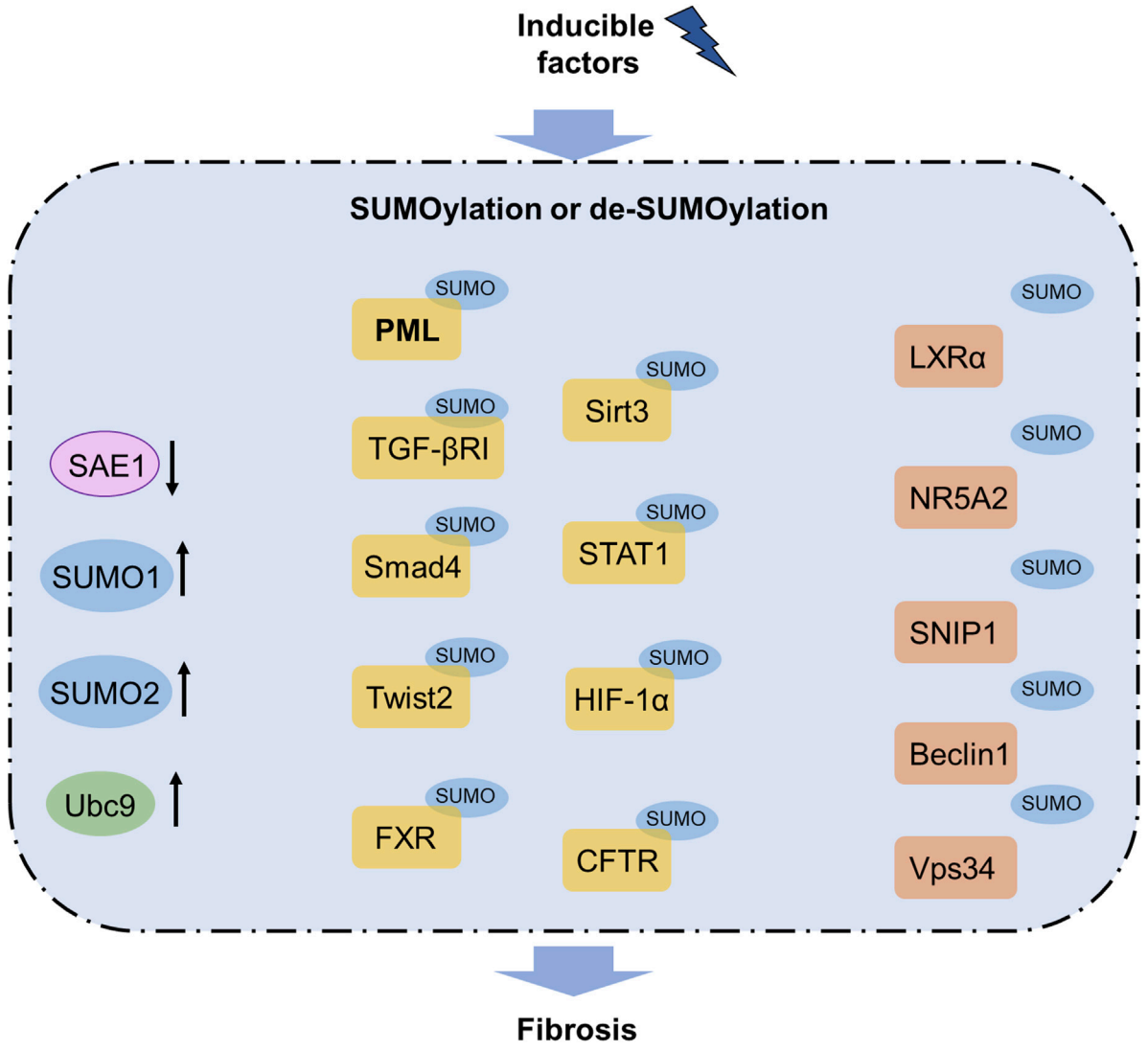

FIGURE 2
SUMOylation pathway in fibrosis. Increased levels of SUMO1, SUMO2, and Ubc9 as well as decreased level of SAE1 are observed in fibrosis. SUMOylation of PML, TGFβRI, Smad4, Twist2, FXR, Sirt3, STAT1, HIF-1α, and CFTR contribute to fibrosis, whereas SUMOylation of LXRα, NR5A2, SNIP1, Beclin1, and Vps34 can inhibit fibrosis. Notably, SUMOylated PML has been reported to participate in several organ fibrosis and functions as a promising therapeutic target for fibrosis treatment. CFTR, cystic fibrosis transmembrane conductance regulator; FXR, farnesoid X receptor; HIF-1α, hypoxia-inducible factor-1α; LXRα, liver X receptor α; NR5A2, nuclear receptor subfamily 5 group A member 2; PML, promyelocytic leukemia; SAE1, SUMO-activating enzyme subunit 1; SNIP1, Smad nuclear-interacting protein 1; TGFβRI, transforming growth factor-β receptor I; Ubc9, ubiquitin-conjugating enzyme 9.

describe only a few of the important findings concerning different types of organ fibrosis for the sake of brevity. In the liver, the human PML protein is a key organizer of nuclear bodies that participates in fibrosis. Silencing Ubc9, the only known E2-conjugating enzyme in SUMOylation, alleviates hepatic stellate cell activation, while silencing RNF4, an E3 ubiquitin ligase family member, facilitates the TGF-β/Smad pathway and causes liver fibrosis by enhancing SUMOylated PML accumulation (Dai et al., 2020; Li et al., 2024a). The TGF-β pathway is essential in fibrogenesis, and SUMOylation has been proven as an important target of the TGF-β pathway to treat fibrosis (Ungefroren, 2019). SUMOylation of the TGF-β receptor I occurs at the Lys385 and Lys389 residues, while SUMOylation of Smad4 occurs at Lys113 in the MH1 domain and Lys159 in the linker segment (Wang et al., 2021). Moreover, SAE1 is a promising therapeutic target for suppressing ferroptosis against liver fibrosis by reducing SUMOylation (Zhang et al., 2024a). SUMOylation of the farnesoid X receptor (FXR) occurs at the Lys122, Lys275, and Glu277 residues, and inhibited SUMOylation of FXR promotes its transactivity and suppresses hepatic stellate cell activation against liver fibrosis (Zhou et al., 2020b). The orphan nuclear receptor small heterodimer partner (SHP) alleviates chronic hepatitis C virus (HCV)-induced hepatic fibrosis; SHP regulates gluconeogenesis through Forkhead box O1 acetylation via histone deacetylase 9 (HDAC9) and controls lipogenesis by upregulating the sterol regulatory element binding protein 1c via SUMOylation of the liver X receptor α (Chen et al., 2019). These findings confirm the regulatory effects of SUMOylation in liver fibrosis.

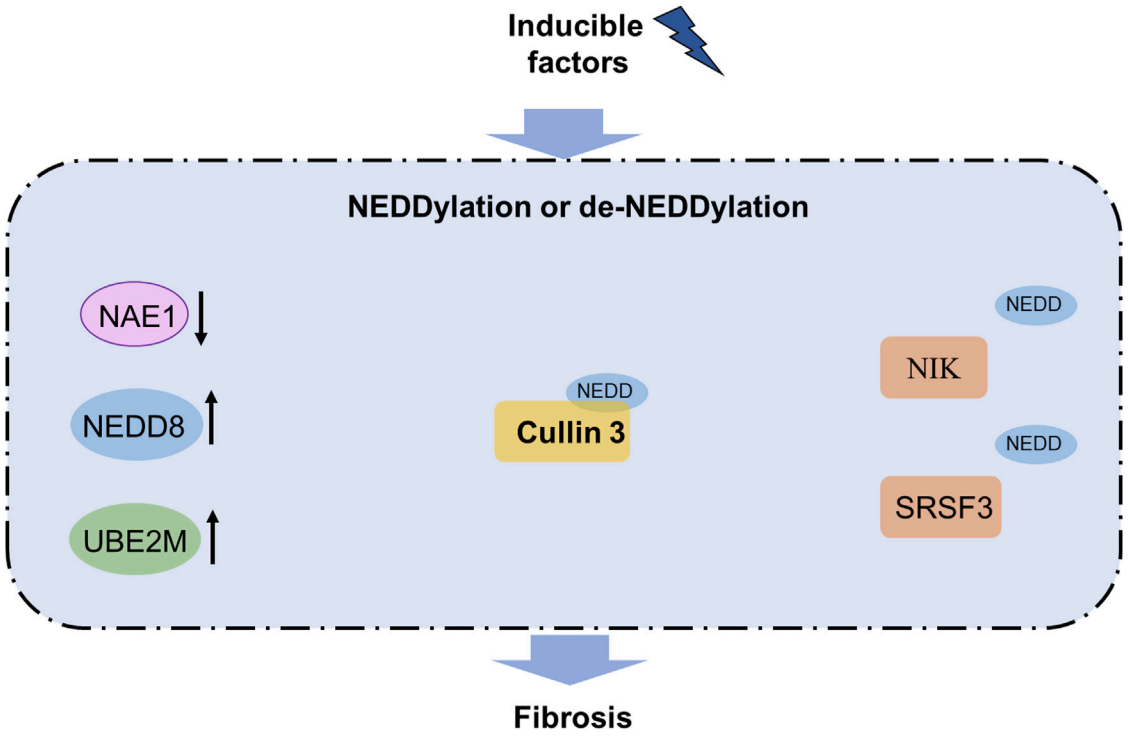

FIGURE 3
NEDDylation pathway in fibrosis. Increased levels of NEDD8 and UBE2M as well as decreased UBE2M are observed in fibrosis. NEDDylation of cullin3 contributes to fibrosis, while NEDDylation of NIK and SRSF3 can block fibrosis. Notably, NEDDylated PML has been reported to participate in organ fibrosis and functions as a promising therapeutic target for fibrosis treatment. NAE, NEDD8-activating enzyme; NIK, NF-κB-inducing kinase; SRSF3, serine-rich splicing factor 3; UBE2M, ubiquitin-conjugating enzyme E2 M.

SUMOylation also has notable roles in pulmonary diseases, including hypoxic pulmonary hypertension, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease (Zheng et al., 2023) (Table 1). Upregulation of SUMO1 and Ubc9 has been observed in human bronchial epithelial cells after exposure to cigarette smoke extract in chronic obstructive pulmonary disease (Zhou et al., 2020a), and upregulation of SUMO1, SUMO2, and Ubc9 has been noted in the lung tissues of patients with idiopathic pulmonary fibrosis (Yu et al., 2022b). Inhibition of SUMO1 blocks idiopathic pulmonary fibrosis (Yu et al., 2022b), highlighting that SUMOylation has an important role in pulmonary fibrosis.

In renal fibrosis, the nuclear receptor subfamily 5 group A member 2 (NR5A2) and Ubc9 are highly expressed in the kidney, while the K224R mutation of SUMOylated NR5A2 fails to upregulate calreticulin to drive fibrosis (Arvaniti et al., 2016; Politis and Charonis, 2022). DeSUMOylation of Sirt3 by SENP1 was found to control macrophage polarization and metabolic stress (Wang et al., 2019; Zhou et al., 2022b). The covalent binding of SUMO1 and Sirt3 increases during acute kidney injury (AKI), and the mutation of lysine to arginine significantly attenuates AKI while minimizing fibroblast-induced repair in a genetically modified mouse model (Zhu et al., 2023). Additionally, renal fibrosis is mainly involved in HIF-1α SUMOylation and SUMO-mediated regulation of the TGF-β/Smad and NF-κB pathways (Li et al., 2019; Yang et al., 2019). STAT1 activation delays epithelial–mesenchymal transitions (EMTs) after high glucose stimulation in the renal tubular epithelial cells, while high glucose levels promote STAT1 SUMOylation to suppress STAT1 activity and accelerate EMT (Gu et al., 2023a). These findings prove that SUMOylation may be a potential therapeutic target against renal fibrosis.

In cardiac fibrosis, obvious TGF-β1, prolyl isomerase NIMA-interacting 1 (Pin1) upregulation, and increased PML SUMOylation have been observed. TGF-β1 stimulation facilitates PML SUMOylation, nuclear body formation, and transformation of Pin1 into the nuclear body to interact with PML (Wu et al., 2019). Another study showed that SUMOylated PML has the ability to control p53 expression as p53 is vital for PML nuclear body formation in cardiac fibroblasts (Huang et al., 2023). The knockout of a poly-SUMO-specific E3 ubiquitin ligase RNF4 has been shown to aggravate interstitial fibrosis and cardiac dysfunction in the animal model of myocardial infarction. RNF4 knockout and PML overexpression facilitate PML SUMOylation as well as p53 recruitment and activation to exacerbate cardiomyocyte apoptosis. The interactions among RNF4, PML, and p53 could be potential therapeutic targets against cardiac fibrosis and apoptosis in myocardial infarction (Qiu et al., 2020). PML overexpression and RNF4 knockdown by small interfering RNA (siRNA) enhance PML SUMOylation, promote p53 recruitment and activation, and exacerbate H_2O_2/ATO-induced cardiomyocyte apoptosis, which could be partially reversed by knockdown of p53. Ubc9 has been proven to be a novel therapeutic target for protecting cardiomyocytes from ischemic stress while obviously alleviating cardiomyocyte apoptosis, fibrosis, and improving

TABLE 1 Profibrotic and antifibrotic effects of SUMOylation and NEDDylation substrates as well as their target organs, related diseases, and biochemical functions.

Target organ	Related disease	Substrates/related proteins	Biological functions	References
SUMOylation				
Liver	Arsenic-trioxide-induced liver fibrosis	PML	Facilitating TGF-β/Smad pathway and accumulating liver fibrosis	Dai et al. (2020), Li et al. (2024a)
	—	TGF-β receptor I at Lys385 and Lys389	Regulating the generation of myofibroblasts and EMTs	Wang et al. (2021)
		Smad4 at Lys 113 and Lys159	Regulating the generation of myofibroblasts and EMTs	Wang et al. (2021)
	Non-alcoholic steatohepatitis (NASH)	FXR at Lys122, Lys275 and Glu277	Suppressing FXR transactivity and hepatic stellate cell activation	Zhou et al. (2020b)
	Thioacetamide-induced liver fibrosis	SAE1 downregulation	Antifibrotic effect	Zhang et al. (2024a)
	Chronic hepatitis C virus-induced liver fibrosis	Liver X receptor α	Regulating lipogenesis and alleviating liver fibrosis	Chen et al. (2019)
	Chronic obstructive pulmonary disease	SUMO1 and Ubc9 upregulation	Profibrotic effect	Zhou et al. (2020a)
Lung	Idiopathic pulmonary fibrosis	SUMO1, SUMO2, and Ubc9 upregulation	Profibrotic effect	Yu et al. (2022b)
	Unilateral ureteric obstruction	NR5A2 at Lys224	Upregulating calreticulin to drive fibrosis	Arvaniti et al. (2016), Politis and Charonis (2022)
Kidney	Folic acid and ischemia–reperfusion-induced acute kidney injury	Sirt3	Suppressing fibroblast-induced repair and promoting fibrosis	Zhu et al. (2023)
	Unilateral ureteric obstruction	HIF-1α	Regulating TGF-β/Smad and NF-κB pathways	Li et al. (2019), Yang et al. (2019)
	Diabetic kidney disease	STAT1	Suppressing STAT1 activity and accelerating EMTs	Gu et al. (2023a)
	Transverse-aortic-constriction-induced cardiac fibrosis	PML	Facilitating PML nuclear body formation and further transforming Pin1 into nuclear to interact with PML, thus promoting fibrosis	Wu et al. (2019)
Heart	Myocardial infarction	PML	Controlling p53 expression to facilitate PML-nuclear-body formation and regulating fibrosis	Huang et al. (2023)
			Promoting p53 recruitment and activation to exacerbate cardiac fibrosis	Qiu et al. (2020)
	Myocardial ischemic injury	Ubc9	Inhibiting apoptosis under oxygen and glucose deprivation against fibrosis	Xiao et al. (2020)
		Vps34 and Beclin1	Facilitating the protein assembly of PI3K-III complexes I and II to inhibit fibrosis	
	Transaortic constriction	Sirt1	Blocking the transformation of cardiac fibroblasts into myofibroblasts to delay fibrosis	Luo et al. (2022)
	Transverse aortic constriction	SUMO2	Dual regulation of SUMO2 and STAT1 to affect fibrosis, hypertrophy, and inflammation	Rangrez et al. (2020)
CFBE41o- airway cells	Cystic fibrosis	CFTR	Modulating biogenesis and degradation	Gong et al. (2019)
Intestine	Crohn's disease	SNIP1	Inhibiting EMTs and intestinal fibrosis	Chen et al. (2024c)
MCF10A cell	—	Twist2 at Lys129	Accelerating EMTs and promoting mesenchymal phenotypes	Zeng et al. (2021)

(Continued on following page)

TABLE 1 (*Continued*) Profibrotic and antifibrotic effects of SUMOylation and NEDDylation substrates as well as their target organs, related diseases, and biochemical functions.

Target organ	Related disease	Substrates/related proteins	Biological functions	References
NEDDylation				
Liver	CCl_4-induced liver fibrosis	Global NEDDylation	Promoting the kinase activity of Eph receptor tyrosine kinase EphB1 to trigger fibrosis	Li et al. (2023)
	Bile-duct ligation and CCl_4-induced liver fibrosis	Global NEDDylation	Promoting chemokine (C-X-C motif) ligand 1 and CCL2 expressions to promote apoptosis and fibrosis	Zubiete-Franco et al. (2017)
	NAFLD	NEDD8	Ameliorating liver fibrosis, lipid peroxidation, lipid accumulation, and inflammation	Serrano-Maciá et al. (2021)
	NASH	Cullin 3	Driving Nrf2 dysfunction and AGER1 downregulation to promote fibrosis	Dehnad et al. (2020)
	Acute liver failure	NIK	Suppressing abnormal NIK activation, aggressive hepatocyte damage, fibrosis, and inflammation	Xu et al. (2022)
	NAFLD and NASH	SRSF3 at Lys11	Promoting SRSF3 degradation and alterations in RNA splicing to alleviate hepatic steatosis, fibrosis, and inflammation	Kumar et al. (2019)
Pancreas	Chronic pancreatitis	UBE2M	Suppressing CCL5 and CD163 expression to drive fibrosis	Lin et al. (2021)
Lung	Cystic fibrosis	NEDD8	Promoting ΔF508-CFTR-induced cystic fibrosis	Ramachandran et al. (2016)

cardiac function. Ubc9 overexpression attenuates cardiomyocyte apoptosis, while Ubc9 knockout aggravates apoptosis under oxygen and glucose deprivation. A mechanical study showed that Ubc9 promotes SUMOylation of Vps34 and Beclin1, which are two core proteins in the class III phosphatidylinositol 3-kinase (PI3K-III) complex, and facilitates the protein assembly of the PI3K-III complexes I and II (Xiao et al., 2020). Sirt1 SUMOylation blocks the transformation of cardiac fibroblasts into myofibroblasts by suppressing fibrogenesis via the AKT/GSK3β pathway (Luo et al., 2022). The HECT domain containing E3 ubiquitin protein ligase 3 (HectD3) ameliorates pathological hypertrophy, macrophage infiltration, and cardiac fibrosis induced by pressure overload; HectD3 exhibits dual regulation of SUMO2 and STAT1 against hypertrophic and inflammatory effects in cardiomyocytes (Rangrez et al., 2020). These results indicate that SUMOylation is an important tool for treating cardiac fibrosis.

SUMOylation of the cystic fibrosis transmembrane conductance regulator (CFTR) involves its degradation and is a potential therapeutic target in the treatment of cystic fibrosis. Mechanically, the E3 ligase enzyme, which is the protein inhibitor of activated STAT 4 (PIAS4), mediates covalent binding of CFTR to SUMO1 but suppresses such binding to SUMO2/3 (Gong et al., 2019). Other studies have shown that inhibition of SUMOylation can attenuate cystic fibrosis via CFTR (Borgo et al., 2024; Peters et al., 2021), confirming that CFTR is a vital therapeutic target against cystic fibrosis. The long non-coding RNA MSC-AS1 is highly expressed in EMT and intestinal fibrosis through modulation of the Smad nuclear-interacting protein 1 (SNIP1); MSC-AS1 also directly interacts with SENP1 to deSUMOylate and inactivate SNIP1 (Chen et al., 2024c).

Emerging evidence also shows the vital role of SUMOylation in fibrogenesis *in vitro*. Twist2 is a key transcription factor in EMT that contributes to fibrosis. The SUMO2/3-specific E3 ligase zinc finger protein 451 (ZNF451) has been identified as a regulator of Twist2 to maintain its stability. Mechanistic studies show that a direct bond between ZNF451 and Twist2 results in Twist2 SUMOylation at the Lys129 residue and hinders the Ub-dependent degradation of Twist2. Ectopic expression of ZNF451 promotes Twist2 expression and EMT, whereas knockout of ZNF451 inhibits the mesenchymal phenotypes. ZNF functions as a novel mediator in fibrosis by facilitating Twist2 SUMOylation (Zeng et al., 2021). The RAN GTPase-activating protein 1 (RanGAP1) has been identified as a functional partner of SUMOs in fibrogenesis. Mechanically, the RanGAP1-SUMO1 complex mediates nuclear Smad4 accumulation by dissociating Smad4 and CRM1 (Lin et al., 2023). SUMOylation triggers modulation of aldosterone-activated mineralocorticoid receptor transactivation to regulate fibrosis (Gadasheva et al., 2021). The SUMO1-RanGAP1 complex has been proven to be a key molecule for amplification of the TGF-β/Smad and HIF-1 pathways. During fibrogenesis, SUMOylation is activated so that HIF-1α is SUMOylated by SUMO1 at Lys391 and Lys477 (Lin et al., 2020).

3.2 Role of NEDDylation in fibrosis

NEDDylation has been proven to be an important regulator of liver fibrosis (Table 1). Hepatic NEDDylation dysfunction causes oxidative stress, inflammation, fibrosis, hepatocyte reprogramming, and liver injury in acute and chronic liver diseases. NEDDylation

can be considered a novel therapeutic target against liver fibrosis, and MLN4924 as an inhibitor of NAE shows promising potential in the treatment of liver fibrosis (Xu et al., 2022; Yao et al., 2020). The upregulation of EphB1 is accompanied by increased NEDDylation in activated hepatic stellate cells. This enhanced NEDDylation promotes the kinase activity of the Eph receptor tyrosine kinase EphB1 by preventing its degradation to facilitate proliferation and migration of the hepatic stellate cells. These results indicate that EphB1 could be a promising therapeutic target of liver fibrosis (Li et al., 2023). In liver fibrosis, enhanced NEDDylation is positively related to increased caspase 3 activity to induce hepatic stellate cell apoptosis, whereas inhibited NEDDylation reduces chemokine (C-X-C motif) ligand 1 and C-C motif chemokine ligand 2 (CCL2) expressions to ameliorate apoptosis. Chemokine receptors and cytokines are increased in activated macrophages but decreased in the mouse Kupffer cells after NEDDylation inhibition. These findings indicate that enhanced NEDDylation contributes to liver fibrosis and that NEDDylation may be a promising therapeutic target for treating liver fibrosis (Zubiete-Franco et al., 2017). In the progression of non-alcoholic fatty liver disease (NAFLD) and liver fibrosis, the serum NEDD8 levels are closely related to hepatic NEDDylation. Inhibition of NEDDylation through NEDD8 suppression was shown to ameliorate liver fibrosis, lipid peroxidation, lipid accumulation, and inflammation in the NAFLD mouse model. Deptor is upregulated in NAFLD and liver fibrosis accompanied by suppressed mTOR signaling, increased fatty acid oxidation, and decreased lipid content while its silencing counteracts the antisteatotic effects of NEDDylation inhibition. These results indicate the important roles of Deptor in NEDDylation-inhibition-treated NAFLD and liver fibrosis (Serrano-Maciá et al., 2021). NEDDylation of cullin3 is involved in Nrf2 dysfunction and advanced glycation end product receptor 1 (AGER1) downregulation in liver fibrosis. Overexpression of Nrf2 in the hepatocytes blocks AGER1 decrease and reduces the advanced glycation end-product levels, in addition to suppressing inflammation and fibrosis in the mouse model of non-alcoholic steatohepatitis (NASH) (Dehnad et al., 2020). Dysregulation of NAE1, a regulatory subunit of NAE E1, has been observed in human acute liver failure; loss of NAE in the hepatocytes results in hepatocyte death, inflammation, fibrosis, and eventually liver dysfunction in the mouse model. Notably, NF-κB-inducing kinase (NIK) NEDDylation facilitates its ubiquitination and degradation, whereas inhibition of NIK NEDDylation leads to abnormal NIK activation, aggressive hepatocyte damage, and inflammation in adult male mice with acute liver failure (Xu et al., 2022). Serine-rich splicing factor 3 (SRSF3) modulates liver function, and the loss of SRSF3 deteriorates liver fibrosis and injury. SRSF3 is reduced in human liver samples with NAFLD and NASH along with alterations in the RNA splicing of known SRSF3 target genes. The conjugation of NEDD8 protein with SRSF3 and subsequent proteasome-mediated degradation are induced by palmitic acid. The NEDDylation of SRSF3 occurs at Lys11, and the mutation of SRSF3 (SRSF3-K11R) prevents its degradation and alteration in RNA splicing, which alleviates hepatic steatosis, fibrosis, and inflammation (Kumar et al., 2019).

NEDDylation is involved in fibrogenesis in the lungs, the kidneys, chronic pancreatitis, and cystic fibrosis (Table 1). In pulmonary fibrosis, the cullin-associated and NEDDylation-dissociated 1 (CAND1) level is negatively related to cullin1 NEDDylation in EMT, while the interaction between CAND1 and cullin1 enables the Skp-cullin-F-box protein (SCF) ubiquitin ligase system to boost protein ubiquitination (Zhou et al., 2022c). Familial hyperkalemic hypertension is a monogenic disease caused by mutations in the genes encoding WNK kinases, ubiquitin scaffold protein cullin3, or substrate adapter kelch-like 3 (KLHL3). Compared with wild-type (WT) cullin3, mutant cullin3 Δ403-459 retains the ability to bind and ubiquitylate WNK kinases and KLHL3 while being more NEDDylated and activated. The activated cullin3 Δ403-459 exhausts KLHL3 and prevents WNK degradation, while the loss of cullin3 aggravates FHHt and accelerates renal fibrosis in the murine model (McCormick et al., 2014). Chronic pancreatitis is characterized by irreversible fibrotic and inflammatory disease. Compared with normal healthy controls, UBE2M is remarkably decreased in human chronic pancreatitis tissues accompanied by increased CCL5 and CD163 (markers of M2-type macrophages), indicating the important role of NEDDylation in the pathogenesis of chronic pancreatitis (Lin et al., 2021). In addition, knockout of the ubiquitin ligase SYVN1 or NEDD8 partially restores ΔF508-CFTR-mediated Cl- transport in human cystic fibrosis airway epithelia, indicating the important role of NEDD8 in ΔF508-CFTR-induced cystic fibrosis (Ramachandran et al., 2016). The E3 ubiquitin ligase enzyme Parkin and NEDD4 also have the potential to regulate fibroblast activation during fibrogenesis (Shen et al., 2021).

4 Roles of SUMOylation and NEDDylation activators and inhibitors in fibrosis

4.1 Roles of SUMOylation activators and inhibitors in fibrosis

Efforts have been made to determine the underlying effects and action mechanisms of SUMOylation inhibitors and activators in organ fibrosis. Recent studies have shown the protective roles of SUMOylation activators against liver fibrosis through activation of SUMOylation (Table 2). Sclareol isolated from *Salvia sclarea* is a potential SUMOylation activator that downregulates SENP1. Treatment with sclareol has been shown to substantially suppress hepatic stellate cell activation, attenuate liver fibrosis, and improve liver function in two mouse models. Mechanistic studies show that sclareol decreases SENP1 expression to inhibit vascular endothelial growth factor receptor 2 (VEGFR2) SUMOylation in LX-2 cells by affecting VEGFR2 intracellular trafficking (Ge et al., 2023). Meanwhile, SUMOylation inhibition attenuates hepatic fibrosis by modulating the profibrotic or antifibrotic factors. Ginkgolic acid is a SUMOylation inhibitor that reduces the expression of SAE1. Mechanically, ginkgolic acid downregulates SAE1 to induce ferroptosis of the hepatic stellate cells, ultimately leading to antihepatic fibrosis effects (Zhang et al., 2024a). FXR is a promising therapeutic target against liver fibrosis whose enhanced SUMOylation weakens the effect of obeticholic acid (FXR receptor agonist) against hepatic stellate cell activation. The triple mutation of FXR at Lys122, Lys275, and Glu277 facilitates its activity. Interestingly, coadministration of obeticholic acid and

TABLE 2 Profibrotic and antifibrotic effects of SUMOylation and NEDDylation activators and inhibitors as well as their target organs, related diseases, and biochemical functions.

Target organs	Related diseases	Compounds	Activators or inhibitors	Target substrates/ related proteins	Biological functions	References
SUMOylation						
Liver	Bile-duct ligation and CCl_4-induced liver fibrosis	Sclareol	Activator	VEGFR2	Decreasing SENP1 expression to inhibit VEGFR2 SUMOylation against fibrosis	Ge et al. (2023)
	CCl_4 and thioacetamide-induced liver fibrosis	Ginkgolic acid	Inhibitor	SAE1	Downregulating SAE1 to induce ferroptosis of hepatic stellate cells against fibrosis	Zhang et al. (2024a)
	NASH	Ginkgolic acid Spectinomycin	Inhibitor	Global SUMOylation	Modulating STAT3 phosphorylation against fibrosis	Zhou et al. (2020b)
	Polycystic liver diseases	SAMe	Inhibitor	Ubc9	Interrupting SUMO1 to suppress proteasome hyperactivity to activate unfolded protein response and apoptosis against fibrosis	Lee-Law et al. (2021)
Lung	1-NP instillation in lung	1-NP	Activator	ALKBH5	Facilitating ALKBH5 SUMOylation and then causing its ubiquitination and proteasomal degradation to accelerate fibrosis	Li et al. (2024b)
	Idiopathic pulmonary fibrosis	Ginkgolic acid	Inhibitor	SUMO1	Decreasing SUMO1/2/3 and increasing SENP overexpression to suppress Smad4 SUMOylation and regulate EMTs and ROS production against fibrosis	Ding et al. (2022), Yu et al. (2022b)
Heart	Myocardial ischemic Injury	Puerarin	Activator	SUMO2	Facilitating SUMO2 expression and then activating ER/ERK pathway against fibrosis	Zhao et al. (2021)
	Transaortic constriction	(−)-Epicatechin	Activator	Sirt1	Promoting Sirt1 SUMOylation to suppress fibrogenesis via AKT/GSK3β pathway	Luo et al. (2022)
	Transverse aortic constriction	QFYXF	Activator	SERCA2a	Boosting β-arrestin2-mediated SERCA2a SUMOylation and expression	Wang et al. (2024)
	Transverse aortic constriction	LY364947 Juglone	Inhibitor	PML	Reducing the mRNA and protein expression of TGF-β1 and Pin1 to delay cardiac fibrosis process	Wu et al. (2019)
	Myocardial infarction	Ginkgolic acid	Inhibitor	SUMO1	Controlling TGF-β1-induced PML/p53 interaction to suppress cardiac fibrosis	Huang et al. (2023)
	Myocardial ischemic Injury	Arsenic trioxide	Inhibitor	PML	Downregulating RNF4 and PML SUMOylation to suppress myocardial apoptosis and fibrosis	Qiu et al. (2020)
NEDDylation						
Liver	Bile-duct ligation and CCl_4-induced liver fibrosis	MLN4924	Inhibitor	NAE	Modulating the accumulation of c-Jun against fibrosis	Zubiete-Franco et al. (2017)
	Bleomycin-induced pulmonary fibrosis				suppressing NF-κB responses and MAPK activity against fibrosis	Deng et al. (2017)

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TABLE 2 (*Continued*) Profibrotic and antifibrotic effects of SUMOylation and NEDDylation activators and inhibitors as well as their target organs, related diseases, and biochemical functions.

Target organs	Related diseases	Compounds	Activators or inhibitors	Target substrates/ related proteins	Biological functions	References
	Acute liver failure	N-acetylcysteine	Inhibitor	NAE	Reducing hepatic NAE1 expression to prevent liver inflammation, fibrosis and injury	Xu et al. (2022)
Lung	Human pulmonary fibroblasts	Celastrol	—	Cullin1	Facilitating the interactions between CAND1 and cullin1 to suppress EMTs against fibrosis	Zhou et al. (2022c)
	CCl_4-induced liver fibrosis	HZX-960	Inhibitor	Cullin3	Blocking the interaction of DCN1 (co-E3 ligase) and Ubc12 and inhibiting cullin3 NEDDylation against liver fibrosis	Zhou et al. (2022a)
Heart	Doxorubicin-induced cardiac fibrosis	MLN4924	Inhibitor	NAE	Maintaining mitochondrial function, alleviating fibrosis, cardiomyocyte apoptosis and oxidative stress damage, and boosting cardiac contractile function	Chen et al. (2024b)
	Pressure overload-cardiac fibrosis	DN-2	Inhibitor	Cullin3	Inhibiting cullin3 NEDDylation to reverse cardiac fibroblast activation	He et al. (2022)
Pancreas	Chronic pancreatitis	MLN4924	Inhibitor	NAE	Promoting CCL5-mediated M2 macrophage infiltration, and the blockage of CCL5 to aggravate fibrosis	Lin et al. (2021)

SUMOylation inhibitors (ginkgolic acid and spectinomycin) has been found to significantly alleviate liver fibrosis (Zhou et al., 2020b). Treatment with S-adenosylmethionine (SAMe), a natural Ubc9-dependent SUMOylation inhibitor, shows obvious hepatic protection through inhibition of hepatic cystogenesis and fibrosis along with decreased liver/body weight ratio and liver volume. Mechanically, SAMe interrupts SUMO1 to suppress proteasome hyperactivity while activating unfolded protein response and stress-related apoptosis (Lee-Law et al., 2021), indicating that it could be a candidate for treating liver fibrosis.

Exposure to 1-nitropyrene (1-NP) has been found to trigger pulmonary fibrosis in mice, and 1-NP is also identified as an activator of AlkB homolog 5 (ALKBH5) SUMOylation. Mechanically, 1-NP facilitates ALKBH5 SUMOylation followed by ALKBH5 ubiquitination and proteasomal degradation in mouse lung epithelial-12 cells (Li et al., 2024b). Interestingly, inhibition of SUMO1 exhibits protection against pulmonary fibrosis. Ginkgolic acid functions as a SUMO1 inhibitor to block idiopathic pulmonary fibrosis. Mechanically, ginkgolic acid suppresses upregulation of SUMO1/2/3 and promotes SENP overexpression. SENP1 inhibits Smad4 SUMOylation while regulating EMT and reactive oxygen species (ROS) production (Ding et al., 2022; Yu et al., 2022b). These results provide solid evidence that SUMOylation inhibitors and activators are potential candidates against pulmonary fibrosis.

Both SUMOylation inhibitors and activators exert antifibrotic effects in heart tissues (Table 2). Puerarin alleviates cardiac inflammation and cardiac fibrosis by reducing lactate dehydrogenase, COX-2, galectin-3, and cleaved PARP-1. Mechanically, puerarin facilitates SUMO2 expression and SUMOylation before activating the ER/ERK pathway to exert cardioprotective effects (Zhao et al., 2021). (-)-Epicatechin blocks the transformation of cardiac fibroblasts to myofibroblasts against cardiac fibrosis in a Sirt1-dependent manner. The underlying mechanism involves Sirt1 activation by the transcription specificity protein 1 and Sirt1 SUMOylation to suppress fibrogenesis via the AKT/GSK3β pathway (Luo et al., 2022). The Qifu Yixin formula (QFYXF) of traditional Chinese medicine exhibits cardiac protection via restoration of cardiac function as well as amelioration of myocardial fibrosis and hypertrophy. The effects of QFYXF are related to enhanced sarcoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2a) expression and SUMOylation. Molecular docking results show that the main active compounds in QFYXF have high affinities to β-arrestin2, SERCA2a, and SUMO1, with SERCA2a having high affinity to SUMO1. QFYXF exerts antifibrotic effects by boosting β-arrestin2-mediated SERCA2a SUMOylation and expression (Wang et al., 2024). Furthermore, inhibition of PML SUMOylation by LY364947 or Juglone significantly reduces the mRNA and protein expressions of TGF-β1 and Pin1 to delay cardiac fibrosis (Wu et al., 2019). Pharmacological inhibition of the SUMO pathway by the SUMO1 inhibitor ginkgolic acid can substantially control TGF-β1-induced PML/p53 interactions to suppress cardiac fibrosis (Huang et al., 2023). Treatment with arsenic trioxide, which is an ROS inhibitor, reduces RNF4 expression and PML SUMOylation to suppress myocardial apoptosis and fibrosis against myocardial infarction (Qiu et al., 2020). Various natural products have also been identified as vital regulators in

SUMOylation (Liu et al., 2022). These findings provide potential candidates against cardiac fibrosis through the regulation of SUMOylation.

4.2 Roles of NEDDylation activators and inhibitors in fibrosis

NEDDylation activators and inhibitors have also been found to modulate liver fibrosis (Table 2). MLN4924, also called as pevonedistat, is a first-in-class NAE inhibitor. The inhibition of NEDDylation by MLN4924 triggers hepatic stellate cell apoptosis to prevent liver injury, inflammation, and fibrosis through the accumulation of c-Jun (Zubiete-Franco et al., 2017). MLN4924 controls the NEDDylation of CRLs to delay liver fibrosis progression by suppressing NF-κB responses and MAPK activity (Deng et al., 2017). Treatment with N-acetylcysteine, a glutathione surrogate and antioxidant, has been found to significantly reduce hepatic NAE1 expression to prevent liver inflammation, fibrosis, and injury in the acute liver failure mouse model (Xu et al., 2022).

Celastrol is a pentacyclic triterpene compound isolated from *Tripterygium wilfordii* as a novel treatment for pulmonary fibrosis; it exhibits antifibrotic effects through the covalent linkage of CAND1 at the Cys264 residue. Celastrol treatment influences cullin1 NEDDylation; celastrol also exerts antifibrotic effects in a CAND1-dependent manner and facilitates interactions between CAND1 and cullin1 to activate the Skp1/cullin1/F-box ubiquitin ligases that control EMTs (Zhou et al., 2022c). Additionally, HZX-960 has been identified as an inhibitor that blocks the interaction of DCN1 (co-E3 ligase) with Ubc12 and inhibits cullin3 NEDDylation against liver fibrosis. HZX-960 attenuates liver fibrotic signaling by suppressing collagen I and α-SMA while promoting Nrf2, HO-1, and NQO-1, indicating that it is a promising therapeutic candidate against liver fibrosis (Zhou et al., 2022a).

In the heart, MLN4924 exerts antifibrotic properties (Table 2); MLN4924 mitigates doxorubicin-induced cardiotoxicity by maintaining mitochondrial function, alleviating cardiomyocyte apoptosis, suppressing oxidative-stress-induced damage, boosting cardiac contractile function, inhibiting cardiac fibrosis, and impeding cardiac remodeling. Mechanistically, MLN4924 delays cardiac NEDDylation and offers cardiac protection by limiting NAE activity (Chen et al., 2024b). The antifibrotic effects of DCN1 have also been demonstrated in cardiac fibrosis, where DCN1 is upregulated in the cardiac fibroblast and pressure overloaded mouse hearts. The compound DN-2 has been optimized as a potent DCN1-Ubc12 inhibitor and shown to have high affinity to DCN1; DN-2 effectively reverses cardiac fibroblast activation by inhibiting cullin3 NEDDylation (He et al., 2022). These results highlight the potential of DCN1 as a promising therapeutic target against organ fibrosis. The inhibition of global NEDDylation by MLN4924 obviously aggravates chronic pancreatitis by promoting CCL5-mediated M2 macrophage infiltration, and the blockage of CCL5 counteracts MLN4924-mediated chronic pancreatitis. A mechanistic study showed that inactivation of CRLs stabilizes the level of HIF-1α to facilitate CCL5 upregulation and transactivation (Lin et al., 2021).

5 Conclusion and perspectives

PTMs enhance the functional diversity of proteins by modulating the covalent modifications of the functional groups or proteins to induce slicing or degradation, thereby influencing the physiological and pathophysiological processes (Schepers et al., 2023; Wang and Tong, 2023c). Ubiquitination controls protein degradation, transcriptional regulation, cell survival, protein–protein interactions, and intracellular trafficking (González et al., 2023; Gu et al., 2023b). The three-step process of ubiquitination involves activation, conjugation, and ligation through the E1 activating, E2 conjugating, and E3 ligase enzymes. The processes of SUMOylation and NEDDylation are also similar to ubiquitination, but their enzymes are distinguishable from ubiquitination. In SUMOylation, the E1 activating enzyme consists of the SAE1 and SAE2 subunits, and Ubc9 is the only E2 enzyme. In NEDDylation, the NAE E1 activating enzyme consists of the NAE1 and Uba3 subunits. SUMOylation participates in nuclear–cytoplasmic transfer, genomic integrity, translational regulation, and cell-cycle regulation, while NEDDylation contributes to DNA replication and repair, chromatin structure, translational regulation, and caryomitosis (Ren et al., 2024; Tan et al., 2023; Xu et al., 2023; Zou et al., 2023). SUMOylation and NEDDylation can be reversed by SENPs and NEDP1, respectively.

Emerging evidence has shown that SUMOylation and NEDDylation play pivotal and diverse roles in organ fibrosis by mediating the PTMs of profibrotic or antifibrotic factors. In the liver, SUMOylation of FXR at Lys122, Lys275, and Glu277 along with the liver X receptor α has been found to attenuate fibrosis (Chen et al., 2019; Zhou et al., 2020b) while SAE1 contributes to fibrosis. Upregulation of SUMO1, SUMO2, and Ubc9 have been reported to aggravate pulmonary fibrosis (Yu et al., 2022b; Zhou et al., 2020a). In the kidneys, SUMOylation of NR5A2 at Lys224 and that of STAT1 have been found to promote EMT and fibrosis (Arvaniti et al., 2016; Gu et al., 2023a; Politis and Charonis, 2022), whereas SUMOylation of Sirt3 has been shown to suppress fibroblast-induced repair and fibrosis (Zhu et al., 2023). SUMOylation of HIF-1α is also involved in renal fibrosis through regulation of the TGF-β/Smad pathway (Li et al., 2019; Yang et al., 2019). SUMOylation of Vps34, Beclin1, and Sirt1 have been noted to obviously suppress cardiac fibrosis (Luo et al., 2022; Xiao et al., 2020), while SUMO2 was observed to affect cardiac fibrosis through dual regulation along with STAT1 (Rangrez et al., 2020). Additionally, SUMOylation of CFTR and SNIP1 was found to facilitate fibrosis (Chen et al., 2024c; Gong et al., 2019), while SUMOylation of Twist2 at Lys129 was noted to accelerate fibrosis *in vitro* (Zeng et al., 2021).

Notably, although the roles of SUMOylation and NEDDylation are diverse in different organs, their regulation of PML, HIF-1α, and TGF-β are common in fibrogenesis. SUMOylation of PML promotes fibrosis in the lung and heart tissues, and the underlying mechanism is involved in facilitating PML nuclear body activation of the TGF-β/Smad pathway as well as recruitment and activation of p53 (Dai et al., 2020; Huang et al., 2023; Li et al., 2024a; Qiu et al., 2020; Wu et al., 2019). Further investigations have highlighted the vital role of SUMOylation in the TGF-β/Smad pathway; SUMOylation of the TGF-β receptor I at Lys385 and Lys389 as well as Smad4 at

Lys113 and Lys159 can control the generation of myofibroblasts and EMTs to influence hepatic fibrosis (Wang et al., 2021). Ubc9 was found to participate in pulmonary and cardiac fibrosis; upregulation of Ubc9 was observed in fibrotic pulmonary tissues, whereas overexpression of Ubc9 was noted to suppress cardiomyocyte apoptosis against fibrosis (Xiao et al., 2020; Zhou et al., 2020a). These studies have proved the vital role of SUMOylation in organ fibrosis and its function as a potential target against organ fibrosis.

Global NEDDylation was found to exacerbate liver fibrosis through activation of the Eph receptor tyrosine kinase EphB1 as well as upregulation of the chemokine (C-X-C motif) ligand 1 and CCL2 expression to accelerate fibrosis (Li et al., 2023; Zubiete-Franco et al., 2017). NEDDylation of cullin3 induces Nrf2 dysfunction and AGER1 downregulation to trigger fibrosis (Dehnad et al., 2020; Kumar et al., 2019), and NEDDylation of NIK SRSF3 at Lys11 can alleviate liver fibrosis (Xu et al., 2022). UBE2M has been found to drive fibrogenesis in chronic pancreatitis by suppressing CCL5 and CD163 expressions (Lin et al., 2021). Interestingly, NEDD8 ameliorates liver fibrosis but promotes cystic fibrosis in the lungs (Ramachandran et al., 2016; Serrano-Maciá et al., 2021), indicating the diverse roles of NEDDylation in different organ fibrosis. These results provide solid evidence and highlight NEDDylation as a potential therapeutic target for treating organ fibrosis.

The activators and inhibitors of SUMOylation are potential candidates that can influence organ fibrosis. Sclareol activates VEGFR2 SUMOylation against hepatic fibrosis, and SAMe inhibits Ubc9 to interrupt SUMO1 and activate unfolded protein responses against hepatic fibrosis (Ge et al., 2023; Lee-Law et al., 2021). The SUMOylation activator 1-NP promotes ALKBH5 SUMOylation and subsequent ubiquitination as well as proteasomal degradation to trigger lung fibrosis (Li et al., 2024b). In the heart, puerarin functions as a SUMOylation activator to facilitate SUMO2 expression and activate the ER/ERK pathway against fibrosis (Zhao et al., 2021). (-)-Epicatechin promotes Sirt1 SUMOylation to suppress cardiac fibrogenesis by modulating the AKT/GSK3β pathway (Luo et al., 2022). QFYXF promotes SERCA2a SUMOylation and expression in the treatment of cardiac fibrosis (Wang et al., 2024), while LY364947 and juglone obviously suppress PML SUMOylation to reduce the mRNA and protein expressions of TGF-β1 and Pin1 to delay cardiac fibrosis (Wu et al., 2019). Arsenic trioxide helps PML SUMOylation to reduce RNF4 against myocardial apoptosis and fibrosis (Qiu et al., 2020). Notably, ginkgolic acid alleviates fibrosis in the liver, lungs, and heart by downregulating SAE1, modulating STAT3 phosphorylation, and influencing PML/p53 interactions (Ding et al., 2022; Huang et al., 2023; Yu et al., 2022b; Zhang et al., 2024a; Zhou et al., 2020b). MLN4924 as a first-line NEDDylation inhibitor exhibits antifibrotic properties in the liver, heart, and pancreas. MLN4924 exerts antifibrotic effects by modulating c-Jun accumulation, NF-κB responses and MAPK activity, mitochondrial functions, and CCL5-mediated M2 macrophage infiltration (Deng et al., 2017; Lin et al., 2021; Zubiete-Franco et al., 2017). N-acetylcysteine reduces hepatic NAE1 expression to prevent hepatic inflammation and fibrotic injury (Xu et al., 2022), while celastrol targets cullin1 to facilitate the interactions between CAND1 and cullin1 to suppress EMTs and pulmonary fibrosis (Zhou et al., 2022a). Both DN-2 and HZX-960 inhibit cullin3 NEDDylation against fibrosis; DN-2 inhibits cullin3 NEDDylation to reverse cardiac fibroblast activation, while HZX-960 targets cullin3 to block the interaction of DCN1 (co-E3 ligase) and Ubc12 as well as inhibit cullin3 NEDDylation against liver fibrosis (Zhou et al., 2022c). Even though they target different profibrotic/antifibrotic factors, the activators and inhibitors of SUMOylation/NEDDylation exhibit therapeutic properties against organ fibrosis, suggesting them as potential candidates in the treatment of organ fibrosis.

However, some limitations hinder the recognition and extensive use of SUMOylation/NEDDylation activators and inhibitors in the treatment of organ fibrosis. The primary drawback is the limited number of clinical and preclinical investigations on SUMOylation/NEDDylation activators and inhibitors, especially the lack of high-quality evidence identifying their therapeutic effects and mechanisms. Few clinical studies have shown the therapeutic effects and mechanisms of the SUMOylation and NEDDylation activators and inhibitors in the treatment of organ fibrosis. Notably, some clinical trials have been designed to investigate the effects of MLN4924 in the treatment of advanced solid tumors, acute myeloid leukemia, and myelodysplastic syndromes (Adès et al., 2022; Saliba et al., 2023; Sarantopoulos et al., 2016; Short et al., 2023); the SAE inhibitor subasumstat (TAK-981) was designed to treat head and neck carcinomas (Derry et al., 2023). Although these clinical trials have not targeted organ fibrosis, they can provide references for their potential use in organ fibrosis. Another limitation is the identification of potentially efficient SUMOylation and NEDDylation substrates. For example, the roles of TGF-β and PML are vital in organ fibrosis, and their SUMOylation/NEDDylation are considered as important regulators of activity in organ fibrosis. Hence, efficient substrates need to be identified and verified, through which we could also obtain references beyond the research scope of organ fibrosis. We can use bioinformatics methods like feature extraction and machine learning (Zhao et al., 2022) to predict SUMOylation sites. The level of NEDD8 can be a potential marker of organ fibrosis. The Cancer Genome Atlas (TCGA) database and tissue arrays can be used to evaluate the clinical relevance of NEDD8 expression in disease, and quantitative proteomic analyses may be helpful for exploring the knockdown of disturbed biological pathways (Xian et al., 2021). Biotinylated NEDD8 (^{bio}NEDD8) transgenic mice can be used in the pull-down of NEDDylated liver proteins and characterization of NEDDylomes in liver injury models (Serrano-Maciá et al., 2023) as promising strategies for fast selection and identification of NEDDylated proteins. Additionally, the use of new methods in cancer research allows SUMOylation-related genes as potential novel prognostic signatures and predictors of organ fibrosis (Sun et al., 2023; Wang et al., 2023a). Although clinical and related studies on SUMOylation and NEDDylation as well as their activators and inhibitors are limited, the potential of SUMOylation/NEDDylation has been verified in organ fibrosis treatment. Overall, SUMOylation and NEDDylation are promising therapeutic targets for organ fibrosis, so deeper investigations and clinical trials are needed to verify the therapeutic benefits of their activators and inhibitors to patients.

Author contributions

JH: writing–original draft and writing–review and editing. JW: writing–original draft and writing–review and editing. W-TK: writing–review and editing. L-NX: writing–review and editing. Y-LT: writing–review and editing. D-LZ: writing–review and editing. PL: project administration, supervision, and writing–review and editing. D-QC: conceptualization and writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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VASN knockout induces myocardial fibrosis in mice by downregulating non-collagen fibers and promoting inflammation

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Myocardial fibrosis (MF) is an important cause of heart failure and cardiac arrest. Vasorin knockout (VASN$^{-/-}$) leads to pathological cardiac hypertrophy (PCH); however, it is not yet clear whether this PCH transitions to MF in mice. VASN-knockout mice showed typical pathological, imaging, and molecular features of MF upon hematoxylin and eosin staining, Masson staining, Sirius red staining, quantitative polymerase chain reaction (qPCR), immunohistochemistry-paraffin (IHC-P), and immunofluorescence analyses. RNA was extracted from mouse heart tissue, identified, and sequenced *in vitro*. Differential analysis of the genes showed that the extracellular matrix (ECM) genes (*COL6A1*, *COL9A1,* and *FRAS1*) had strong correlations while their expression levels were significantly reduced by qPCR, IHC-P, and Western blotting. The expression levels of the ECM genes were significantly reduced but those of the inflammatory factors (IL1β and IL6) were significantly upregulated in the heart tissues of VASN-knockout mice. These preliminary results reveal that VASN knockout induces MF by regulating the non-collagen fibers and inflammation.

KEYWORDS

myocardial fibrosis, vasorin, non-collagen fibers, inflammation, mice

1 Introduction

Myocardial fibrosis (MF) is a key stage of heart failure that can exacerbate the associated symptoms and lead to severe outcomes, such as cardiac arrest or sudden death (López et al., 2021). MF is typically caused by prolonged pressure on or damage to the myocardial cells and often causes cardiac hypertrophy. MF is a complex pathological process that is closely related to the extracellular matrix (ECM), immune responses, signaling pathways, and various cardiac cells (Li et al., 2022). Damaged myocardial cells can activate local inflammatory reactions and release pro-inflammatory cytokines, such as interleukin (IL) 1, IL6, IL11, IL17, and tumor necrosis factor alpha (TNFα). Fibroblasts are activated and transformed into myofibroblasts in the heart tissues, which then synthesize and secrete large amounts of collagen and ECM components (Liu et al., 2021). Collectively, these risk factors contribute to MF.

Vasorin (VASN), also known as slit-like 2 (slitl2), contains two exons, of which exon 2 is the main coding region (Pintus et al., 2018). VASN is a transmembrane glycoprotein composed of 673 amino acids and is located on the cell surface (Bonnet et al., 2018). VASN is highly expressed in the cardiovascular system, including the heart, vascular smooth muscles, and umbilical vein endothelial cells (Bonnet et al., 2018; Pintus et al., 2018). Upregulation of VASN expression prevents smooth muscle cell calcification through specific binding to the transforming growth factor (Luong et al., 2019). Downregulation of VASN expression can alleviate adverse reactions to vascular wall injury (Li et al., 2015). However, overexpression or knockout of VASN has been found to cause developmental abnormalities in the heart and blood vessels of zebrafish (Chen et al., 2005). VASN-knockout ($VASN^{-/-}$) mice have been reported to die suddenly 3 weeks after birth (Ikeda et al., 2004). Our previous study showed that a VASN-knockout mouse model exhibited pathological cardiac hypertrophy symptoms (Sun et al., 2022).

In the present study, VASN-knockout mice showed the pathological, molecular, and protein features of MF. RNA from the mouse heart tissue was extracted, identified, and sequenced *in vitro*. Bioinformatic analysis then showed significantly decreased expressions of key ECM genes (*COL6A1*, *COL9A1*, and *FRAS1*); however, the expressions of inflammatory factors IL1β and IL6 were significantly upregulated in the heart tissues of VASN-knockout mice. Our results thus reveal that VASN knockout induces MF by affecting the ECM and inflammation.

2 Materials and methods

2.1 Preparation and identification of VASN-knockout mice

All mouse experiments were approved by the Ethics Committee of Guangxi Medical University (approval no. 202209200). C57BL/6J mice were obtained from the Laboratory Animal Center of Guangxi Medical University (SCXK GUI 2020–0003, SYXK GUI 2020–0004). When the $VASN^{-/-}$ mice were 28 days old and exhibited behavioral and morphological characteristics, such as arched backs, sparse hair, reduced body sizes, and immobility, the $VASN^{+/+}$, $VASN^{+/-}$, and $VASN^{-/-}$ mice from the same batch were divided into three groups for subsequent experiments. The hydroxyproline (HYP) assay was then performed according to manufacturer instructions (A030-2-1; Nanjing Jiancheng) (Sun et al., 2022).

2.2 Hematoxylin and eosin (HE) staining

HE staining was performed on the tissue samples from the mice according to a previously described protocol (Sun et al., 2022).

2.3 Masson staining

The heart samples were fixed in Bouin's solution and embedded in paraffin. The slices were then dewaxed, oxidized with 1% potassium permanganate for 5 min, bleached with oxalic acid for 1 min, stained with azure blue for 5 min, dried with Mayer's hematoxylin for 3–5 min, rinsed under running water for 5–10 min, stained with Lichun red picric acid saturated solution for 5 min, differentiated using 1% phosphomolybdic acid for approximately 5 min, dried with 1% light green for 30 s, differentiated using 95% alcohol, dehydrated with anhydrous ethanol, made transparent with xylene, and lastly sealed with neutral gum.

2.4 Sirius staining

The wax layers were first removed from the paraffin sections. Then, iron hematoxylin staining solution was applied to each section for 5–10 min followed by washing with distilled water for 10–20 s. The samples were then soaked in tap water for 5–10 min and cleaned with distilled water thrice for 5–10 s each time. Sirius red staining solution was then applied for 15–30 min, and each section was rinsed gently with running water to remove the surface dye. The slices were rapidly dehydrated using 80%, 95%, and anhydrous ethanol. Finally, the slices were sequentially made transparent in three cylinders of xylene for 3 min before being sealed with neutral gum.

2.5 Transcriptome sequencing and bioinformatics analysis

Transcriptome sequencing of the hearts from the three groups was performed at the Wuhan Genome Institute (BGI-Shenzhen), where a total of 12 RNA samples (three mice per group) were sequenced. Data from the whole transcriptome were collected and compared with the ribosome database to identify known transcripts (mRNA), perform quantitative analysis of the known and new mRNAs, and analyze differences between the samples (at least two samples) and groups (at least two samples with at least three biological repeats in each group). The differentially expressed genes (DEGs) were analyzed using the DAVID database through gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) functional enrichment analyses based on the miRNA target genes.

2.6 Quantitative polymerase chain reaction (qPCR) analysis

RNA reverse transcription and qPCR were performed according to a previous study (Sun et al., 2022) with primers (Table 1) obtained from Sangon Biotech (Shanghai, China). Each mRNA was subjected to 40 cycles of PCR, and this process was repeated thrice. The expression levels of the endogenous *GAPDH* genes were compared, and the relative mRNA expressions were compared using the $2^{-\triangle\triangle CT}$ method.

2.7 Western blotting (WB) analysis

WB was performed according to a previous protocol (Sun et al., 2022) using the primary antibodies COL6A1 (17023-1-AP, Protein,

TABLE 1 List of primer sequences.

Gene	Forward/reverse	Sequence
COL1A1	Forward	CTGACTGGAAGAGCGGAGAG
	Reverse	ACATTAGGCGCAGGAAGGTC
COL3A1	Forward	AGCCTTCTACACCTGCTCCT
	Reverse	CGGATAGCCACCCATTCCTC
CTGF	Forward	AGAACTGTGTACGGAGCGTG
	Reverse	GTGCACCATCTTTGGCAGTG
COL6A1	Forward	ATGTGCTCCTGCTGTGAGTG
	Reverse	TCTTGCATCTGGTTGTGGCT
COL9A1	Forward	CGACCGACCAGCACATCAA
	Reverse	AGGGGGACCCTTAATGCCT
FRAS1	Forward	GCTTGTCTGTATCAGGGCTCC
	Reverse	CTTCTCCCTTCTCAAAGGCAC
COL2A1	Forward	AAGGGAGAGACTGGACCTGC
	Reverse	GAATCCACGGTTGCCAGGAG
IL1β	Forward	TGCAGCTGGAGAGTGTGGA
	Reverse	GGCTTGTGCTCTGCTTGTGA
IL6	Forward	CTGCAAGAGACTTCCATCCAG
	Reverse	AGTGGTATAGACAGGTCTGTTGG
TNF	Forward	GACGTGGAACTGGCAGAAGAG
	Reverse	TTGGTGGTTTGTGAGTGTGAG
IL10	Forward	ACTATGCCGTCAGCGATACAG
	Reverse	GGCACCAGCTTTGAATAATACGA
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	AGGAGCGAGACCCCACTAACA

1:300), COL9A1 (12507-1-AP, Protein, 1:300), FRAS1 (29654-1-AP, Protein, 1:300), COL2A1 (A19308, ABclonal, 1:300), IL1β (D220820, Sangon Biotech, 1:300), IL6 (26404-1-AP, Protein, 1:300), IL10 (60269-1-Ig, Protein, 1:300), TNFα (17590-1-AP, Protein, 1:300), endogenous protein tubulin (AC001, ABclonal, 1:500), and secondary antibodies (AS014, ABclonal, 1:1000). The expressions of the target proteins were calculated using an automatic analysis system (Image Lab 6.0).

2.8 Immunohistochemistry (IHC) and immunofluorescence (IF) analyses

IHC-paraffin (IHC-P) and IF analyses were performed according to previously described protocols (Sun et al., 2022; Sun et al., 2021) using the primary antibodies against COL1A1 (A22090, ABclonal, 1:300), COL3A1 (22734-1-AP, Protein, 1:300), and CTGF (25474-1-AP, Protein, 1:300) as well as secondary antibodies (AS014, ABclonal, 1:500). Primary antibodies against α-SMA (67735-1-Ig, protein, 1:200) and a horseradish peroxidase (HRP)-conjugated secondary antibody (AS014, ABclonal, 1:500) were also used.

2.9 Statistical analysis

All experiments were performed in triplicate. The data were presented as mean ± standard deviation (SD) and analyzed statistically using one-way analysis of variance (ANOVA) in SPSS software. The value $p < 0.05$ was considered to indicate a significant difference, and $p < 0.01$ indicated an extremely significant difference.

3 Results

3.1 VASN knockout induces MF

HE staining showed that the thickness of the heart wall in a $VASN^{-/-}$ mouse was significantly higher than those in $VASN^{+/+}$ and $VASN^{+/-}$ mice (Figures 1A, E2). Significantly higher areas were observed for the cardiac cells of the $VASN^{-/-}$ mice; however, no abnormalities were observed in the heart tissues of the $VASN^{+/+}$ and $VASN^{+/-}$ mice (Figure 1B). These experimental results are consistent with those of our previous report (Sun et al., 2022). Masson and Sirius staining showed that cardiac interstitial fibrosis was significantly enhanced in the $VASN^{-/-}$ mice (Figures 1C, D), but no obvious abnormalities were observed in the heart tissues of the $VASN^{+/+}$ and $VASN^{+/-}$ mice. HYP expressions were significantly increased in the $VASN^{-/-}$ and $VASN^{+/-}$ mice (Figure 1F). qPCR and IHC-P showed that the expression levels of COL1A1, COL3A1, and CTFG were significantly higher in the heart tissues of the $VASN^{+/+}$ and $VASN^{+/-}$ mice (Figures 1G, H). IF analysis showed that the expression level and fluorescence intensity of α-SMA were significantly higher in the VASN-knockout mice (Figure 1I). These results confirmed that the VASN-knockout mice exhibited typical symptoms of MF.

3.2 Bioinformatics analysis to explore key molecules involved in MF

DEGs were identified based on the criteria of a false diagnosis rate (FDR) of <0.05, and |log2 (fold change)| >1.5 (Figures 2A, B). Cluster Profiler (R version 3.5.1, University of Auckland, Auckland, New Zealand) and GO (http://www.geneontology.org; accessed 20 August 2024) were used to enrich and analyze the DEGs. The volcano plot of the DEGs revealed key genes (Figure 2C), among which WT-VS-HO had the highest fold difference and was upregulated. These upregulated genes may be associated with cardiac hypertrophy and fibrosis. The GO enrichment analysis revealed the functional roles of 1,217 DEGs in WT-HO (Figure 2D). Cellular component analysis was used to obtain the localization of the top-10 DEGs. The ECM is one of the main structural components of myocardial tissue, and abnormal expression of the ECM may lead to MF, resulting in cardiac dysfunction.

KEGG enrichment analysis was used to find the top-10 enriched entries for all DEGs, including 786 enriched entries for

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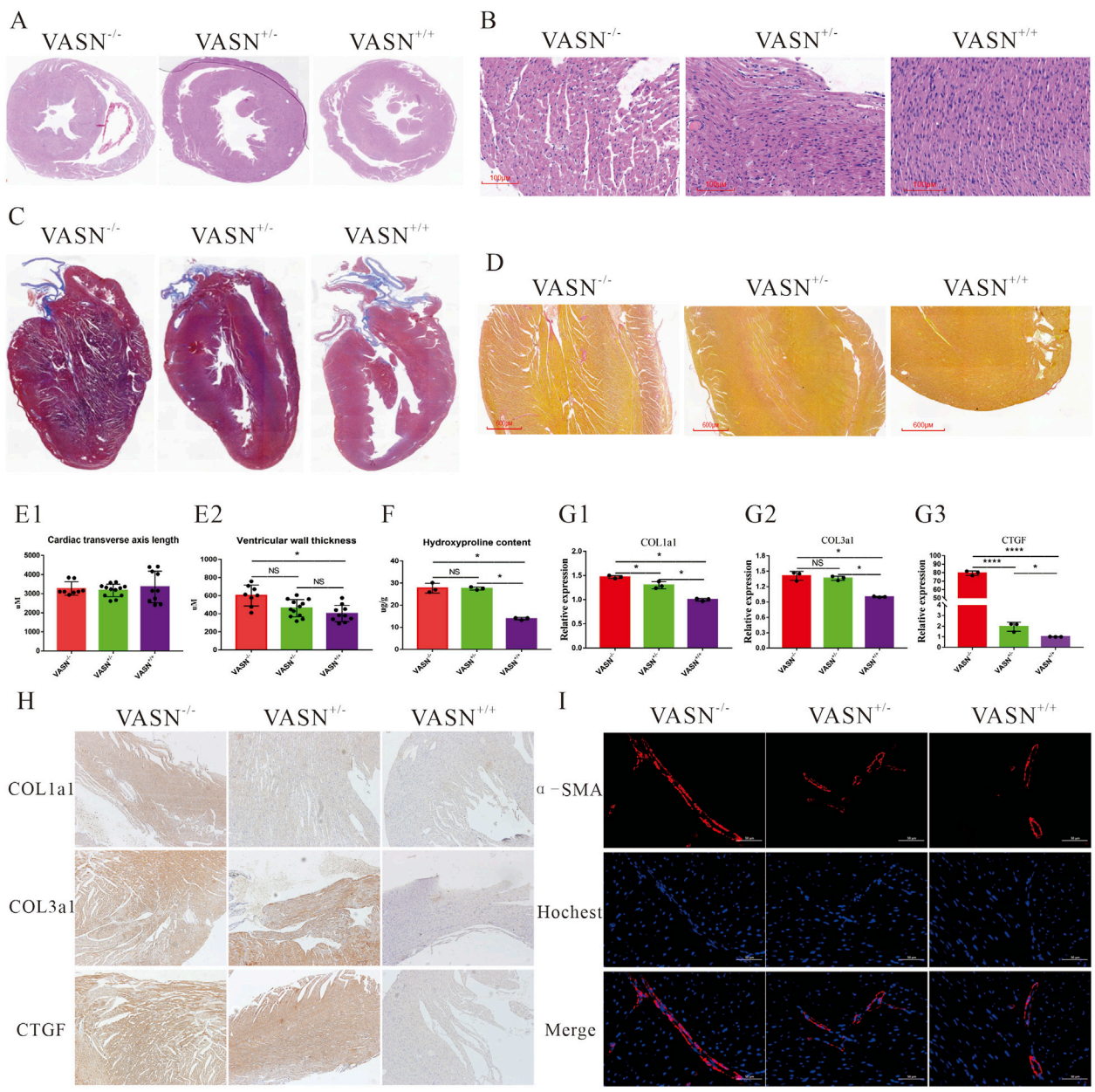

FIGURE 1
Typical characteristics of myocardial fibrosis (MF) in VASN-deficient mice: **(A)** overall morphology of VASN mouse heart under HE staining; **(B)** changes in the cardiac hypertrophy of VASN mice under HE staining; **(C)** changes in the MF of VASN mice under Masson staining; **(D)** changes in the MF of VASN mice under Sirius staining; **(E1, E2)** changes in the cardiac transverse axis length and ventricular wall thickness in VASN mice; **(F)** changes in the HYP expression levels in VASN mice; **(G1–G3)** changes in the MF markers of VASN mice in qPCR analysis; **(H)** changes in the MF markers of VASN mice in IHC-P analysis; **(I)** changes in the MF markers of VASN mice in IF analysis. $p < 0.05$ indicates significant difference, $p < 0.0001$ indicates extremely significant difference, $p > 0.05$ indicates no difference, and the subtables are represented by superscripts *, ****, and ^{NS}.

downregulated genes (Figure 2E); these also recruit genes related to the ECM. According to the interaction diagram of the top-8 downregulated genes in KEGG analysis, *COL6A1* (12,839) and *COL9A1* (12,833) were both involved with the ECM and cytoskeleton in the muscle cell pathways (Figure 2F). Protein–protein interaction (PPI) network mapping of the differential genes in the ECM pathway revealed close interactions between *COL6A1*, *COL9A1*, and *FRAS1* (Figure 2G); here, *COL6A1* and *COL9A1* are upstream genes that regulate *FRAS1* expression via ITGA8.

3.3 VASN knockout reduces expression of non-collagen fibers

Functional verifications were performed to investigate whether the expression of non-collagen fibers was downregulated in the heart tissue of VASN-knockout mice. HE staining showed that the gaps between the myocardial cells significantly increased in the heart tissue of VASN$^{-/-}$ mice (Figure 3A); qPCR showed that the mRNA expression levels of *CAL6A1*, *CAL9A1*, and *FRAS1* were significantly lower in the VASN$^{-/-}$ hearts (Figure 3B). IHC-P and WB showed

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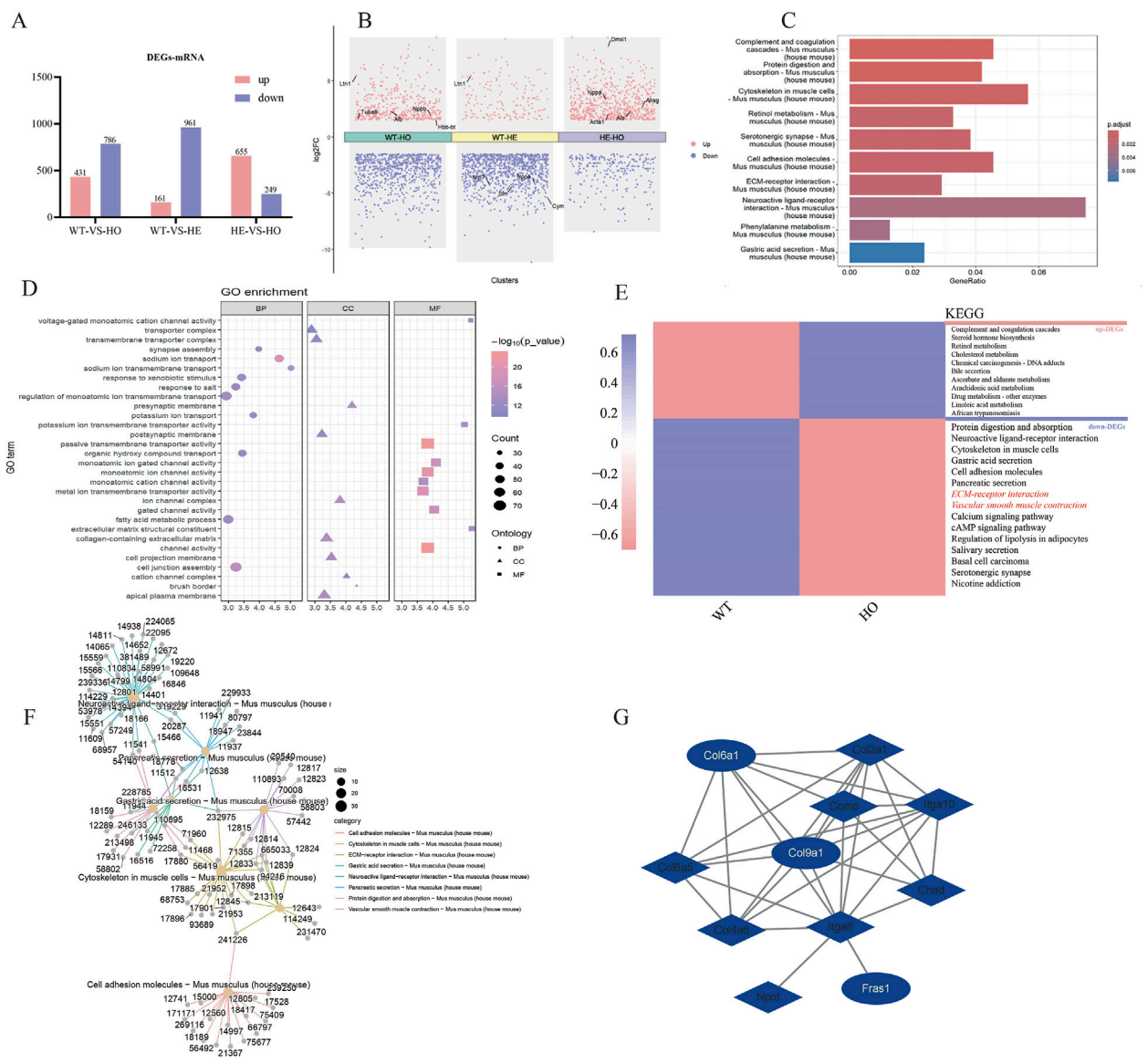

FIGURE 2
Transcriptome analysis of MF in VASN-deficient mice. The present study entailed heart tissues from VASN$^{+/+}$ (n = 3), VASN$^{+/-}$ (n = 3), and VASN$^{-/-}$ (n = 3) mice for transcriptome sequencing. **(A)** Differentially expressed genes (DEGs) based on miRNA transcriptome data; **(B)** volcano plot presenting the key genes involved in differential expression; **(C)** KEGG enrichment plot of all DEGs in WT-HO; **(D)** GO enrichment map of all DEGs in WT-HO; **(E)** WT-HO differential gene heatmap and KEGG annotation; **(F)** interaction diagram of the top-8 entries in KEGG for all DEGs in WT-HO; **(G)** extracellular matrix pathway gene protein interaction network diagram.

that the protein expression levels of CAL6A1, CAL9A1, and FRAS1 were significantly lower in the VASN$^{-/-}$ hearts (Figures 3C, D). These preliminary results imply that the downregulated expression of non-collagen fibers (*CAL6A1*, *CAL9A1*, and *FRAS1*) plays an important role in MF in the VASN$^{-/-}$ mouse hearts.

3.4 VASN knockout promotes cardiac inflammation

To investigate whether myocardial cell inflammation is exacerbated in MF, the inflammatory factors were identified. Accordingly, HE staining showed hypertrophy or atrophy of the myocardial cells, nuclear condensation, diffuse vacuolization of the myocardial cells, myocardial scars, and significantly increased immune cells in the heart tissues of VASN$^{-/-}$ mice (Figure 4A); qPCR showed that the mRNA expression levels of IL1β and IL6 were significantly upregulated in the VASN$^{-/-}$ hearts (Figure 4B). IHC-P and WB showed that the protein expression levels of IL1β and IL6 were significantly higher in the VASN$^{-/-}$ hearts (Figures 4C, D). These results indicate that intensified inflammation could cause MF in VASN$^{-/-}$ mouse hearts.

4 Discussion

MF is a complex pathological process that plays a crucial role in the occurrence and development of cardiovascular disease. MF and

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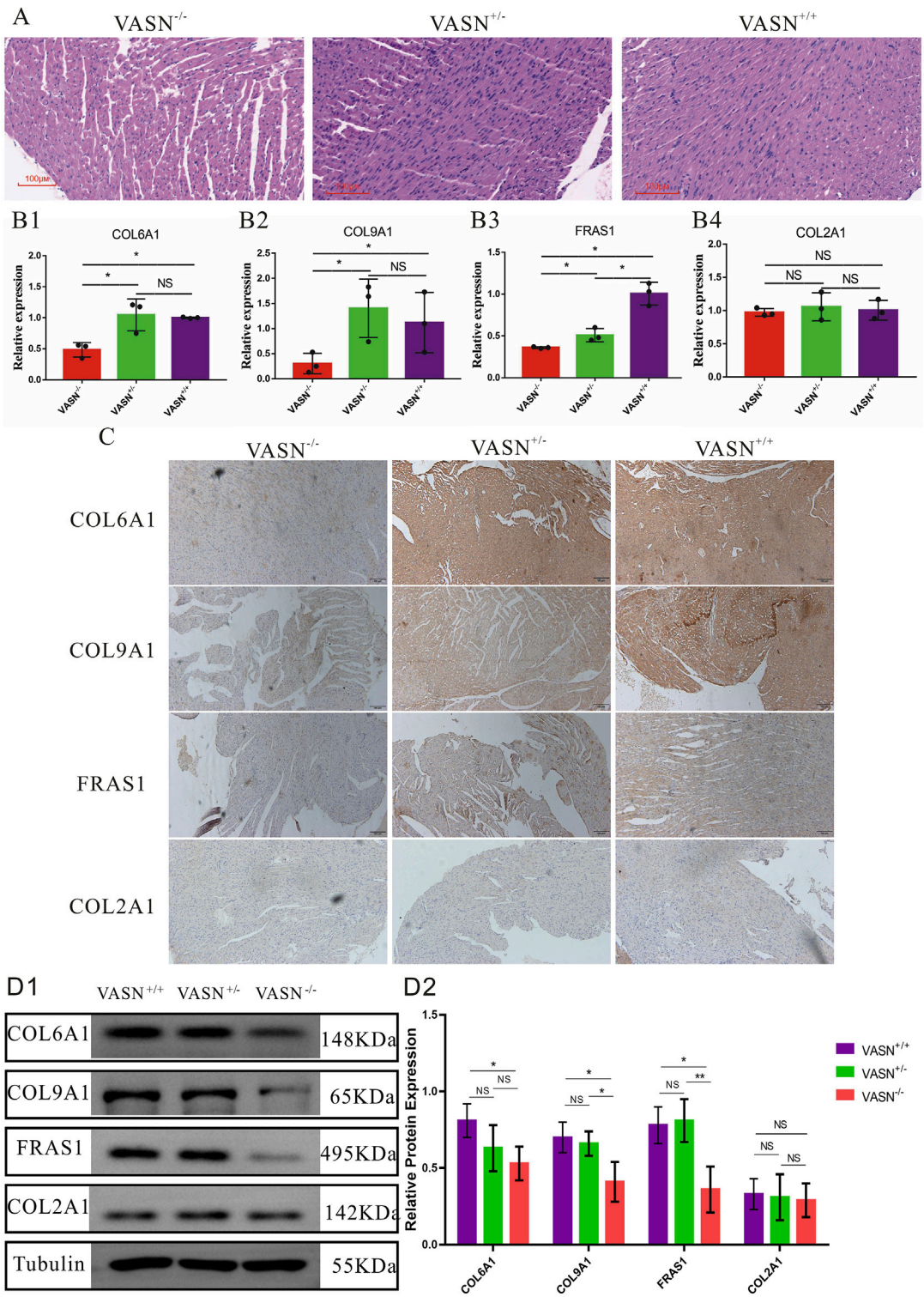

FIGURE 3
Changes in the non-collagen fibers of MF in VASN-deficient mice. **(A)** Changes in the MF of VASN mice under HE staining; **(B)** qPCR validation of the expression levels of the target genes in heart tissue; **(C)** IHC-P validation of the expression levels of the target genes in heart tissue; **(D)** WB validation of the expression levels of the target genes in heart tissue. $p < 0.05$ indicates significant difference, $p > 0.05$ indicates no difference, and the subtables are represented by superscripts * and ^{NS}.

cardiac hypertrophy often coexist and interact with each other (Pagourelias et al., 2021), and cardiac hypertrophy could lead to MF. Under long-term pressure or increased volume load, the cardiac cells undergo pathological hypertrophy (Figure 5). However, cardiac hypertrophy is often accompanied by remodeling of the myocardial ECM, including collagen deposition and fibrosis (Detterich, 2017).

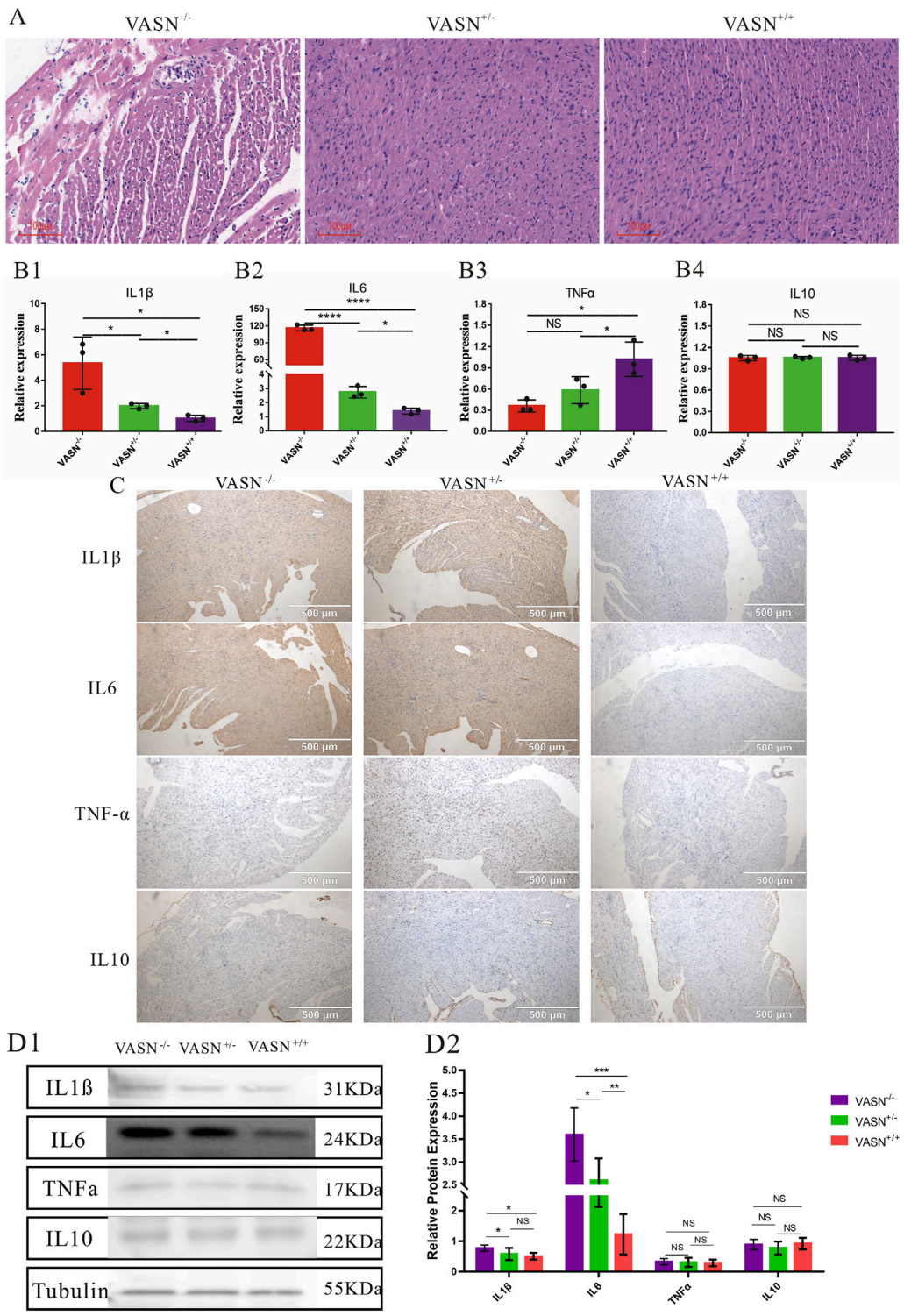

FIGURE 4
Changes in the inflammatory factors of MF in VASN-deficient mice. **(A)** Changes in the MF of VASN mice under HE staining; **(B)** qPCR validation of the expression levels of the target genes in heart tissue; **(C)** IHC-P validation of the expression levels of the target genes in heart tissue; **(D)** WB validation of the expression levels of the target genes in heart tissue. $p < 0.05$ indicates significant difference, $p < 0.01$ indicates extremely significant difference, $p < 0.001$ indicates extremely significant difference, $p < 0.001$ indicates extremely significant difference, $p > 0.05$ indicates no difference, and the subtables are represented by superscripts *, **, ***, ****, and ^{NS}.

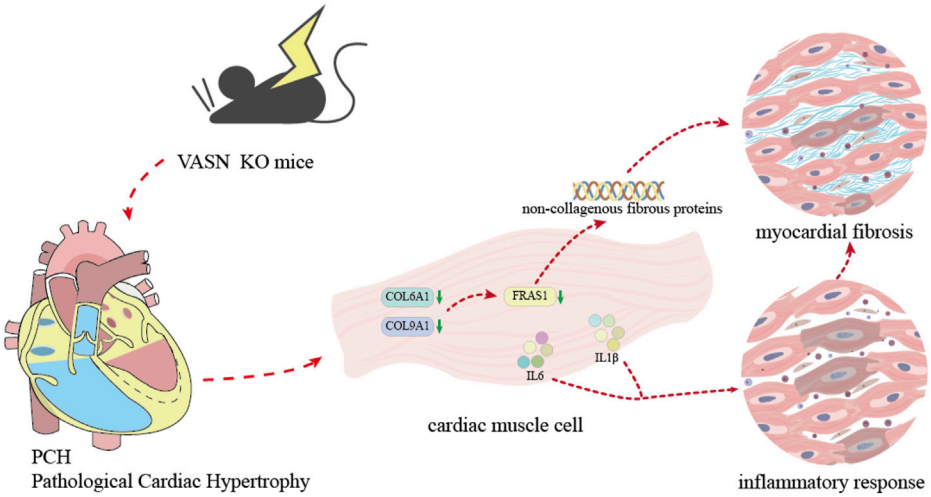

FIGURE 5
Diagram showing the mechanism by which VASN knockout induces MF by regulating the non-collagen fibers and inflammation.

MF caused by cardiac hypertrophy may be closely related to multiple mechanisms, such as activation of the neuroendocrine system (Qiu et al., 2019), inflammatory responses (Bacmeister et al., 2019), and oxidative stress (Wang et al., 2017). MF exacerbates the progression of cardiac hypertrophy as the stiffening and reduced compliance of the fibrotic myocardial tissue impair both diastolic and systolic heart functions (Lafuse et al., 2020). To maintain the pumping function, the cardiac cells are further enlarged, thereby exacerbating the degree of cardiac hypertrophy. MF can also affect the electrophysiological properties of the myocardial cells, thereby increasing the risk of arrhythmias (Baggett et al., 2023).

One of the typical features of MF is the adverse repair response of the cardiac tissue to various damaging factors. HE staining showed that the gaps between the myocardial cells widened during MF and that there was proliferation of pale pink fibrous tissue in the interstitium (Karur et al., 2024). As the degree of fibrosis worsened, the fibrous tissue increased gradually, and focal or diffuse fibrous cord-like structures became more pronounced. Masson staining showed significantly larger blue areas in the MF heart tissue, indicating greater deposition of collagen fibers. The myocardial interstitium in the fibrotic area was stained dark blue, forming a sharp contrast with the red color (muscle fibers) of normal myocardial tissue (Flori et al., 2024). Sirius staining of MF tissue showed large numbers of type I collagen fibers that appeared strongly positive in red or yellow color, whereas type III collagen fibers were relatively fewer and showed lighter staining (Qi et al., 2022). HYP was increasingly expressed in the fibrotic cardiac tissues (Yang et al., 2021). Collagen fiber types I and III are shown to be significantly increased in MF tissues (Xing et al., 2024). The expression level of α-SMA is reported to be low in normal myocardial cells but high in fibrotic cardiac tissues (Hsieh et al., 2022). Our experimental results are consistent with the findings of the above literature, indicating that VASN-knockout mice exhibit typical symptoms of MF.

The *VASN* gene is important for the occurrence and development of cardiovascular diseases. In atherosclerosis, abnormal expression of the *VASN* gene can cause endothelial dysfunction, reduce the resistance of the vascular endothelium to lipid deposition, and promote the formation of atherosclerotic plaques (Louvet et al., 2022). VASN may regulate the expression of adhesion molecules on the surfaces of endothelial cells, increase the adhesion of leukocytes to the vascular walls, and trigger inflammatory reactions to accelerate atherosclerosis (Huang et al., 2015). After myocardial infarction, local tissue ischemia and hypoxia can trigger a series of pathophysiological changes, and VASN is known to be involved in regulating the balance between apoptosis and regeneration of the myocardial cells (Pintus et al., 2018). Abnormal VASN expression leads to increased apoptosis of the myocardial cells, hindered myocardial repair and regeneration capabilities, exacerbated myocardial injury, and pump dysfunction (Shamhart and Meszaros, 2010). Under hypertension, the pressure on the vascular wall increases, and VASN affects the tension and compliance of blood vessels by regulating the contraction and relaxation of the vascular smooth muscle cells (Qin et al., 2024). Owing to dysregulation of VASN expression, the vascular smooth muscles contract excessively, further increasing the blood pressure and exacerbating the burden on the cardiovascular system (Wang et al., 2024).

MF is closely related to the occurrence and development of non-collagenous fibers, which play important roles in normal cardiac tissues. Non-collagen fibers together with collagen fibers form the ECM of the myocardial cells, providing structural support and mechanical stability to the cells (Shamhart and Meszaros, 2010). Non-collagen fibers include various components, such as elastic fibers, fibronectin, and laminin. In MF, changes to the non-collagen fibers often occur before significant changes to the collagen fibers. In the early stages of MF, fibronectin may respond to myocardial injury (Ning et al., 2017). Non-collagen fibers are shown to promote the adhesion, migration, and activation of cardiac fibroblasts, laying the foundation for excessive deposition of collagen fibers (Luther et al., 2012). Elastic fibers endow the myocardium with a certain degree of elasticity in a normal heart, which is beneficial for relaxation and contraction of the heart (Hiesinger et al., 2012). However, these elastic fibers are damaged or replaced by collagen fibers in MF,

leading to decreased elasticity and compliance of the heart (Lin et al., 2022). In addition, non-collagen fibers can participate in the regulation of the MF process by interacting with cytokines and growth factors.

Inflammatory factors are another trigger of MF, and there are numerous inflammatory factors that can directly induce MF. Both IL1β and IL6 were observed to play important roles in MF; IL1β activates the nuclear factor kappa B (NF-κB) signaling pathway to promote the production of more profibrotic factors by the cardiac fibroblasts, thereby accelerating MF (Sun et al., 2023); IL6 promotes the activation of cardiac fibroblasts and collagen synthesis through various pathways, such as the downstream signal transduction and transcription activating protein 3 (STAT3) (Rao et al., 2024). MF also triggers inflammatory reactions, resulting in a vicious cycle. As MF progresses, the structure and functions of the myocardial tissue induce local tissue hypoxia, metabolic disorders, and other conditions. Inflammatory cells such as macrophages then aggregate in the fibrotic myocardial tissues, releasing more inflammatory factors like IL1β and IL6 to exacerbate the severity of MF (Fu et al., 2024). The inflammatory factors interact with other signaling pathways to promote MF; they also affect the survival and functions of the myocardial cells, thereby promoting the development of MF (Liu et al., 2020).

VASN deletion leads to MF in mice with cardiac hypertrophy. VASN-knockout mice exhibit typical pathological, imaging, and molecular features of MF. Differential analysis of the various genes involved, especially the ECM genes (*COL6A1*, *COL9A1,* and *FRAS1*), showed strong correlations even as their expression levels decreased significantly in the heart tissues of VASN-knockout mice. The expression levels of inflammatory factors IL1β and IL6 were significantly upregulated in the heart tissues of VASN-knockout mice. These preliminary results reveal that VASN knockout leads to MF by downregulating the non-collagen fibers and promoting inflammation.

Data availability statement

The data presented in the study are deposited in the Harvard Dataverse repository, Harvard Dataverse tracking link https://doi.org/10.7910/DVN/NDTQEP. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by The Animal Care and Welfare Committee of Guangxi Medical University. The study was conducted in accordance with all local legislations and institutional requirements.

Author contributions

JS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. SY: Data curation, Investigation, Methodology, Software, Writing–original draft. QL: Data curation, Formal Analysis, Resources, Writing–original draft. JZ: Investigation, Supervision, Validation, Visualization, Writing–original draft. XG: Conceptualization, Investigation, Project administration, Resources, Writing–original draft. NY: Writing–original draft, Data curation, Software. BH: Software, Writing–original draft, Conceptualization. YO: Conceptualization, Writing–review and editing. QH: Conceptualization, Supervision, Visualization, Writing–review and editing. MH: Conceptualization, Investigation, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Roles of retinoic acid-related orphan receptor α in high glucose-induced cardiac fibroblasts proliferation

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Diabetic cardiomyopathy, characterized by myocardial fibrosis, is a common complication of diabetes. Retinoic acid-related orphan receptor α (RORα) participates in various pathological and physiological cardiovascular processes. The current research aims to elucidate the roles and mechanisms of RORα in high glucose induced cardiac fibroblasts proliferation. Primary neonatal cardiac fibroblasts were isolated from Sprague-Dawley rats, and pre-administrated with RORα antagonist SR3335 (20 μM) or RORα agonist SR1078 (10 μM) followed by the stimulation with normal glucose (5.5 mM) or high glucose (33.3 mM) respectively. Lactate Dehydrogenase (LDH) release into culture medium, cellular adenosine-triphosphate (ATP), and cell number were detected. Expressions of Collagen I, Collagen III, proliferating cell nuclear antigen (PCNA), α-smooth muscle actin (α-SMA), receptor-interacting protein kinase 1 (RIPK1) and receptor-interacting protein kinase 3 (RIPK3) were evaluated. The extent of oxidative stress was also assessed. Our study found that high glucose elevated LDH release, reduced cellular ATP production, increased cells numbers, elevated expression of Collagen I, Collagen III, PCNA, α-SMA, RIPK1 and RIPK3, decreased mitochondrial membrane potential, strengthened intensity of dihydroethidium (DHE) and MitoSOX fluorescence. Above effects were all further exacerbated by SR3335 but significantly reversed by SR1078. In conclusion, RORα antagonist SR3335 promoted cell injury and proliferation, enhanced collagen synthesis, facilitated oxidative stress and necroptosis in cardiac fibroblasts with high glucose stimulation, whereas RORα agonist SR1078 showed opposing effects. Our study proposed RORα as a novel target against high glucose-induced cardiac fibroblasts proliferation, which is beneficial to clarify ideal therapeutic implication for diabetic cardiomyopathy.

KEYWORDS

cardiac fibroblasts, retinoic acid-related orphan receptor α, proliferation, necroptosis, oxidative stress

1 Introduction

Diabetic cardiomyopathy (DC) was originally characterized as the presence of structural or functional abnormalities of the myocardium associated with diabetes mellitus (DM) in the absence of hypertension, coronary heart disease, and/or obesity (Song et al., 2021). However, this characterization lacks robust evidence as only a limited number of diabetic patients meet these criteria, rendering its clinically impractical. Recently,

the Heart Failure Association of the European Society of Cardiology (ESC), in collaboration with the Working Group on Myocardial and Pericardial Diseases, has published a consensus statement proposing that DC should be defined as the presence of myocardial diastolic and/or systolic dysfunction related to diabetes (Seferović et al., 2024). From the standpoint of heart failure progression, the asymptomatic functional and structural cardiac anomalies in patients with DC can be considered as precursors to heart failure. Nonetheless, therapeutic options for DC remain limited in clinical practice. Furthermore, the role of glycemic control in the prevention of heart failure among diabetic patients is not well understood. Some studies have indicated a U-shaped relation between blood glucose levels and the incidence of heart failure, suggesting that glycemic control alone may be insufficient to prevent the progression of DC to heart failure (Parry et al., 2015). Consequently, clarifying pathogenesis of DC and exploring rational and effective treatment strategies will be beneficial in prevention and management of DC.

Myocardial fibrosis is a prominent pathological feature observed in DC, manifested primarily as an excessive accumulation of collagen fibers, a marked increase in collagen content or abnormal alterations in collagen composition. These changes lead to an elevated number of cardiac fibroblasts within the extracellular matrix (ECM) of the myocardium (Frangogiannis, 2022). Cardiac fibroblasts play a crucial role in maintaining ECM homeostasis. Upon activation by several stimuli or damaging factors, such as ischemia, pressure overload, metabolic disorders, and neurohormonal release, cardiac fibroblasts differentiate into myofibroblasts, which are instrumental in driving pathological cardiac remodeling (Liu T. et al., 2024; González et al., 2024; Yu et al., 2024; Zhang et al., 2023a). Myofibroblasts exhibit proliferative capabilities and contribute to ECM turnover and collagen deposition. However, there remains a significant gap in effective strategies to prevent excessive proliferation of cardiac fibroblasts in the context of DC.

Necroptosis, a form of programmed cell death identified as an alternative to apoptosis following the binding death structural domains to receptor, playing a significant role in myocardial hypertrophy, myocardial infarction, atherosclerosis, and neurodegenerative diseases (Cai et al., 2024; Zhang et al., 2023b; Sheng et al., 2023; Cao and Mu, 2021; Khan et al., 2021). Necroptosis is characterized by rupture of cell membranes, swelling of organelles, enlargement of cell volume, and breakdown of cytoplasm and nucleus, while exhibiting minimal alterations in nuclear chromatin. Increasingly, studies have shown that the necroptotic pathway is mediated by the canonical death receptor comprising of receptor-interacting protein kinase 1 (RIPK1) and receptor-interacting protein kinase 3 (RIPK3) (Zhou et al., 2019; Shao et al., 2021). Active RIPK1 participates in the formation of oligomeric complexes that involve caspase-8, caspase-10 and Fas-associated protein with death domain (FADD). In detail, RIPK1 phosphorylates RIPK3, which subsequently phosphorylates mixed lineage kinase domain-like protein (MLKL), leading to the formation of necrosomes. Following this, MLKL oligomers translocate to phosphatidylinositol phosphate (PIP)-rich region of plasma membrane, resulting in the formation of large pores, causing a substantial influx of ions, lysis of the cell membrane, permeabilization of lysosomal membrane and uncontrolled release of intracellular contents, culminating in necroptosis (Koerner et al., 2024; Liu S. et al., 2024). Moreover, our previous studies have established a correlation between necroptosis and mitochondrial dysfunction, oxidative stress, and inflammation during DC (Song et al., 2021; Gong et al., 2022; Zhang S. et al., 2023), indicating that necroptosis inhibition may protect against cardiac fibroblasts proliferation in DC.

Retinoic acid-related orphan receptor (ROR) is classified within nuclear hormone receptor superfamily, which integrates nutritional, pathophysiological, hormonal signaling and gene regulation (Zheng et al., 2024; Sajinovic and Baier, 2023). Three primary isoforms in mammals are recognized: RORα, RORβ, and RORγ, each of which is capable of forming multiple variants through selective splicing. RORα has been associated with various functions, including neurodevelopment, cellular differentiation, immunoregulation, metabolism, and the regulation of circadian rhythms. Recent studies have revealed that RORα exerts a protective impact against cardiovascular disorders such as myocardial hypertrophy, myocardial ischemia-reperfusion injury, and atherosclerosis (Chen et al., 2023). Our previous research demonstrated a significant reduction in RORα expression in diabetic hearts, and lack of RORα exacerbated diabetes-induced systolic dysfunction and cardiac remodeling (Zhang S. et al., 2023). These findings suggest that RORα may possess an inhibitory role in DC. However, the specific influence of RORα on cardiac fibroblast proliferation during DC remains inadequately understood.

Therefore, in our current study, the primary cardiac fibroblasts were isolated and subsequently subjected to high glucose stimulation. The study aimed to elucidate the effects and potential mechanisms of RORα antagonist and RORα agonist on cardiac proliferation, with a focus on oxidative stress and necroptosis. It is conducive to provide innovative insights for clinical prevention and treatment of DC.

2 Materials and methods

2.1 Culture and treatment of primary cardiac fibroblasts

Hearts from Sprague-Dawley rats aging one to 3 days were excised and rapidly taken off using sterilized surgical scissors. After rinsing three times in cold phosphate buffered saline (PBS) solution, the hearts were cut into approximately 1–3 mm^3 cubes and transferred into a 50 mL of conical bottle. About 1.5–2.0 mL of Dulbecco's modified eagle medium (DMEM, Wisent Inc., Montreal, QC, Canada) having trypsin was added into the conical bottle placed on an incubator with shaking for 5 min at 37°C to start digestion. The first digestion's supernatant was discarded. Then, the precipitate underwent further digestion for 3 min at a time and repeated for about 10 times. All digested supernatants were collected into a beaker containing DMEM with 10% fetal bovine serum (FBS, Gibco, Thornton, NSW, Australia). The cell suspension after filtering with a cell sieve was centrifuged in a centrifuge tube for 5 min at 1,200 r/min. Following removing the supernatant, the cells in the precipitate were re-suspended and inoculated into a new culture dish with DMEM having 10% FBS. The cells were placed at 37°C in 5% CO_2 incubator, and the differential adhesion method to acquire cardiac

fibroblasts was performed. In detail, culture medium containing cardiomyocytes was removed after cells had adhered to the plate for 180 min. The cardiac fibroblasts that remained attached to the plate were digested and cultured in fresh DMEM having 10% FBS. The cardiac fibroblasts were sub-cultured basing on their growth conditions, and the cardiac fibroblasts of 3rd to 4th generation were seeded into plates in the present study. After starvation for 12 h, the cardiac fibroblasts were pre-administrated with RORα antagonist SR3335 (5, 10, 20 and 40 μM, MedChemexpress, Rahway, NJ, United States) or RORα agonist SR1078 (2.5, 5, 10 and 20 μM, MedChemexpress) followed by 48 h of stimulation with normal glucose (NG) or high glucose (HG) respectively (Liang et al., 2021; Chen D. et al., 2024; Wahyuni et al., 2021; Zhang Y. et al., 2022; Xiong et al., 2020; Shen et al., 2023). Cardiac fibroblasts under the normal glucose (5.5 mM) group and high glucose (11.1 mM, 22.2 mM and 33.3 mM) group were exposed to 27.8 mM, 22.2 mM, 11.1 mM mannitol and 0 mM mannitol respectively to balance the osmotic pressure (Gong et al., 2022; Zhang S. et al., 2023; Tian et al., 2021; Lu et al., 2023).

The study was conducted according to National Institutes of Health guidelines for the Care and Use of Laboratory Animals, and approved by Committee of Nantong University (approval no. S20210227-011 on 27 February 2021). The study was conducted in accordance with the local legislation and institutional requirements.

2.2 Lactate dehydrogenase (LDH) release detection

After treatment, the centrifugation was made for cell culture medium at 400 g for 5 min. The supernatants of 120 μL were collected and transferred to 96-well plate followed by incubation at 25°C for 30 min with 60 μL of LDH test solution (Beyotime, Shanghai, China) without light. The absorbance at 490 nm, representing LDH release level, were recorded by microplate-reader (BioTek, Winooski, VT, United States) and standardized by the value obtained from the normal glucose group value.

2.3 Adenosine-triphosphate (ATP) level measurements

After treatment, 100 μL ATP assay reagent (Beyotime, Shanghai, China) was utilized to incubate cardiac fibroblasts at 25°C for 10 min. Then, microplate-reader was employed to record the luminescence intensity. The relative ATP levels were standardized by the value obtained from the normal glucose group.

2.4 Cell counting kit-8 (CCK-8) assay

After treatment, 10 μL CCK-8 reagent (Beyotime, Shanghai, China) was added to cardiac fibroblasts in 96-well plates and incubated at 37°C for 1 h without light. The optical density (OD), which correlates with the cell number, was recorded for each sample by a microplate-reader at 450 nm.

2.5 EdU (5-ethynyl-2′-deoxyuridine) staining

After treatment, EdU (50 μM, RiboBio, Guangzhou, China) was added to cardiac fibroblasts in 24-well plates and incubated for 2 h without light. Next, the cells were washed twice with PBS. Then, PBS containing 4% paraformaldehyde was used to fix the cells for 30 min. Glycine (2 mg/mL) was then added and the mixture was agitated on a shaker for 5 min. After washing, EdU penetrant (PBS containing 0.5% TrixonX-100) was used to incubate the cells for 10 min, and washed by PBS once for 5 min. Cells were incubated with EdU penetrant once again for an additional 10 min after the application of Apollo fluorescence staining solution for 30 min 4′,6-diamidino-2-phenylindole (DAPI, blue) was used to stain the nuclei. EdU red fluorescence was monitored and imaged with a confocal laser microscope (Leica, Wetzlar, Germany). ImageJ software was employed to count the EdU positive cells.

2.6 Immunofluorescence staining

After treatment, the cardiac fibroblasts in 24-well plates were fixed at 25°C for 30 min with immunofluorescence fixative. Following fixation, the cells were washed with PBS and incubated for 1 h with blocking solution. The primary antibodies, including RORα (1:200, Abcam, Cambridge, United Kingdom), α-smooth muscle actin (α-SMA, 1:1,000, Boster Biological Technology, Dublin, CA, United States), Collagen I and Collagen III (1:200, Proteintech, Rosemont, IL, United States) were applied and incubated overnight at 4°C. PBS was used for washing followed by the incubation of cells with Alexa Fluor 488 (green) or Cy3 (red) conjugated IgG dilution (1: 500, Beyotime, Shanghai, China) on a shaker for 2 h without light at 25°C. DAPI (blue) was used to stain the nuclei. The fluorescence was monitored and imaged with a confocal laser microscope.

2.7 Dihydroethidium (DHE) staining

After treatment, DHE (2 μM, Beyotime, Shanghai, China) was added to incubate the cardiac fibroblasts at 37°C without light for 30 min in 24-well plates placed in oven. DAPI was employed to stain the nuclei. Red fluorescence reflecting superoxide anion levels were monitored and imaged with a confocal laser microscope.

2.8 MitoSOX staining

After treatment, MitoSOX Red (5 μM, Yeasen, Shanghai, China) and MitoTracker Green (200 nM, Beyotime, Shanghai, China) were added to incubate cardiac fibroblasts at 37°C in 24-well plates for 20 min without light in an oven. DAPI was employed to stain the nuclei. Red fluorescence reflecting mitochondria reactive oxygen species (ROS) levels were monitored and imaged with a confocal laser microscope.

2.9 JC-1 staining

After treatment, JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide) working solution (Beyotime, Shanghai,

China) was added to incubate cardiac fibroblasts in 24-well plates at 37°C for 20 min in an oven without light. DAPI was employed to stain the nuclei. JC-1 monomers show green fluorescence reflecting impaired mitochondria and JC-1 aggregates show red fluorescence reflecting normal mitochondria with less and higher mitochondrial membrane potentials, respectively. They were detected and imaged with a confocal laser microscope.

2.10 Quantitative real-time PCR

TRIzol reagent (Takara, Kyoto, Japan) was used to extract RNA from cardiac fibroblast and reverse transcription was performed. Then, SYBR Green qPCR mixture (Takara) was employed to amplify cDNA in the Real-time PCR systems (ABI 7500, Carlsbad, CA, United States). The sequences of primers (Sangon Biotech, Shanghai, China) were as follows: rat α-SMA mRNA (F, 5′-CATCAGGAACCTCGAGAAGC-3′ and R, 5′-TCGGATACTTCAGGGTCAGG-3′), rat Collagen I mRNA (F, 5′-AGGGTCATCGTGGCTTCTCT-3′ and R, 5′-CAGGCTCTTGAGGGTAGTGT-3′), rat Collagen III mRNA (F, 5′-AGCGGAGAATACTGGGTTGA -3′ and R, 5′-GATGTAATGTTCTGGGAGGC-3) and 18S mRNA (F, 5′-AGTCCCTGCCCTTTGTACACA-3′ and R, 5′-CGATCCGAGGGCCTCACTA-3′). Standardization was made for the experimental Ct values by those in normal glucose group.

2.11 Western blot

After washing with PBS 2–3 times, lysis solution was added into the cardiac fibroblasts and incubated for 40 min on ice. Then, cells were scraped off using a cell spatula, collected into centrifuge tubes and continued to lysis for an additional 40 min. Next, the cells were centrifuged with 12,000 rpm at 4°C for 15 min to collect the supernatant and stored at −80°C for subsequent experiments. Protein quantification (BCA method) was made to determine the protein concentration and sample volume for measurement was calculated.

Next, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate the proteins followed by transferring to polyvinylidene fluoride (PVDF) membrane. Then, 5% non-fat milk was employed to incubate the membranes for 2 h at 25°C. After washing for 10 min by Tris-buffered saline Tween-20 (TBST), RORα (1:1,000, Abcam, Cambridge, United Kingdom), proliferating cell nuclear antigen (PCNA, 1:1,000, ABclonal, Wuhan, China), α-SMA (1:2000, Boster Biological Technology, Dublin, CA, United States), Collagen I and Collagen III (1:200, Proteintech, Rosemont, IL, United States), RIPK1 and RIPK3 (1:1,000, Cell Signaling Technology, Danvers, MA, United States), GAPDH (1:5,000, Sigma-Aldrich, St. Louis, MO, United States), and β-tubulin (1:3,000, CMCTAG, Milwaukee, WI, United States) antibodies were incubated at 4°C overnight. Next day, TBST was used to wash the membrane three times for 10 min each. A secondary antibody was then added followed by incubation for 2 h at 25°C on a shaker. Finally, blots were visualized using an enhanced chemiluminescence (ECL, Thermo Fisher Scientific Inc., Rockford, IL, United States) solution.

2.12 Statistical analysis

The data were presented as mean ± standard deviation (SD), and statistically evaluated by one-way ANOVA followed by the Student-Newman-Keuls test with Stata 15.0. p-value of <0.05 was set as statistically significant.

3 Results

3.1 High glucose promoted cell proliferation but inhibted RORα expressions in cardiac fibroblasts

Initially, a concentration-response curve was established to assess the relation between glucose concentration and cell number. The data demonstrated that glucose concentrations of 11.1, 22.2 and 33.3 mM significantly increased cell number, with the most pronounced effects observed at a concentration of 33.3 mM (Figure 1A). Then, a time-dependent experiment showed that stimulation with 33.3 mM glucose for durations of 24 h, 48 h and 72 h increased cell numbers, with the maximum enhancement begining at 48 h (Figure 1B). Therefore, a 48 h exposure to 33.3 mM glucose was selected for subsequent experiments aimed at promoting cell proliferation, consistent with previous studies (Gong et al., 2022; Zhang S. et al., 2023; Tian et al., 2021; Lu et al., 2023).

Our previous research confirmed that high glucose decreased RORα expression in cardiomyocytes (Zhang S. et al., 2023). In alignment with these findings, the current study confirmed that high glucose also reduced RORα expression in cardiac fibroblasts (Figures 1C, D). To elucidate the role of RORα in cardiac fibroblast proliferation, the effects of RORα antagonist and RORα agonist on primary cardiac fibroblasts with high glucose stimulation were further investigated.

3.2 SR3335 promotes cell injury and proliferation in high glucose stimulated cardiac fibroblasts

To evaluate the impact of RORα on cell injury induced by high glucose, LDH release and ATP level were measured. And cardiac fibroblast number was assessed through OD obtained from the cell CCK-8 assay. The data demonstrated that compared to high glucose stimulation alone, RORα antagonist SR3335 at different concentration (10 μM, 20 μM and 40 μM) further increased LDH release in the medium, reduced the cellular ATP production but enhanced OD value in cardiac fibroblasts (Figures 2A–C). These findings suggested that SR3335 promoted cell injury and increased cell number in high glucose stimulated cardiac fibroblasts. Notably, the most pronounced effects were observed at a concentration of 20 μM, which was selected for subsequent experiments.

EdU is capable of infiltrating the DNA that is newly synthesized. Thus, EdU staining is a sensitive and effective method for evaluating cell proliferation (Zhang et al., 2021; Zhang Y. et al., 2024). It was found that EdU positive cells were enhanced in response to high

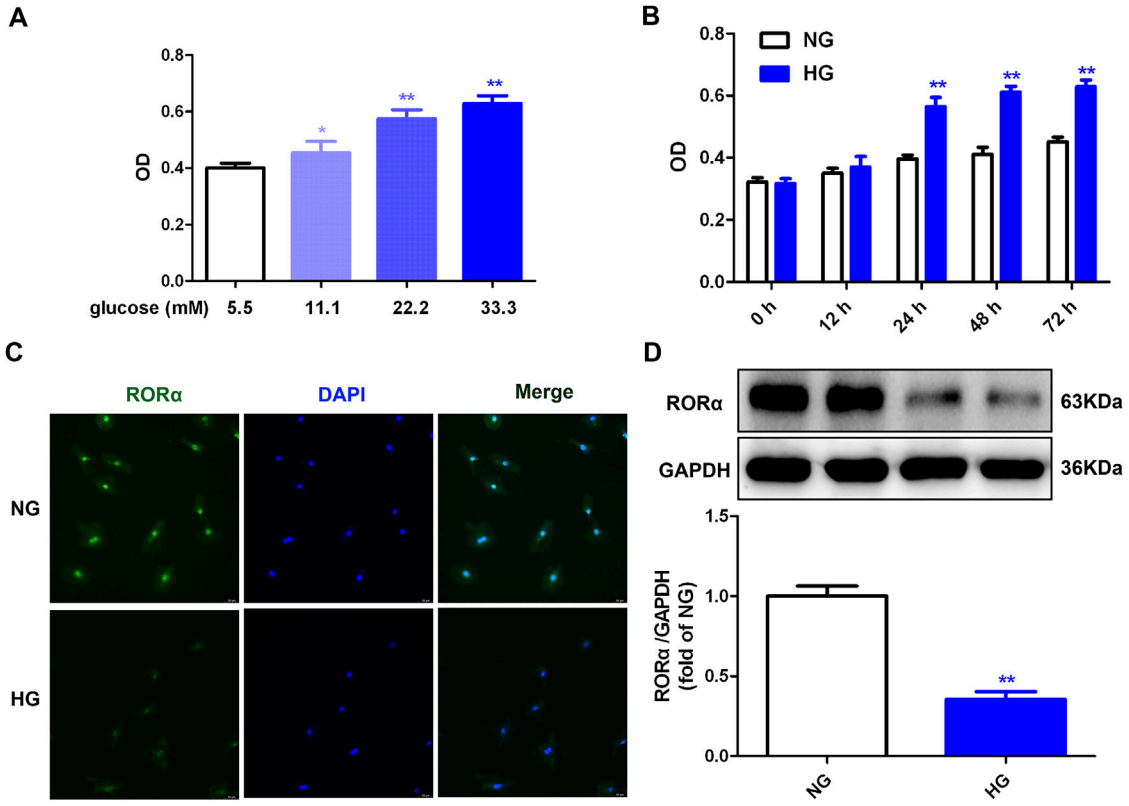

FIGURE 1
High glucose promoted cell proliferation but inhibted RORα expressions in cardiac fibroblasts. **(A)** After stimulation with glucose of different concentration (5.5, 11.1, 22.2 and 33.3 mM) and mannitol with different concentration (27.8 mM, 22.2 mM, 11.1 mM and 0 mM respectively) for 48 h, OD value obtained from CCK-8 assay was measured. *$p < 0.05$, **$p < 0.01$ verses 5.5 mM glucose, n = 6. **(B)** After stimulation with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for different times (0 h, 12 h, 24 h, 48 h and 72 h), OD value obtained from CCK-8 assay was measured. **$p < 0.01$ verses 5.5 mM glucose with the same stimulation time, n = 6. **(C)** After stimulation with NG or HG for 48 h, RORα was immunofluorescence stained with Alexa Fluor 488 (green) conjugated IgG. The nuclei were stained with DAPI (blue). Bar = 50 μm. **(D)** The protein expression of RORα was measured by Western blot. GAPDH was serviced as a control. **$p < 0.01$ verses NG, n = 6.

glucose stimulation, with further enhancement by SR3335 (Figures 2D, E). PCNA, a crucial protein associated with DNA polymerase and cell proliferation (He et al., 2024). Western blot showed increased PCNA expression after stimulation by high glucose was further augmented by SR3335 (Figure 2F). Additionally, Western blot, Real-time PCR, and immunofluorescence demonstrated that α-SMA, another sensitive indicator of cell proliferation, was enhanced after stimulation by high glucose, with further promotion by SR3335 (Figures 2G–I). Taken together, SR3335 promoted cell proliferation in high glucose stimulated cardiac fibroblasts.

3.3 SR3335 enhances synthesis of collagen in high glucose stimulated cardiac fibroblasts

Obviously, increased cardiac fibroblasts during cell proliferation will secret a large amount of collagen. Real-time PCR, Western blot, and immunofluorescence demonstrated that Collagen I and Collagen III, two predominant types of fibroblasts in the myocardium, were enhanced after stimulation by high glucose. This effect was further augmented by SR3335 (Figure 3), suggesting that SR3335 enhanced synthesis of collagen in high glucose stimulated cardiac fibroblasts.

3.4 SR3335 facilitates oxidative sstress in high glucose stimulated cardiac fibroblasts

Reported studies suggested that oxidative stress played a vital role in cardiac fibroblasts proliferation (Zhang Q. et al., 2022; Li et al., 2023). The present research found that red fluorescence of DHE was significantly enhanced after high glucose stimulation, which was further amplified by SR3335 (Figure 4A). This suggested that SR3335 boosted cellular superoxide anion in high glucose stimulated cardiac fibroblasts. ROS in the mitochondria was further measured using MitoSOX staining. Similarly, MitoSOX fluorescence was dramatically strengthened by high glucose, with further enhancement by SR3335 (Figure 4B).

The impairment of mitochondrial membrane potential not only leads to cell injury but also induces oxidative stress (Drăgoi et al., 2024; Chen S. et al., 2024). JC-1 staining demonstrated that JC-1 monomers' green fluorescence was enhanced while JC-1 aggregates' red fluorescence was attenuated in cardiac fibroblasts with high glucose stimulation, indicating that high glucose had impaired the mitochondrial membrane potential. SR3335 further enhanced green fluorescence but alleviated red fluorescence of JC-1 staining for high glucose stimulated cardiac fibroblasts (Figure 4C). Collectively, SR3335 facilitated oxidative stress in high glucose stimulated cardiac fibroblasts.

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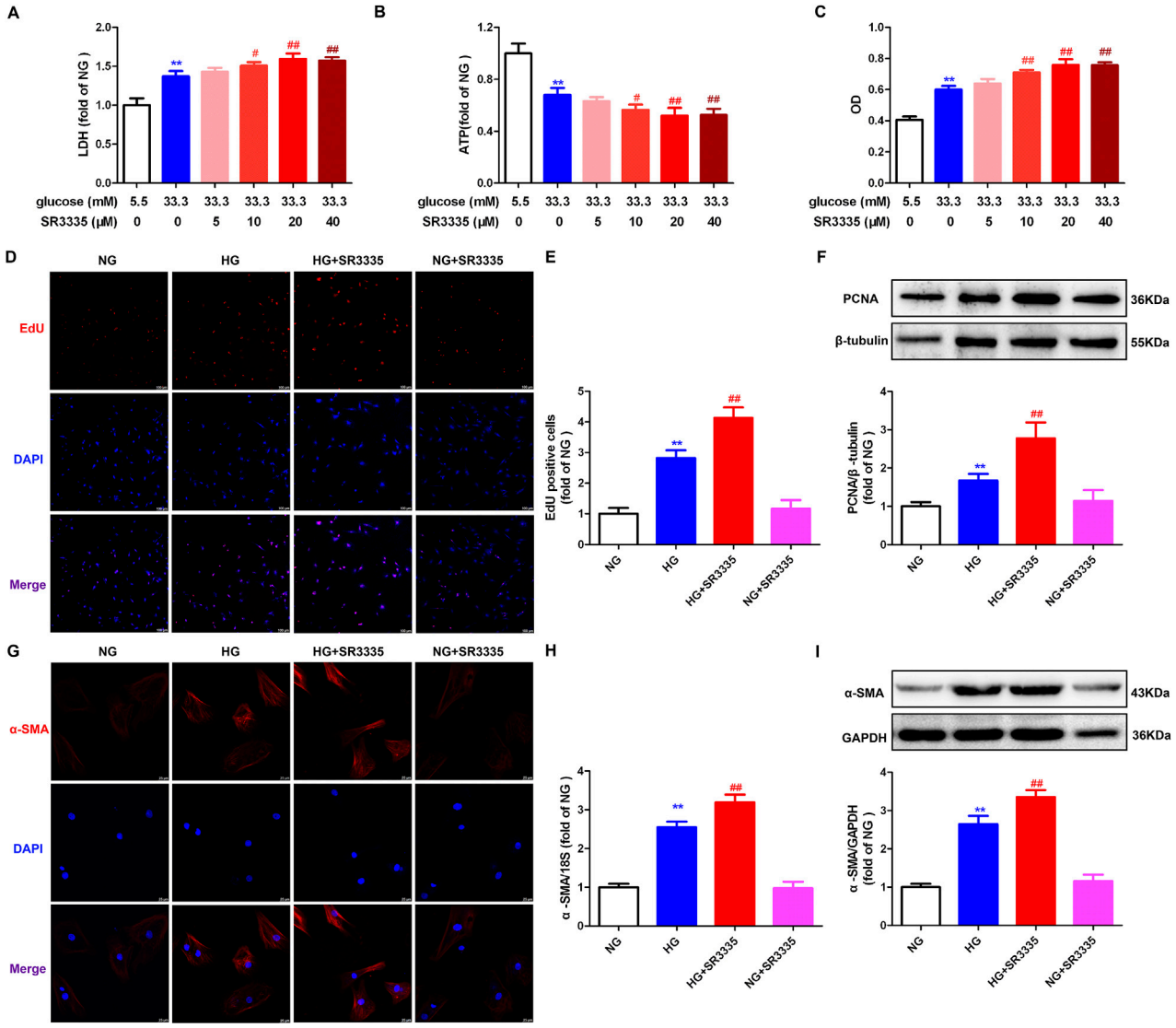

FIGURE 2
SR3335 promoted cell injury and proliferation in high glucose stimulated cardiac fibroblasts. **(A–C)** After pre-administration with different concentration of SR3335 (5 μM, 10 μM, 20 μM and 40 μM) for 4 h, the cardiac fibroblasts were stimulated with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for 48 h. LDH release in the medium **(A)**, cellular ATP in the cardiac fibroblasts **(B)**, and OD value obtained from the CCK-8 assay **(C)** were measured. $^{**}p < 0.01$ verses 5.5 mM glucose; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ verses 33.3 mM glucose, n = 6. **(D, E)** After pre-administration with SR3335 (20 μM) for 4 h, the cardiac fibroblasts were stimulated with NG or HG for 48 h. EdU staining with red fluorescent was performed to evaluate cardiac fibroblasts proliferation. Bar = 100 μm. EdU positive cells were quantitatively analyzed. **(F)** The protein expression of PCNA was measured by Western blot. β-tubulin was serviced as a control. **(G)** α-SMA was immunofluorescence stained with Cy3 (red) conjugated IgG. The nuclei were stained with DAPI (blue). Bar = 25 μm. **(H)** The mRNA expression of α-SMA was measured by Real-time PCR. **(I)** The protein expression of α-SMA was measured by Western blot. GAPDH was serviced as a control. $^{**}p < 0.01$ verses NG; $^{\#\#}p < 0.01$ verses HG, n = 6.

3.5 SR3335 promotes necroptosis in high glucose stimulated cardiac fibroblasts

A significant release of cellular content following cell injury can trigger necroptosis, thereby aggravating cell damage and promoting cell proliferation (Zhou et al., 2024). This current work demonstrated that RIPK1 and RIPK3 expressions, two hallmark proteins associated with necroptosis, were enhanced after stimulation by high glucose. This effect was further augmented by SR3335 (Figure 5), suggesting that SR3335 promoted necroptosis in high glucose stimulated cardiac fibroblasts.

3.6 SR1078 attenuates cell injury and proliferation in high glucose stimulated cardiac fibroblasts

The aforementioned data verified that RORα antagonist SR3335 promoted oxidative stress and necroptosis to

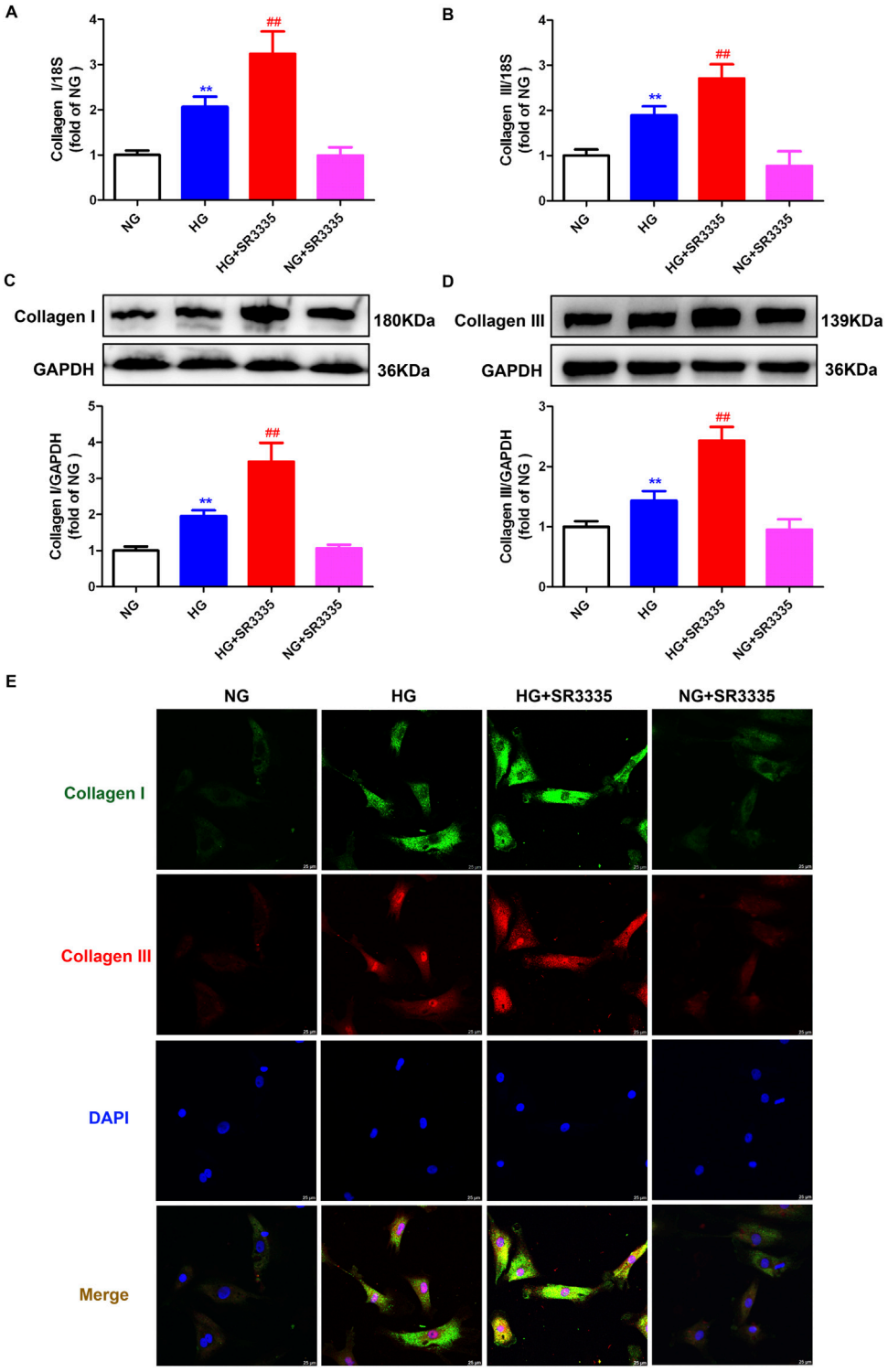

FIGURE 3
SR3335 enhanced collagen synthesis in high glucose stimulated cardiac fibroblasts. After pre-administration with SR3335 (20 μM) for 4 h, the cardiac fibroblasts were stimulated with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for 48 h **(A, B)** The mRNA expressions of Collagen I and Collagen III were measured by Real-time PCR. **(C, D)** The protein expressions of Collagen I and Collagen III were measured by Western blot. GAPDH was serviced as a control. **(E)** Collagen I and collagen III were immunofluorescence stained with Alexa Fluor 488 (green) and Cy3 (red) conjugated IgG, respectively. The nuclei were stained with DAPI (blue). Bar = 25 μm $^{**}p < 0.01$ verses NG; $^{\#\#}p < 0.01$ verses HG, n = 6.

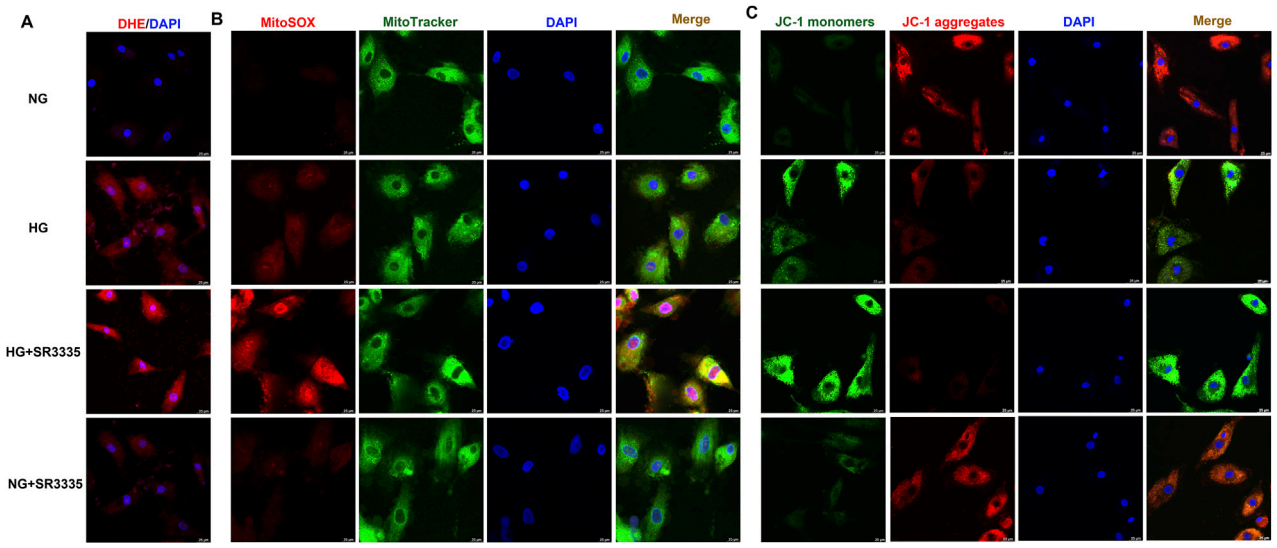

FIGURE 4
SR3335 facilitated oxidative stress in high glucose stimulated cardiac fibroblasts. After pre-administration with SR3335 (20 μM) for 4 h, the cardiac fibroblasts were stimulated with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for 48 h **(A)** DHE staining with red fluorescent was performed to measure superoxide anion production in cardiac fibroblasts. Bar = 25 μm. **(B)** MitoSOX staining with red fluorescent was performed to measure Mitochondrial ROS production. MitoTracker with green fluorescent was stained to co-localize Mitochondria. Bar = 25 μm. **(C)** Mitochondrial membrane potential was measured by JC-1 staining. Bar = 25 μm.

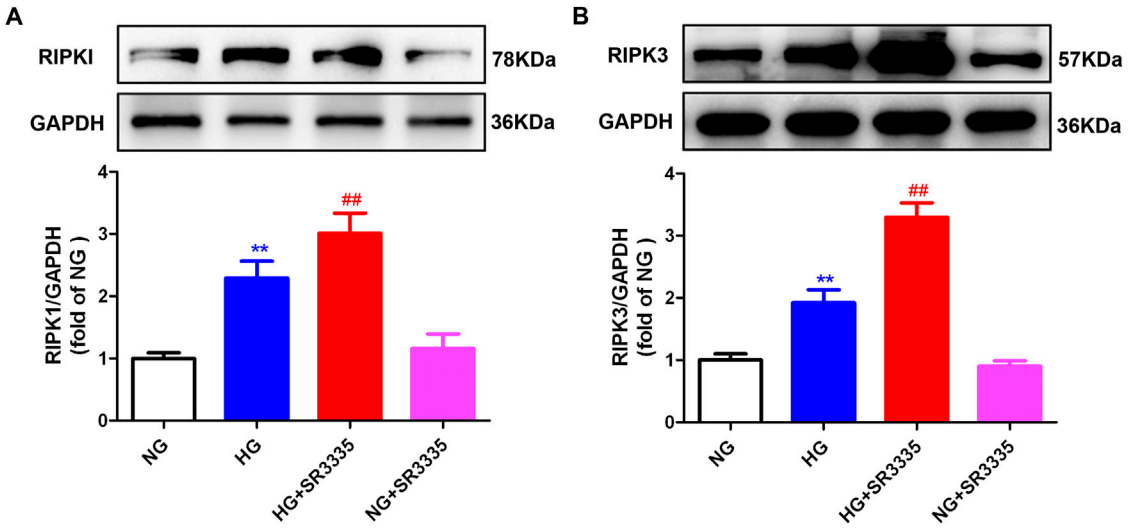

FIGURE 5
SR3335 promoted necroptosis in high glucose stimulated cardiac fibroblasts. After pre-administration with SR3335 (20 μM) for 4 h, the cardiac fibroblasts were stimulated with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for 48 h. Expression of RIPK1 **(A)** and RIPK3 **(B)** protein was measured by Western blot. GAPDH was serviced as a control. $^{**}p < 0.01$ verses NG; $^{\#\#}p < 0.01$ verses HG, n = 6.

accelerate proliferation after high glucose stimulation in cardiac fibroblasts. Nonetheless, the potential of RORα agonists to resist proliferation in cardiac fibroblasts under similar conditions remains to be elucidated. Our study demonstrated that RORα agonist SR1078 at different concentration (5 μM, 10 μM and 20 μM) significantly reduced LDH release in the medium, elevated the cellular ATP production and decreased OD value in cardiac fibroblasts with high glucose stimulation (Figures 6A–C). These findings suggested that SR1018 attenuated cell injury and decreased cell number in high glucose stimulated cardiac fibroblasts. Notably, SR1078 exhibited the most reversal effects at a concentration of 10 μM, which was chosen for subsequent experiments.

Additionally, the enhanced number of EdU positive cells were restrained by SR1078 in high glucose stimulated cardiac fibroblasts (Figures 6D, E). Moreover, elevated expressions PCNA and α-SMA

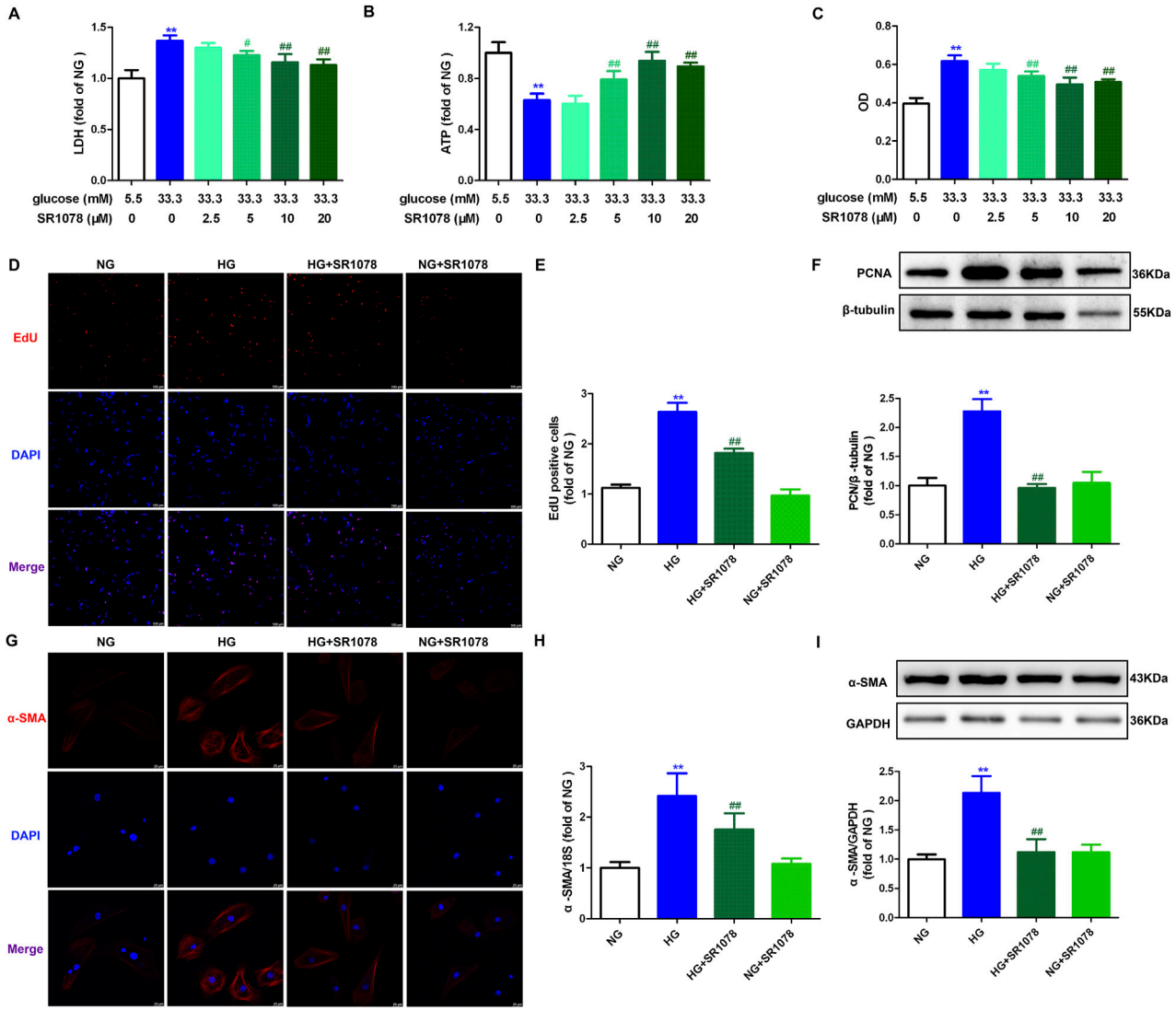

FIGURE 6
SR1078 attenuated cell injury and proliferation in high glucose stimulated cardiac fibroblasts. **(A–C)** After pre-administration with different concentration of SR1078 (2.5 μM, 5 μM, 10 μM and 20 μM) for 4 h, the cardiac fibroblasts were stimulated with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for 48 h. LDH release in the medium **(A)**, cellular ATP in the cardiac fibroblasts **(B)**, and OD value obtained from CCK-8 assay **(C)** were measured. $^{**}p < 0.01$ verses 5.5 mM glucose; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ verses 33.3 mM glucose, n = 6. **(D, E)** After pre-administration with SR1078 (10 μM) for 4 h, the cardiac fibroblasts were stimulated with NG or HG for 48 h. EdU staining with red fluorescent was performed to evaluate cardiac fibroblasts proliferation. Bar = 100 μm. EdU positive cells were quantitatively analyzed. **(F)** The protein expression of PCNA was measured by Western blot. β-tubulin was serviced as a control. **(G)** α-SMA was immunofluorescence stained with Cy3 (red) conjugated IgG. The nuclei were stained with DAPI (blue). Bar = 25 μm. **(H)** The mRNA expression of α-SMA was measured by Real-time PCR. **(I)** The protein expression of α-SMA was measured by Western blot. GAPDH was serviced as a control. $^{**}p < 0.01$ verses NG; $^{\#\#}p < 0.01$ verses HG, n = 6.

were also suppressed by SR1078 in these cells (Figures 6F–I). Taken together, SR1078 attenuated cell proliferation in high glucose stimulated cardiac fibroblasts.

3.7 SR1078 reduces synthesis of collagen inHigh glucose stimulated cardiac fibroblasts

Real-time-PCR, Western blot, and immunofluorescence demonstrated that enhanced Collagen I and III Collagen syntheses in high glucose stimulated cardiac fibroblasts were suppressed by SR1078 (Figure 7), suggesting that SR1078 reduced synthesis of collagen in these cells.

3.8 SR1078 suppresses oxidative stress in high glucose stimulated cardiac fibroblasts

DHE staining showed that stronger red fluorescence was weakened by SR1078 in high glucose stimulated cardiac fibroblasts (Figure 8A), suggesting SR1078 inhibited cellular superoxide anion production in these cells. MitoSOX staining demonstrated that stronger MitoSOX fluorescence was attenuated

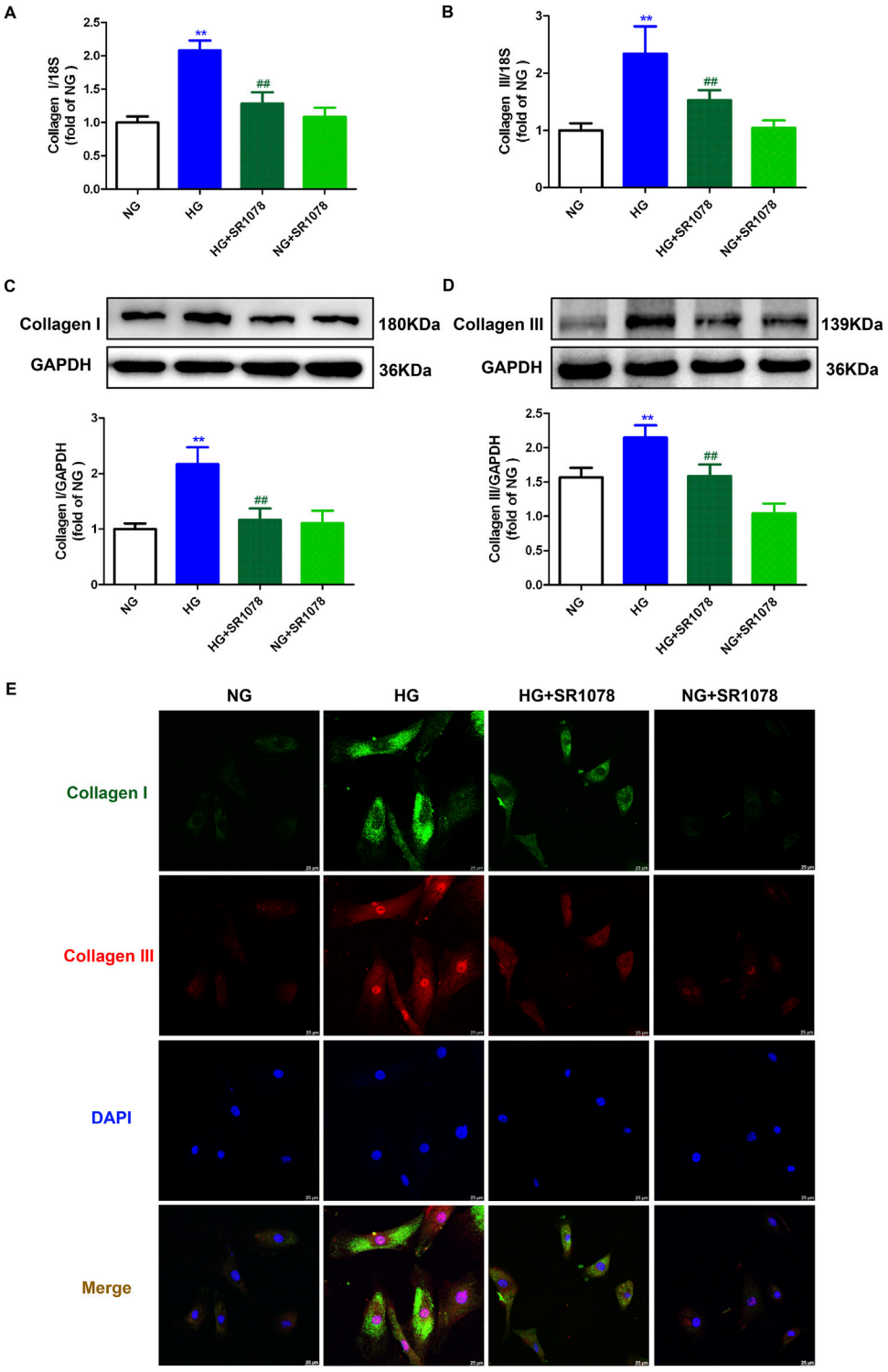

FIGURE 7
SR1078 reduced collagen synthesis in high glucose stimulated cardiac fibroblasts. After pre-administration with SR1078 (10 μM) for 4 h, the cardiac fibroblasts were stimulated with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for 48 h **(A, B)** The mRNA expressions of Collagen I and Collagen III were measured by Real-time PCR. **(C, D)** The protein expressions of Collagen I and Collagen III were measured by Western blot. GAPDH was serviced as a control. **(E)** Collagen I and collagen III were immunofluorescence stained with Alexa Fluor 488 (green) and Cy3 (red) conjugated IgG, respectively. The nuclei were stained with DAPI (blue). Bar = 25 μm $^{**}p < 0.01$ verses NG; $^{\#\#}p < 0.01$ verses HG, n = 6.

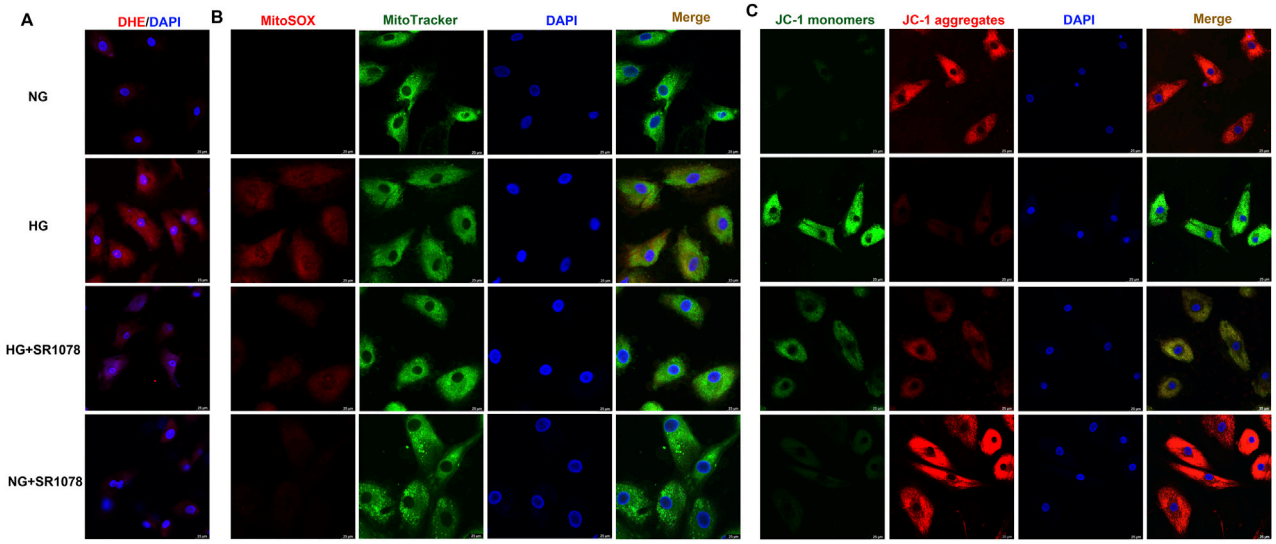

FIGURE 8
SR1078 suppressed oxidative stress in high glucose stimulated cardiac fibroblasts. After pre-administration with SR1078 (10 μM) for 4 h, the cardiac fibroblasts were stimulated with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for 48 h **(A)** DHE staining with red fluorescent was performed to measure superoxide anion production in cardiac fibroblasts. Bar = 25 μm. **(B)** MitoSOX staining with red fluorescent was performed to measure Mitochondrial ROS production. MitoTracker with green fluorescent was stained to co-localize Mitochondria. Bar = 25 μm. **(C)** Mitochondrial membrane potential was measured by JC-1 staining. Bar = 25 μm.

by SR1078 in high glucose stimulated cardiac fibroblasts (Figure 8B), suggesting SR1078 suppressed mitochondrial ROS production in this context. JC-1 staining indicated that stronger green fluorescence was alleviated, while weaker red fluorescence was strengthened by SR1078 in high glucose stimulated cardiac fibroblasts (Figure 8C), suggesting that mitochondrial membrane potential was enhanced by SR1078. Taken together, SR1078 suppressed oxidative stress in high glucose stimulated cardiac fibroblasts.

3.9 SR1078 alleviates necroptosis in high glucose stimulated cardiac fibroblasts

Western blot showed that the increased RIPK3 and RIPK1 expressions were reduced by SR1078 in high glucose stimulated cardiac fibroblasts (Figure 9), suggesting SR1078 alleviated necroptosis.

4 Discussion

Our present study firstly investigated the effects and potential mechanisms underlying high glucose-stimulated cardiac fibroblast proliferation in the context of RORα antagonist and agonist. The data verified that activation of RORα, through the inhibition of necroptosis, exerts protective effects against cell proliferation, thereby proposing a novel approach to alleviate DC. Nonetheless, several limitations are present in the current study. Firstly, the level of RORα mRNA or protein in the myocardium of diabetic patients was not detected in the present study. Secondly, neonatal rat cardiac fibroblasts may not serve as an optimal model for assessing diabetes-induced alterations. Utilizing primary cardiac fibroblasts derived from diabetic adult mice would provide valuable insights into the roles of RORα in the pathological process of DC. Thirdly, in addition to high glucose, elevated fatty acids and insulin are also prevalent in the context of diabetes. High glucose alone may not sufficiently replicate the conditions associated with type 2 diabetes. Further studies should consider stimulation with high fatty acid and/or high insulin to more accurately reflect the diabetic environment.

Diabetes mellitus, a metabolic disorder, arises from insulin secretion deficiency or insulin dysfunction. In 2021, it was estimated that approximately 537 million individuals aged 20–79 were living with diabetes globally, with projections indicating an increase to 784 million by 2045 (Zhang S. et al., 2023; Huo et al., 2023). Chronic diabetes can cause various complications including nephropathy, retinal disorders, cardiovascular damage and peripheral neuropathy. Among these complications, DC is distinguished by its unique pathophysiological mechanisms, including early-stage abnormalities in diastolic function of the heart, ultimately progressing to clinical heart failure even in absence of coronary artery disease, hypertension and dyslipidemia (Seferović et al., 2024). The potential pathophysiological contributors to DC include immune dysfunction, impaired nutrient-sensing signaling, insulin resistance, cardiac inflammation, oxidative stress, subcellular component (primarily mitochondria) abnormalities, inappropriate activation of the renin-angiotensin system, and obesity (Huo et al., 2023; Zhang C. et al., 2024; Dhar et al., 2023; Hsuan et al., 2023). Collectively, these factors facilitate interstitial fibrosis of cardiac tissue, increase cardiac stiffness, and lead to subsequent systolic dysfunction, ultimately resulting in heart failure (Pan et al., 2023; Cheng et al., 2023). Despite the availability of various strategies to effectively manage blood glucose levels, the incidence of DC remains high, and progression to heart failure cannot be entirely prevented in certain patients (Parry et al., 2015; Kim et al., 2022). Consequently,

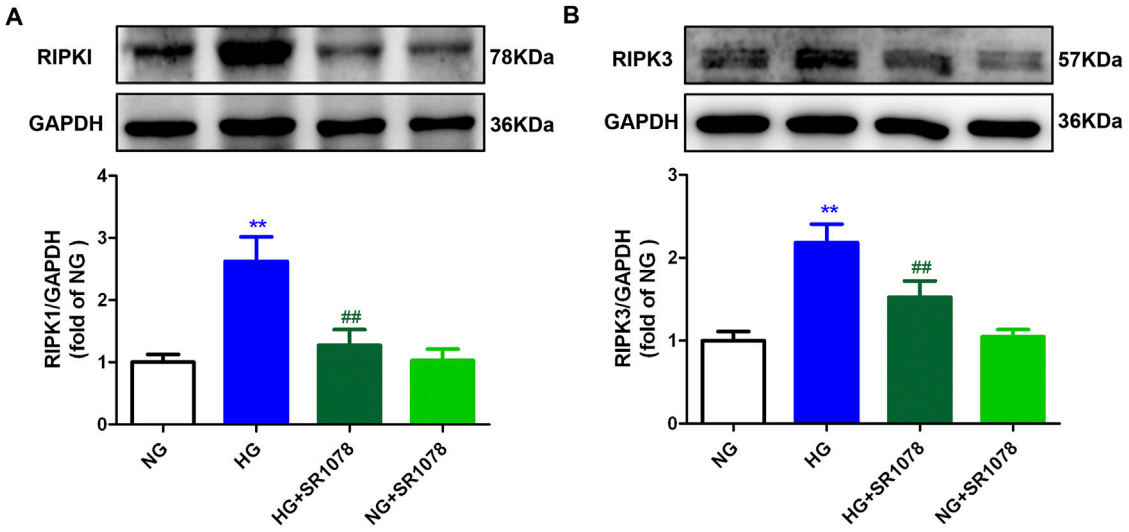

FIGURE 9
SR1078 alleviated necroptosis in high glucose stimulated cardiac fibroblasts. After pre-administration with SR1078 (10 μM) for 4 h, the cardiac fibroblasts were stimulated with normal glucose (NG, glucose 5.5 mM and mannitol 27.8 mM) or high glucose (HG, 33.3 mM) for 48 h. Expression of RIPK1 **(A)** and RIPK3 **(B)** protein was measured by Western blot. GAPDH was serviced as a control. $^{**}p < 0.01$ verses NG; $^{\#\#}p < 0.01$ verses HG, n = 6.

seeking new means to delay or even halt the progression of DC is crucial for reducing the incidence and mortality associated with cardiovascular adverse events in individuals with diabetes.

Myocardial fibrosis, mainly resulting from an imbalance between ECM degradation and production, represents a significant manifestations of DC (Levick and Widiapradja, 2020). Myocardial fibrosis further exacerbates cardiac dysfunction and leads to distinct cardiovascular diseases. Activated myofibroblasts and fibroblasts serve as the principal sources of matrix proteins and act as the primary cellular effectors of myocardial fibrosis. Additionally, cardiomyocytes, vascular cells and immune cells can also attain fibrotic phenotypes in response to stress, ultimately causing the activation of fibroblast populations (Aguado-Alvaro et al., 2024). Various cytokines, including interleukin (IL)-1, IL-4, IL-6, IL-10, and tumor necrosis factor-α, along with neurohumoral pathways, and fibroblast growth factors such as platelet-derived growth factor and transforming growth factor-β can facilitate fibrotic signaling cascades by activating the downstream signaling pathways and interacting with surface receptors (Wang et al., 2023). Our present experiments confirmed that under high glucose (33.3 mM) stimulation, cardiac fibroblasts number was increased, collagen secretion was elevated, and cell proliferation was significantly accelerated, indicating a marked cardiac fibroblasts activation with pronounced fibrotic characteristics. Therefore, there is an urgent need for timely intervention to mitigate the progression of DC.

RORα, a member of orphan nuclear receptor family, exhibits higher tissue specificity and is involved in regulating processes of immunity, inflammation, circadian rhythms, and metabolic homeostasis. Notably, substantial evidence indicates that RORα influences both pathological and physiological within the cardiovascular system, including myocardial hypertrophy, hypertension, atherosclerosis, myocardial ischemia/reperfusion injury, and hypoxia or ischemia (Chen et al., 2023). Prior studies have demonstrated that RORα expression is downregulated in high glucose stimulated cardiomyocytes, as well as in the myocardium of diabetic mice. In streptozocin (STZ)-induced RORα knockout mice, exacerbated myocardial remodeling and cardiac dysfunction were observed, indicating a protective role for RORα against DC (Zhang S. et al., 2023; Zhao et al., 2017). However, the precise function of RORα in fibrosis remains to be elucidated. Furthermore, molecular mechanism underlying the transcriptional regulation pattern of RORα under a high glucose environment is still unclear. We previously found that hydrogen sulfide increased the expression of E2F transcription factor 1 (E2F1), promoted E2F1 binding to the promoter of RORα, increased RORα transcription, and eventually alleviated cell damage in cardiomyocytes with high glucose stimulation via a RORα-dependent manner (Zhang S. et al., 2023). Nevertheless, as a gasotransmitter, the potential of hydrogen sulfide as an effective regulatory molecule for RORα is still not optimistic. Fortunately, recent discoveries of endogenous ligands of RORα suggest that pharmacological modulation of RORα expression or activity through the use of exogenous agonists or antagonists may allow for the precise control of RORα within a physiological range, thereby maintaining the homeostasis of the cardiovascular system (Solt et al., 2011). RORα antagonist SR3335, which is actually one selective RORα inverse agonist of RORα, has demonstrated a substantial capacity to inhibit RORα activity upon its binding (Liang et al., 2021). In contrast, SR1078 functions as a RORα agonist, directly interacting with the ligand-binding domain of RORα, which increases the transcriptional activity of RORα target genes (Moreno-Smith et al., 2021). The present study found that inhibiting RORα activity further aggravated cell damage, increased cell number, upregulated collagen I and collagen III secretion, enhanced EdU-staining positive cells, and elevated PCNA and α-SMA expressions in high glucose stimulated cardiac fibroblasts. Conversely, activating RORα activity mitigates the above manifestations, indicating that adjusting RORα activity through pharmacological means represents an effective strategy for regulating cardiac fibroblasts proliferation with high glucose stimulation.

The pathogenic mechanism underlying DC remain incompletely elucidated, with associations identified between DC and cardiac metabolic disorders, microvascular dysfunction, endoplasmic reticulum stress, inflammation, mitochondrial dysfunction, oxidative stress, impaired Ca^{2+} handling, and apoptosis (Hsuan et al., 2023). Moreover, as of now, the precise protective mechanism of RORα on cardiovascular system is yet to be fully clarified (Chen et al., 2023). Necroptosis is a novel and unique form of regulated and programmed cell death (Newton et al., 2024). Necroptosis is primarily governed by receptor-binding protein kinases (notably RIPK1 and RIPK3). This process involves the sequential activation and phosphorylation of key proteins of necroptosis, culminating in the disruption of plasma membrane integrity and the amplification of inflammatory responses, which contribute to cellular dysfunction (Yuan and Ofengeim, 2024). Specifically, necroptosis may be triggered by various stimuli, predominantly tumor necrosis factor (TNF). In the absence of caspase-8, RIPK1 undergoes auto-phosphorylation at its serine/threonine residue sites and combines to RIPK3 through RIP homotypic interaction motif (RHIM), forming a RIPK1-RIPK3 complex named as necrosome. This complex subsequently recruits and activates the downstream protein MLKL, which is then phosphorylated. Then, phosphorylated MLKL translocates to the cell membrane, resulting in membrane rupture and the release of damage-associated molecular patterns (DAMPs), thereby mediating the occurrence of necroptosis (Aguado-Alvaro et al., 2024; Chaouhan et al., 2022). Importantly, as DC progresses, mitochondrial dysfunction is further exacerbated to enhance oxidative stress, which in turn promotes the process of necroptosis and the release of cellular contents to speed up cardiac fibroblasts' proliferation and synthesis of collagen. Under high glucose stimulation, mitochondrial membrane permeability alters to enhance ROS production and the occurrence of necroptosis, thereby increasing the possibility of oxidative stress burs. In turn, ROS prone to leading to mitochondrial dysfunction and cardiac fibroblasts proliferation, accelerating the myocardial fibrosis during the process of DC (Song et al., 2021; Gong et al., 2022; Zhang S. et al., 2023). That is to say, the mechanism of excessive cardiac fibroblasts proliferation in DC might be attributed to oxidative stress and necroptosis. Our research verified that the inhibition of RORα activity resulted in enhanced oxidative stress levels, reduced mitochondrial membrane potential, promoted necroptosis, and subsequently accelerated cardiac fibroblasts proliferation with high glucose stimulation. Conversely, the enhancement of RORα activity reversed the above manifestations, suggesting that necroptosis and RORα-mediated inhibition of oxidative stress may constitute a protective mechanism regulating the proliferation of cardiac fibroblasts. Interestingly, our study showed that SR1078 attenuated necroptosis while simultaneously inhibiting cell proliferation, a seemingly paradoxical outcome. However, it is plausible that following necroptosis, cardiac fibroblasts release additional cellular contents due to membrane rupture, thereby promoting the proliferation of cardiac fibroblast (Zhang et al., 2021). Thereby the inhibitory effects of SR1078 on cardiac fibroblast proliferation may be ascribed to its capacity to alleviate necroptosis.

In summary, RORα antagonist SR3335 promoted cell injury and proliferation, enhanced collagen synthesis, facilitated necroptosis and oxidative stress in high glucose stimulated cardiac fibroblasts. In contrast, RORα agonist SR1078 attenuated cell injury and proliferation, reduced collagen synthesis, alleviated necroptosis, and suppressed oxidative stress in high glucose stimulated cardiac fibroblasts. Our present study proposed RORα as a novel therapeutic target for addressing high glucose-induced cardiac fibroblasts proliferation, which is beneficial to clarify some other ideal therapeutic implication for DC.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the study was conducted according to National Institutes of Health guidelines for the Care and Use of Laboratory Animals, and approved by Committee of Nantong University (approval no. S20210227-011 on 27 February 2021). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WS: Data curation, Formal Analysis, Investigation, Methodology, Writing–original draft. QZ: Data curation, Formal Analysis, Investigation, Methodology, Writing–original draft. DS: Data curation, Methodology, Writing–original draft. DC: Data curation, Software, Writing–original draft. YC: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing–review and editing. GM: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Deciphering the role of IGFBP5 in delaying fibrosis and sarcopenia in aging skeletal muscle: therapeutic implications and molecular mechanisms

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Introduction: Sarcopenia is a condition characterized by the loss of muscle fibers and excessive deposition of extracellular matrix proteins. The interplay between muscle atrophy and fibrosis is a central feature of sarcopenia. While the mechanisms underlying skeletal muscle aging and fibrosis remain incompletely understood, cellular senescence has emerged as a key contributor. This study investigates the role of D-galactose (D-gal) in inducing fibroblasts senescence and skeletal muscle fibrosis, and aims to find the key regulator of the process to serve as a therapeutical target.

Methods: To discover the role of D-gal in inducing cellular senescence and fibrosis, the senescence markers and the expression of fibrosis-related proteins were assessed after introducing D-gal among fibroblasts, and muscle strength and mass. The severity of muscle atrophy and fibrosis were also verified by using H&E staining and Masson trichrome staining after D-gal treatment via subcutaneous injection among mice. Subsequently, mRNA sequencing (RNA-seq) was performed and the differential expressed genes were identified between under D-gal or control treatment, to discover the key regulator of D-GAL-driven fibroblasts senescence and fibrosis. The role of the key regulator IGFBP5 were then validated in D-GAL treated IGFBP5-knockdown fibroblasts *in vitro* by analyzing the level of senescence and fibrosis-related markers. And the results were further confirmed *in vivo* in IGFBP5-knockdown SAMP8 mice with histological examinations.

Results: D-gal treatment effectively induced cellular senescence and fibrosis in fibroblasts, as well as skeletal muscle atrophy, fibrosis and loss in muscle mass and function in mice. IGFBP5 was identified as a key regulator of D-GAL induced senescence and fibrosis among fibroblasts using RNA-seq. And further validation tests showed that IGFBP5-knockdown could alleviate D-GAL-induced fibroblast cellular senescence and fibrosis, as well as the severity of muscle atrophy and fibrosis in SAMP8 mice.

Discussion: IGFBP5 emerging as a key regulator of D-GAL-induced fibroblast cellular senescence and fibrosis. The findings provide new insights into the

molecular mechanisms underlying age-related skeletal muscle fibrosis and highlight IGFBP5 as a potential therapeutic target. Further research is needed to validate these findings and explore related clinical applications.

KEYWORDS

skeletal muscle fibroblasts, skeletal muscle fibrosis, skeletal muscle aging, fibrosis, sarcopenia

Introduction

Sarcopenia, characterized by the loss of muscle mass and strength, and fibrosis, is a common health issues among the elderly, significantly impacting their mobility and overall health (Di Iorio et al., 2006; Tournadre et al., 2019). With the advent of the aging society, these concerns have garnered widespread attention. Skeletal muscle plays a crucial role in movement, metabolic balance, and heat generation (Argilés et al., 2016). Nevertheless, a range of abnormal health states, including long-term illnesses, malignancies, protracted infections, and the aging process, have the potential to upset the equilibrium between the synthesis and breakdown of muscle proteins. This disruption can subsequently result in the occurrence of muscle atrophy and fibrosis (Kirkendall and Garrett, 1998; Argilés et al., 2016). In the context of sarcopenia, fibrosis poses substantial detrimental effects on patients by escalating muscle stiffness and curtailing their physical activity levels (Argilés et al., 2016; Antar et al., 2023). The excessive accumulation of fibrous tissue can also interfere with the communication between muscle satellite cells and the surrounding cellular milieu, leading to a decline in their myogenic capabilities (Murphy et al., 2011; Serrano et al., 2011; Antar et al., 2023). Therefore, unveiling the mechanisms of fibrosis in aged muscle is fundamental for skeletal muscle health (Serrano et al., 2011; Liu et al., 2018).

Skeletal muscle fibroblasts and Fibro-Adipogenic Progenitors (FAPs) are both important for muscle repair and maintenance but have distinct roles(Molina et al., 2021; Chen et al., 2022; Chapman et al., 2016). Skeletal muscle fibroblasts primarily produce and remodel the extracellular matrix (ECM), supporting tissue structure and wound healing(Chapman et al., 2016; DeLeon-Pennell et al., 2020). In contrast, FAPs are specialized cells within skeletal muscle that aid regeneration by differentiating into adipocytes and fibroblasts in response to injury or disease(Molina, Fabre, and Dumont, 2021). FAPs secrete factors like IL-6 and WNT, which promote muscle repair and create a supportive environment for muscle stem cells (MuSCs) (Madaro et al., 2018; Riparini et al., 2022; Parker and Hamrick, 2021). Skeletal muscle fibroblasts maintain ECM and provide structural support(Gillies and Lieber, 2011), whereas FAPs have a dual role: they aid regeneration by supporting MuSCs but can also contribute to fibrosis or fat buildup in diseases like Duchenne Muscular Dystrophy (DMD) (Chen et al., 2022; Parker and Hamrick, 2021). Additionally, FAPs have broader differentiation potential, allowing them to become adipocytes or fibroblasts, influencing the balance between repair and fibrosis(Judson et al., 2017; Molina et al., 2021). Both cells contribute to muscle health and repair.

Regarding the various pathways involved in muscle fibrosis, oxidative stress and inflammation are significant for muscle atrophy and extracellular matrix (ECM) deposition, capable of activating numerous signal pathways, including the ubiquitin-proteasome system, autophagy-lysosome system, and mTOR (Nishikawa et al., 2021; Gambini and Stromsnes, 2022; Antar et al., 2023). The IGF (insulin-like growth factor) signaling pathway plays a crucial role in skeletal muscle fibrosis and sarcopenia (Clemmons, 2009; Ye et al., 2013; Frost and Lang, 2012; Forbes, Blyth, and Wit, 2020). Among them, IGF-1 is a key factor in this pathway (Hayashi et al., 2004). IGF-1 inhibits inflammation through the Ras/PI3K/IKK/NF-κB pathway, reducing pro-inflammatory cytokine production and promoting tissue repair 6. Chronic inflammation often leads to tissue atrophy due to prolonged cytokine exposure (e.g., TNF-α, IL-6), which disrupts cellular homeostasis. By suppressing NF-κB activation, IGF-1 mitigates inflammatory damage, indirectly preventing muscle or atrophy caused by persistent inflammation (Zhang et al., 2024; Feng et al., 2022; Stitt et al., 2004). Besides, IGF promotes muscle cell growth and differentiation by binding to the IGF-1 receptor and activating the downstream PI3K/Akt/mTOR signaling pathway, thus combating muscle atrophy (Yoshida and Delafontaine, 2020). There may also be an interaction between IGF-1 and TGF-β1, which together influence the process of skeletal muscle fibrosis (Danielpour and Song, 2006; Kjaer et al., 2006).

Insulin-like growth factor binding proteins (IGFBPs) are a group of proteins that bind to insulin-like growth factors (IGFs), finely regulating their biological activity, distribution, and mode of action (Kelley et al., 1996; Baxter, 2023). The IGFBP family includes at least seven different proteins (IGFBP-1 to IGFBP-7), which share similarities in structure and function but also possess some unique characteristics and roles (Kelley et al., 1996; Hwa et al., 1999; Allard and Duan, 2018). IGF binding protein 5 (IGFBP5), as a regulator of IGF-1, can influence the biological activity of IGF-1 and, consequently, the regenerative capacity of muscles (Hwa et al., 1999; Beattie et al., 2006). The biological functions of IGFBP5 remain a subject of debate in scientific research (Duan and Allard, 2020; Waters et al., 2022). Certain investigations propose that IGFBP5 could trigger senescence via the STAT3 pathway or pathways associated with P53. In contrast, other studies observe an increase in IGFBP5 levels in cells that have undergone senescence due to radiation or kinase inhibitor treatment (Alessio et al., 2024). Additionally, some reports associate reduced IGFBP5 expression with senescence (Nojima et al., 2022). The varied and sometimes conflicting biological functions ascribed to IGFBP5 might be due to its participation in multiple signaling pathways (Duan and Allard, 2020). However, the role of IGFBP5 in sarcopenia remains to be elucidated.

In the current study, a series of experiments were conducted *in vitro* and *in vivo* to undermine the mechanisms of skeletal muscle fibrosis under sarcopenic condition (Park et al., 2017; Lim and Frontera, 2023; Nojima et al., 2022). Relying on sequencing and

verifications, IGFBP5 was noticed to be significantly upregulated in aged fibroblasts. Subsequently, we found that reducing the expression of IGFBP5 partially alleviated fibrosis in sarcopenic muscle by moderately potentiating the effects of IGF-1, providing clue to the development of novel anti-fibrosis therapies in sarcopenia.

Materials and methods

Cell culture and induction

Mouse skeletal muscle fibroblast cells (NOR-10) were purchased from the Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained in a humidified incubator at 37°C and 5% CO2 atmosphere. FAPs were isolated from skeletal muscle tissues according to the previous study and cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine (Kang et al., 2024). For the induction of senescence from fibroblast and FAPs, cells were incubated in a D-gal concentration of 20 mg/mL for 3 days, while the negative control (NC) group was treated with an equal amount of PBS.

siRNA structure and design and transfection

Small interfering RNA (siRNA) molecules were designed to specifically target the mRNA of the gene, IGFBP5, to induce RNA interference (RNAi) and achieve gene silencing. The sequences of the siRNA were designed based on the mRNA sequence of IGFBP5 (GenBank Accession No. NM_010518). Cells were seeded in 24-well plates. When cells reached 30%–50% confluence, siRNA was transfected using Lipofectamine 2000. siRNA and Lipofectamine 2000 were diluted in Opti-MEM I, mixed, and incubated for 20 min at room temperature. The complex was added to the cells, incubated at 37°C in 5% CO_2, and after 4–6 h, replaced with complete medium containing 10% FBS. Cells were harvested 48 h later for analysis.

Senescence-associated β-galactosidase(SA-β-gal) staining

The protocol was consistent with the previous study (Shahini et al., 2021), that n = 3 biological replicates were used. Digital camera was used to capture images of the stained cells. ImageJ (Version 1.54 m) was used to count the number of blue-stained senescent cells and the total number of cells in each image.

Transcriptome sequencing (RNA sequencing) and bioinformatic analysis

Raw data was obtained with Feature Extraction software 10.7 and normalized (GSE277119). For fibroblasts induced by D-gal and control group samples (n = 3 in each group), sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA). Raw sequencing reads were processed using FastQC (version 0.11.9) to assess the quality of the sequencing data. Low-quality reads (Phred score <20) were trimmed using Trimmomatic (version 0.39). High-quality reads were retained for further analysis. Genes were considered differentially expressed if they met the following criteria: an adjusted p-value (FDR) < 0.05 and a log2 fold change (log2FC) ≥ 1 or ≤ −1. GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis were performed as the protocol according to the previous study (Sun et al., 2018).

Animals

Healthy male C57BL/6 mice, 10 in total, 6–8 weeks old, with body weights ranging from 20 to 24 g, purchased from Cyagen Biosciences. Mice was randomly divided into experimental and control groups (n = 5), with the experimental group mice receiving D-gal via subcutaneous injection at a dose of 200 mg/kg/d for 8 consecutive weeks. The control group is injected with an equivalent amount of normal saline. The SAMP8 (senescence-accelerated mouse-prone 8) model was chosen for its accelerated aging phenotype, which mimics age-related fibrogenic processes in skeletal muscle. SAMR1 (senescence-accelerated mouse-resistant 1) was used as a control. Healthy male SAMP8, 10 in total, and SAMR1, 5 in total, 24 weeks old, with body weights ranging from 42 to 45 g, purchased from Hangzhou Ziyuan Experimental Anmial Technology Co. Mice was randomly divided into experimental and control groups (n = 5), with the experimental group mice receiving siRNA dissolved in normal saline via tail vein injection at a dose of 100 umol/ml twice a week for 4 consecutive weeks. The control group and the SAMR1 group are injected with an equivalent amount of normal saline. The mice were housed separately, and had sufficient space to meet the growth and behavioral needs of the animals, provided with feed and distilled water. Bedding was kept clean with good air circulation. 1 day after the last injection, mice were placed sacrificed with carbon dioxide,and the CO2 flow was 30% vessel volume per minute to ensure that the animal gradually became consciousness and eventually died before reaching a concentration that could cause pain. The lower limbs of the mouse were carefully amputated, and the muscles (gastrocnemius, tibialis anterior, quadriceps) were dissected away from the bone and surrounding tissues. Department of Shanghai Chedun Experimental Animal Ethics Committee provided full approval for this research (AD2024092).

Western blot

Western blot (WB) was performed following the procedures in a previous publication(n = 3) (Zhang et al., 2023), with primary antibodies identified by the following catalog numbers:

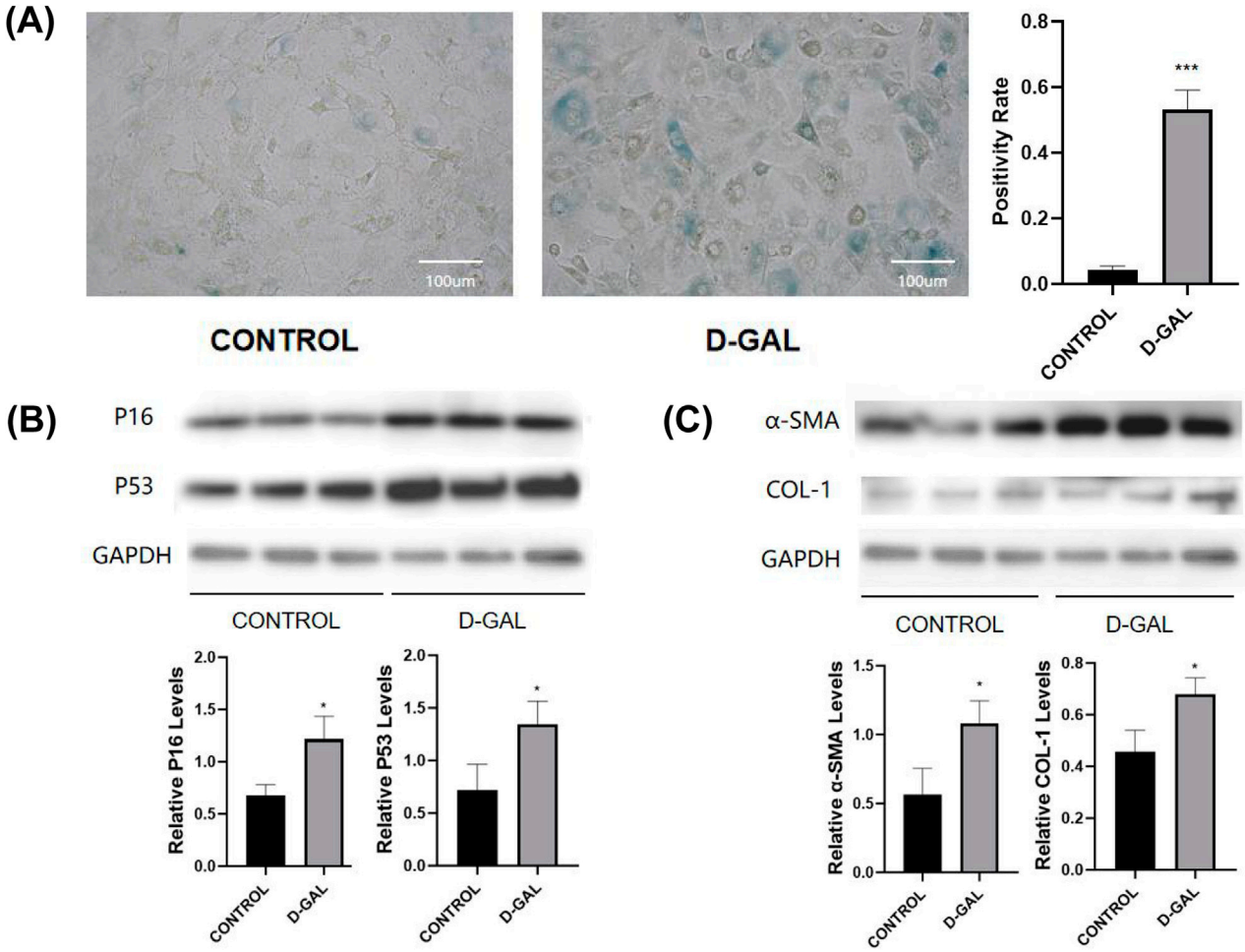

FIGURE 1
(A) SA-β-gal staining of NOR-10 (CONTROL vs. D-GAL) and the statistical analysis **(B)** Western blot of senescence markers (P16, P53) and the statistical analysis **(C)** Western blot of fibrosis-related markers (α-SMA, COL-1) and the statistical analysis (compared to control group, *p < 0.05, **p < 0.01, ***p < 0.001).

P16(10883-1-AP), P53(10442-1-AP), IGFBP5(55205-1-AP), COL-1(14695-1-AP), α-SMA(14395-1-AP).

PCR

The PCR was performed according to the protocols established in a previous study (Mollica, 2010).

HE, MASSON and immunofluorescence staining

All staining protocols were adhered to as described in previous studies(n = 3) (Wang et al., 2017; Van De Vlekkert et al., 2020; Esper et al., 2023), with primary antibodies identified by the following catalog numbers: IGFBP5(55205-1-AP), α-SMA(14395-1-AP), IGF-1(28530-1-AP), TGF-β(26155-1-AP). Fluorescence intensity was measured using a fluorescence microscope and normalized to control.

Statistical analysis

All data are presented as mean ± standard deviation (SD). GraphPad Prism 9.4.1 software (GraphPad, CA, USA) was used for statistical analysis and image construction. For comparisons between two groups, Student's t-test and Paired Samples t-test was used. For comparisons among multiple groups, one-way ANOVA was employed, followed by *post hoc* Tukey's test for pairwise comparisons. All statistical tests were two-tailed, and p-values less than 0.05 were considered statistically significant.

Results

D-gal-induced skeletal muscle fibrosis characteristics

D-gal is a chemical that commonly induces cellular senescence (Azman and Zakaria, 2019). It leads to mitochondrial damage and a decline in energy metabolism, which are associated with aging

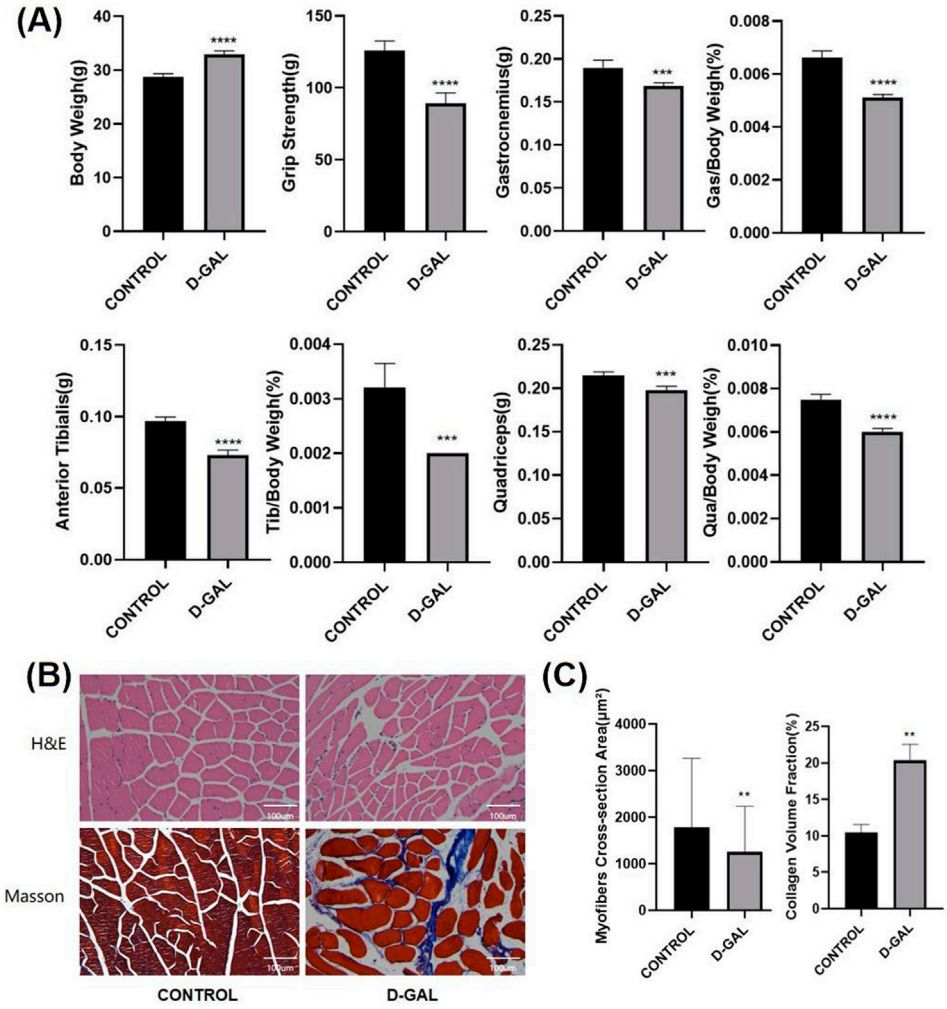

FIGURE 2
(A) Body weight, muscle strength, and the weight of lower limb skeletal muscles (gastrocnemius, tibialis anterior, quadriceps) and their percentage of the body weight of C57BL/6 mice(CONTROL vs. D-GAL) **(B)** The HE and Masson staining of limb skeletal muscle(CONTROL vs. D-GAL) **(C)** the statistical analysis of, myofiber cross sectional area (CSA) and collagen volume fraction (CVF) (compared to control group, *p < 0.05, **p < 0.01, ***p < 0.001, **** *p* < 0.0001).

(Parameshwaran et al., 2010). The first, NOR-10 fibroblasts were treated with D-gal, resulting in significant increases in the protein levels of senescence markers p16 and p53, and SA-β-gal staining confirmed cellular aging (Figures 1A, B). This confirms the successful establishment of an aging model in mouse skeletal muscle fibroblasts post D-gal induction. Western blot of fibrosis-related markers (α-SMA, COL-1) were then detected (Figure 1C). Not surprisingly, a significant elevation was observed after D-gal induction.

According to the research methods in previous articles, D-gal is also widely used to induce skeletal muscle aging (Tian et al., 2022). After 8 weeks of D-gal injection, comparisons were made in terms of body weight, muscle strength, and the weight of lower limb muscles (gastrocnemius, tibialis anterior, quadriceps) and their percentage of the body weight (Figure 2A). The results demonstrated that the D-gal induced group had a significant decrease in muscle strength and slight decline in the weight of individual lower limb muscles. HE and Masson showed a significant reduction in fiber cross-sectional area with, on the other hand, a noticeable increase in ECM in the D-gal induced group (Figures 2B, C).

Identifying IGFBP5 in D-gal-induced skeletal muscle sequencing analysis

To explore the mechanisms underlying the fibrosis of skeletal muscle during its aging process, we performed sequencing on skeletal muscle fibroblasts that had been induced to aging. Compared to the sequencing results of the control group, there were significant differences in mRNA expression (Figures 3A, B). Enrichment analysis was conducted using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and pathways related to skeletal muscle fibrosis were identified as being of particular interest in cellular processes, regulation of biological processes, and metabolism, such as transporter activity, translation regulator activity, ECM-receptor interactions and cell growth and

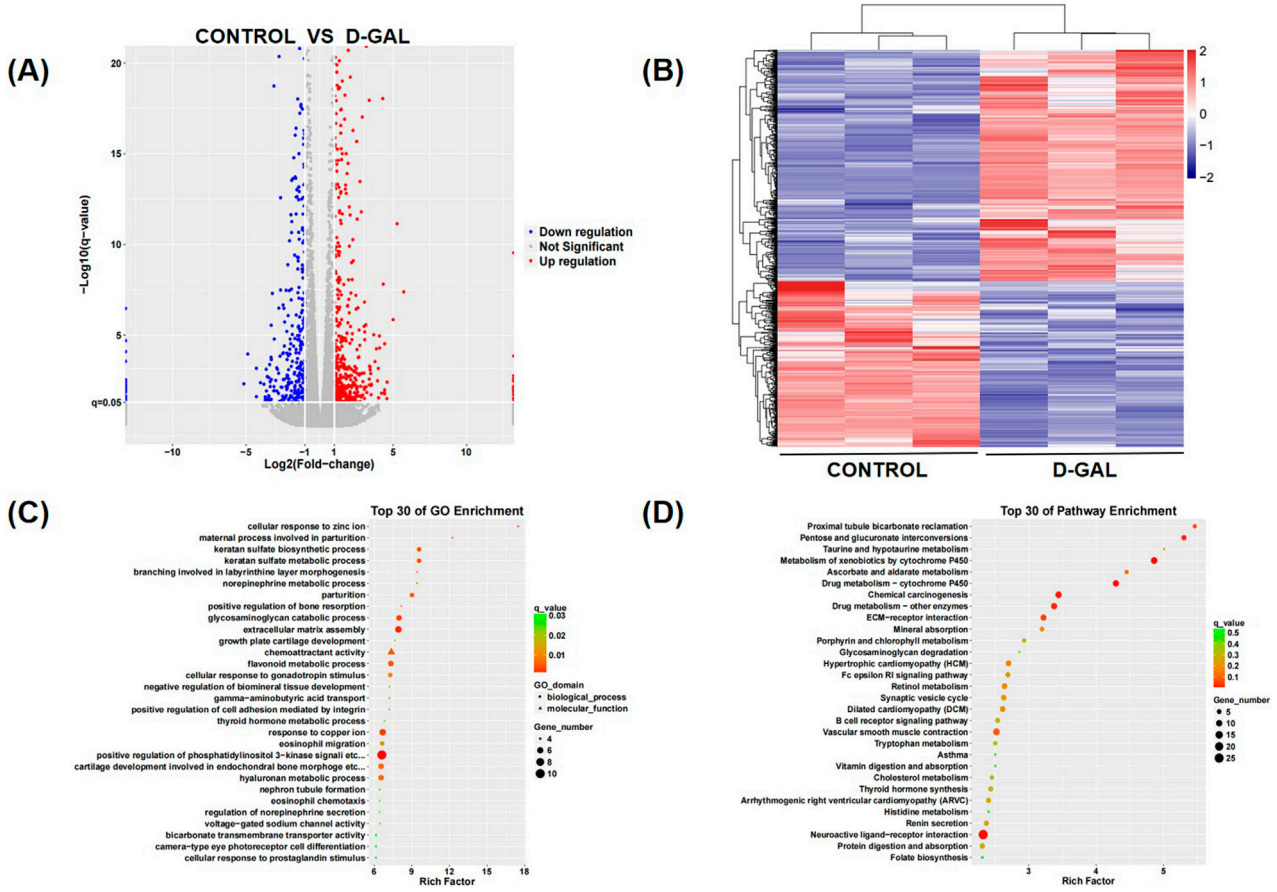

FIGURE 3
(A) The heatmap of differential genes of the sequencing results **(B)** The volcano plot of differential genes of the sequencing results **(C)** The GO enrichment of differential genes of the sequencing results **(D)** The KEGG enrichment of differential genes of the sequencing results.

death (Figures 3C, D). Differential gene expression was selected by data processing, including both upregulated and downregulated genes (Supplementary Figures 1A–D). By further analyzing the gene enrichment results from KEGG and GO, and conducting a search and study of relevant literature and currently published research articles, the IGFBP5 gene has been identified. IGFBP5 was identified as a key candidate gene of differentially expressed genes (DEGs) and the expression of it was significantly upregulated compared to controls (log2 fold change > 2, $p < 0.01$).

IGFBP5 is highly expressed in the senescence

The protein level in cells induced by D-gal of insulin-like growth factor binding protein 5 (IGFBP5) has also exhibited a noticeable elevation, suggesting its potential role in the fibrotic process (Supplementary Figure 2). Additionally, we performed PCR validation using FAPs cells. In FAPs induced by D-gal, the markers of senescence, fibrosis, and adipogenesis were all increased, along with an elevation in IGFBP5 (Supplementary Figure 3). This indicates that within the skeletal muscle aging model, the skeletal muscle not only shows characteristics of fibrosis but also an upregulation in the expression of IGFBP5, aligning with the sequencing results. Immunofluorescence staining in the D-gal-induced aging animal model has revealed a significant increase in the expression of α-SMA. Furthermore, IGFBP5 has shown a more pronounced and widespread distribution in skeletal muscle compared to the control group, indicating a possible association between IGFBP5 expression and the aging process in skeletal muscle (Supplementary Figure 4). These findings suggest that the D-gal-induced aging model is associated with a notable increase in skeletal muscle fibrosis and a high expression of IGFBP5, which may play a role in the fibrotic response to aging.

Knockout of IGFBP5 alleviates fibrosis in the aging model

To investigate the specific mechanisms of action of IGFBP5 at the cellular level, siRNA and plasmids were selected (Figures 4A, B). In NOR-10 cells induced by D-gal, protein level analysis revealed that the fibrosis level in fibroblasts decreased after the knockout of IGFBP5 (Figure 4C). Additionally, the senescence of cells with IGFBP5 knockout was significantly improved (Figure 4D). Moreover, SAMP8 mice were selected for the study. 24-week-old mice was chosen for the experiment, administering siRNA via tail vein

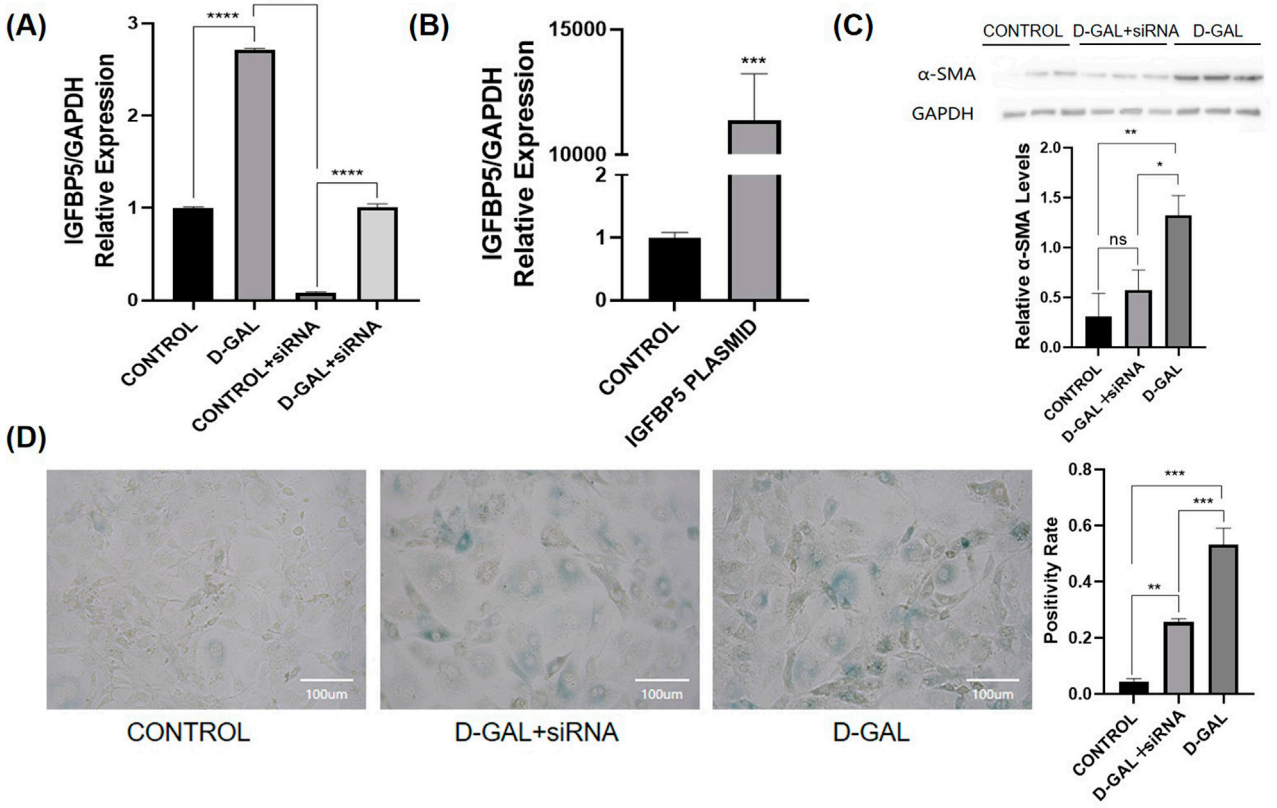

FIGURE 4
(A) The PCR of IGFGP5 (CONTROL vs. D-GAL vs. CONTROL + siRNA vs. D-GAL + siRNA); **(B)** The PCR of IGFBP5 (CONTROL vs. PLASMID) **(C)** Western blot of α-SMA and the statistical analysis **(D)** SA-β-gal staining of NOR-10 and the statistical analysis (CONTROL vs. D-GAL + siRNA vs. D-GAL) (compared to control group, *p < 0.05, **p < 0.01, ***p < 0.001, **** $p < 0.0001$).

injection for 4 weeks to knock down the expression of IGFBP5 in aging mice. At the end of the modeling, consistent with the previous text, the mice's body weight, muscle strength, and the weight of lower limb muscles (gastrocnemius, tibialis anterior, quadriceps) and their percentage related to mice weight were assessed (Figure 5A). The results demonstrated that in the IGFBP5 knockdown group, there was a moderate decrease in body weight, a significant improvement in muscle strength, and a noticeable increase in the weight of the lower limb muscles. The percentage was not as significantly improved, but there was a general upward trend. Tissue section staining with HE and Masson also showed that the degree of fibrosis in skeletal muscle was improved in mice with IGFBP5 knockdown. This was manifested as a significantly larger cross-sectional area of muscle fibers in the siRNA group compared to aging mice, improved gaps between muscle fibers, and relatively less connective tissue compared to aging mice, although it did not reach the condition of normal adult mice (Figures 5B, C).

IGFBP5 regulates skeletal muscle fibrosis through IGF-1

Immunofluorescence staining of muscle tissue from aging mice and mice with IGFBP5 knockout revealed that the expression of the fibrosis marker α-SMA was significantly reduced in mice with IGFBP5 knockout (Figure 6). Notably, IGFBP5 expression was also substantially decreased (Figure 6). This indicates that IGFBP5 can indeed alleviate skeletal muscle fibrosis. IGF-1 can affect TGF-β1 activity, a cytokine linked to fibrosis. It also regulates ECM buildup, key in muscle fibrosis. IGF-1 is the main route for IGFBP5's effects, with IGFBP5 impacting processes both with and without IGF-1. The signaling pathway involves a complex network of genes. This study focuses specifically on investigating whether IGFBP5 can regulate skeletal muscle fibrosis in an IGF-1-dependent manner, without delving into the deeper mechanistic aspects of its action. To verify this, immunofluorescence staining of skeletal muscle tissue was performed again, and it was found that in SAMP8 mice with IGFBP5 knockout, the expression of IGF-1 was increased compared to aging SAMP8 mice (Supplementary Figure 5). TGF- β staining was also performed, and TGF- β expression was reduced in the IGFBP5 knockout mice (Supplementary Figure 5). This suggests that IGFBP5 may modulate the process of skeletal muscle fibrosis by mediating interactions with both IGF-1 and TGF-β pathways.

Discussion

The interplay between muscle atrophy and fibrosis is a central aspect of sarcopenia (Boccardi, 2024). While muscle atrophy

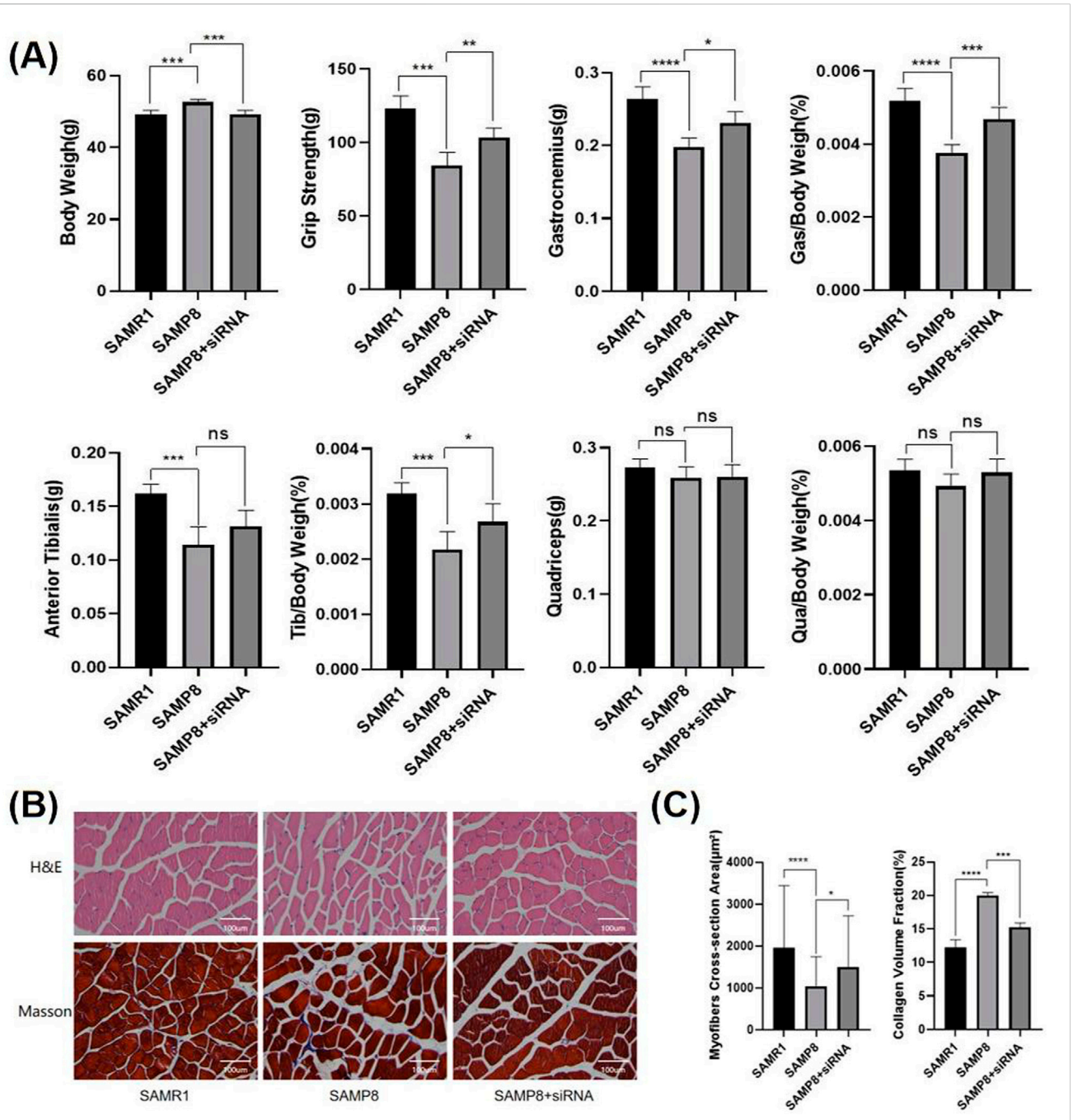

FIGURE 5
(A) Body weight, muscle strength, and the weight of lower limb skeletal muscles (gastrocnemius, tibialis anterior, quadriceps) and their percentage of the body weight of mice(SAMR1 vs. SAMP8 vs. SAMP8+siRNA) **(B)** The HE and Masson staining of limb skeletal muscle(SAMR1 vs. SAMP8 vs. SAMP8+siRNA) **(C)** the statistical analysis of myofiber CSA and CVF (compared to control group, *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001).

involves the loss of muscle fibers, fibrosis refers to the excessive deposition of extracellular matrix (ECM) proteins, particularly collagen, which leads to muscle stiffness and a reduction in physical activity levels (Bonaldo and Sandri, 2013; Sakuma et al., 2014; Mahdy, 2019). This combination not only impairs mobility but also disrupts the communication between muscle satellite cells and their environment, thereby compromising the muscle's regenerative capacity (Blau et al., 2015; Hong et al., 2022).

Skeletal muscle fibroblasts are essential cells within skeletal muscle that play a multifaceted role in maintaining muscle structure, function, and homeostasis (Chapman et al., 2016). These cells are primarily responsible for the synthesis and secretion of extracellular matrix (ECM) components, such as collagen, elastin, and glycosaminoglycans, which provide mechanical support and structural integrity to muscle fibers (Plikus et al., 2021; Chapman et al., 2016). In addition to their

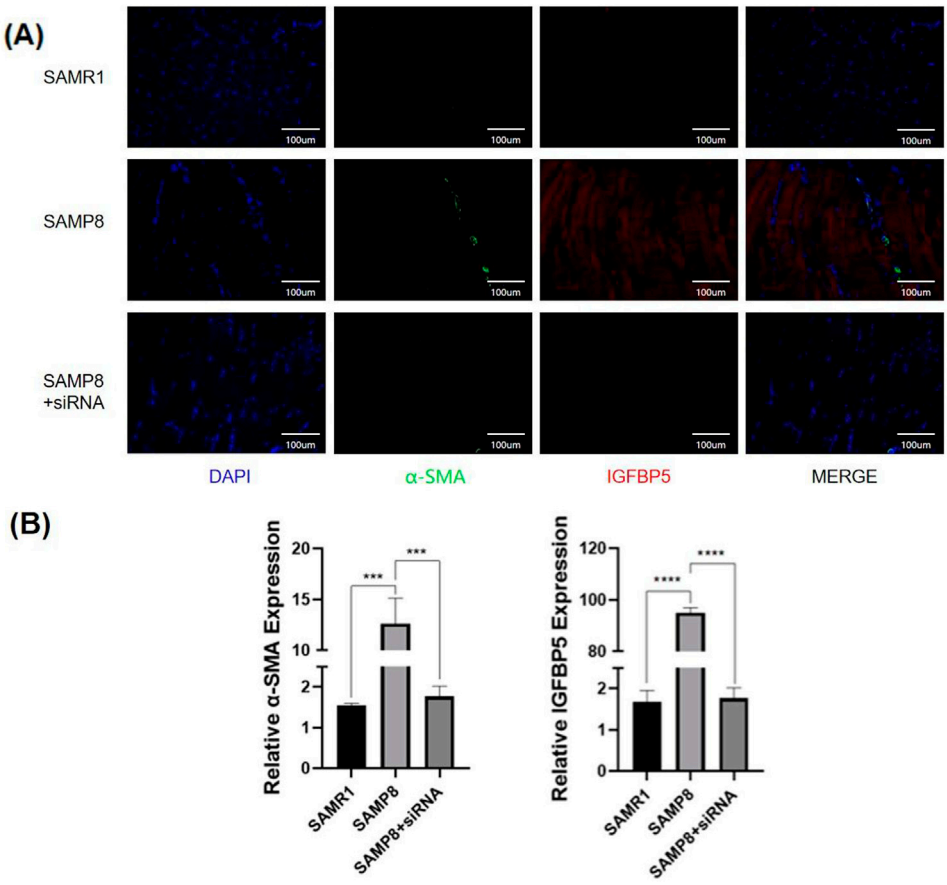

FIGURE 6
(A) The immunofluorescence staining of skeletal muscle (α – SMA, IGFBP5) (SAMR1 vs. SAMP8 vs. SAMP8+siRNA) **(B)** Statistical analysis of the positive expression (compared to control group, *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001).

structural role, skeletal muscle fibroblasts are crucial for tissue repair and regeneration following injury (Tidball, 2011; Younesi et al., 2024). Upon activation, these fibroblasts can differentiate into myofibroblasts, which express contractile proteins like α-smooth muscle actin (α-SMA) and contribute to the formation of scar tissue (Younesi et al., 2024; Hall et al., 2023; Gibb et al., 2020). However, excessive or prolonged activation of myofibroblasts can lead to pathological fibrosis (Schuster et al., 2023; Younesi et al., 2024). Moreover, fibroblasts play a significant role in regulating inflammation and immune responses (Davidson et al., 2021; Chapman et al., 2016). Additionally, skeletal muscle fibroblasts interact closely with muscle cells, influencing their growth, differentiation, and contractile function through the secretion of growth factors like IGF-1 and by providing mechanical signals (Chapman et al., 2016; Murphy et al., 2011; Abdel-Raouf et al., 2021). Their functions extend beyond structural support to include critical roles in immune regulation and cellular communication, highlighting their importance in both physiological and pathological contexts (Chapman et al., 2016).

Fibro-adipogenic progenitors (FAPs) are mesenchymal stromal cells residing in skeletal muscle interstitium, playing dual roles in muscle homeostasis, regeneration, and pathology (Joe et al., 2010; Uezumi et al., 2010). Following muscle injury, FAPs rapidly activate, proliferate, and transiently expand to orchestrate regeneration, which promote muscle satellite cell (MuSCs) proliferation and differentiation into myofibers (Heredia et al., 2013). This post-injury pro-regenerative response was tightly regulated by inflammatory signals such as TNF-α, while anti-inflammatory cytokines such as IL-4 and IL-13 later induce FAPs apoptosis, preventing excessive extracellular matrix (ECM) deposition (Lemos et al., 2015). Dysregulation of this balance leads to pathological outcomes, where FAPs underwent fibro-adipogenic differentiation, replacing functional muscle tissue and impairing contractility (Natarajan et al., 2010).

Notably, FAPs exhibit microenvironment-dependent plasticity. While their crosstalk with MuSCs is essential for repair, aberrant signaling such as TGF-β overactivation shifts FAPs toward a profibrotic state (Contreras et al., 2019). Recent studies highlight their dual nature—indispensable for regeneration yet potential drivers of degenerative diseases. Therefore, FAPs are pivotal regulators of skeletal muscle dynamics, balancing regenerative support with risks of pathological tissue remodeling, making them critical targets for muscle disease therapies. However, their complex mechanisms of action and interactions with numerous other cellular pathways make it challenging to elucidate a singular mechanism. In this study, we focus on skeletal muscle fibroblasts as

the primary cell type for investigation, although FAPs are also employed for some key validations.

D-gal is a widely used chemical to induce cellular senescence (Azman and Zakaria, 2019). Cells undergoing senescence induced by D-gal exhibit mitochondrial structural damage and a decline in energy metabolism, which are highly related to cellular aging studies have confirmed that D-gal can induce senescence, fibrosis, and redox imbalance in skeletal muscle fibroblasts (Wu et al., 2022; Ma et al., 2024). Our results confirm that D-gal effectively induces cellular senescence and skeletal muscle fibrosis in both cellular and animal models. The increase in senescence markers and fibrosis-related proteins, along with the observed decline in muscle strength and mass, are consistent with previous studies that highlight the role of D-gal in modeling aging-associated pathologies. The observed lethargy and reduction in muscle fiber cross-sectional area further validate the model's relevance to sarcopenia research.

The sequencing analysis conducted in our study has unveiled substantial alterations in mRNA expression, pinpointing IGFBP5 as a potential regulator of skeletal muscle fibrosis. This discovery associates with existing literature, which posits that IGFBP5 plays a complex and multifaceted role in cellular processes, particularly in the realms of cell growth and metabolism regulation. The overexpression of IGFBP5 in senescent skeletal muscle fibroblasts, coupled with its association with elevated markers of fibrosis, highlights its potential as a therapeutic target for interventions aimed at combating fibrosis. IGFBP5 is highly conserved in evolution compared to other IGFBP proteins and possesses a variety of biological activities (Duan and Allard, 2020). Existing research has demonstrated that IGFBP5 can play a role in the regulation of cell growth and metabolism by mediating the IGF1 signaling pathway (Ding et al., 2016). However, in addition to its function through the IGF signaling pathway, IGFBP5 also has IGF-independent activity, which adds to the complexity of its regulation of cellular behavior (Duan and Allard, 2020; Dittmer, 2022). We further investigate whether IGFBP5 can affect the fibrotic phenotype of skeletal muscle in an IGF-1-dependent manner.

The intricate role of IGFBP5 extends beyond its interaction with insulin-like growth factors (IGFs) (Beattie et al., 2006). It is known to modulate IGF bioavailability by binding to IGFs, thereby influencing the activity of the IGF signaling pathway (Clemmons, 2016). This pathway is crucial for various physiological processes, including muscle growth and repair. Based on the provided search results, there is no direct evidence discussing the regulation of IGFBP5 expression in fibroblasts and FAPs. However, IGFBP5 were found to be associated with fibrotic pathways in other tissue, suggesting the possibility that the expression of IGFBP5 could also be regulated under muscle pathologies (Contreras et al., 2021; Sorokina et al., 2024; Babaeijandaghi et al., 2023; Li et al., 2025). This study aims to investigate the role of IGFBP5 in the fibrosis of aging skeletal muscle. In the *in vivo* experiments conducted in this paper, it was found that in SAMP8 mice with knockdown of IGFBP5, there was a noticeable improvement in muscle strength, and both the weight and cross-sectional area of the skeletal muscles were improved to some extent. This indicates that the knockdown of IGFBP5 can partially ameliorate the quality of aging skeletal muscle. Staining of the skeletal muscles also showed a reduction in the degree of fibrosis, and the expression of IGF-1 increased to some extent after the knockdown of IGFBP5. This suggests that IGFBP5 can act through the regulation of IGF-1 in the fibrosis of aging skeletal muscle. In previous research related to skeletal muscle, there is literature supporting that IGFBP5 can function as a growth factor regulating skeletal muscle growth and also plays a role in disuse atrophy of skeletal muscle.

Mice and humans share a high degree of similarity in genetic mechanisms and physiological characteristics, which is why mouse models are widely used in medical research on human aging (Breschi et al., 2017). One of the most commonly used strains is the C57BL/6J mouse; almost all biological markers can detect aging changes in mice aged 18–24 months, making it a frequently used model for natural aging (Wu et al., 2024). The D-gal-induced aging model involves the continuous injection of D-gal into animals over a certain period, leading to an increase in galactose concentration within cells (Wang et al., 2023). Under the catalysis of aldose reductase, galactose is reduced to galactitol, which cannot be further metabolized by cells and accumulates, affecting osmotic pressure, causing cell swelling and dysfunction, ultimately leading to aging (Azman and Zakaria, 2019; Azman et al., 2021). Initially used to establish cataract models, this model has been developed through continuous research, and its various biochemical and physiological indicators are similar to natural aging, making it widely used today (Azman and Zakaria, 2019). The senescence-accelerated mouse (SAM) is a kind of premature aging model mouse, including two strains: SAMP (senescence accelerated-prone mouse) and SAMR (senescence accelerated resistant mouse) (Chiba et al., 2009; Takeda, 2009). SAMP exhibits rapid aging characteristics after a normal growth period (Takeda, 2009). SAMP8, a sub-strain of SAMP, is currently recognized as an ideal model for natural aging and dementia (Butterfield and Poon, 2005; Liu et al., 2020). In this article, the D-gal aging model and the SAMP8 premature aging mouse model were selected for their short modeling time and simple operation. Many pathways and targets related to skeletal muscle have been identified in these two models, such as the Wnt/β-catenin signaling pathway and its downstream cascade (Rudolf et al., 2016), the AMPK/TGF-β/SMAD axis (Zhong et al., 2024), and important skeletal muscle-related pathways, as well as targets related to skeletal muscle fibrosis and atrophy, such as CILP2 and TRIM16 (Deng et al., 2024; Guo et al., 2024). This study found that the IGFBP5 target may regulate the progression of fibrosis and sarcopenia in aging skeletal muscle through the IGF-1 pathway.

In this study, we also observed a seesaw effect between IGF-1 and TGF-β. The role of TGF-β in skeletal muscle fibrosis is undoubted. In skeletal muscle fibrotic pathologies, TGF-β1 is highly expressed and plays a key role in the development of skeletal muscle fibrosis (Ismaeel et al., 2019; Budi et al., 2021). It can promote the expression of extracellular matrix (ECM) components such as collagen and fibronectin and inhibit ECM degradation, playing a significant role in cell morphogenesis, proliferation, and differentiation processes (Roberts et al., 1992; Akhurst, 2004; Massagué and Sheppard, 2023). The activation of the TGF-β signaling pathway leads to pathological fibrosis (Meng et al., 2016). IGF-1 also plays a very important positive role in the growth and development of skeletal muscle, can delay various pathological muscle atrophies, and maintain and promote the growth and survival of the nervous system (Yoshida and Delafontaine, 2020; Ahmad et al., 2020). The decline in skeletal muscle mass and strength (sarcopenia) is also related to the reduced activity of the IGF-1/Akt/mTOR signaling pathway (Feng, 2010; López-Caamal

et al., 2012). Both TGF-β and IGF-1 are important factors in skeletal muscle, and our research suggests that IGFBP5 may affect skeletal muscle aging and fibrosis by regulating the dynamic balance between TGF-β and IGF-1 through the expression of regulatory factors.

While our study focuses on the role of IGFBP5 in skeletal muscle fibrosis and sarcopenia, its involvement in fibrosis extends to multiple tissues and disease states, highlighting its potential as a therapeutic target. IGFBP5 is upregulated in idiopathic pulmonary fibrosis (IPF) and contributes to fibroblast activation and ECM remodeling. Elevated IGFBP5 levels in bronchoalveolar lavage fluid correlate with disease severity, suggesting its potential as a biomarker (Sureshbabu et al., 2011). In heart failure and myocardial infarction, IGFBP5 plays a dual role in fibrosis and repair, and also supports angiogenesis and cardiomyocyte survival under stress, highlighting its context-dependent roles (Zhu et al., 2024). IGFBP5 promotes fibroblast-to-myofibroblast transition and collagen synthesis, and interacts with ECM components (e.g., collagen I, III) to stabilize fibrotic lesions (Sureshbabu et al., 2009). Moreover, IGFBP5 has been shown to act independently of IGF-1 in other cell and disease models, indicating a complexity that warrants additional research (Duan and Allard, 2020; Dittmer, 2022). The interplay between TGF-β and IGF1 is not confined to a single pathway and requires further exploration. By elucidating the broader role of IGFBP5 in fibrotic disorders, our study not only advances understanding of its mechanisms in sarcopenia but also highlights its relevance across multiple diseases. This positions IGFBP5 as a promising target for anti-fibrotic therapies, with potential applications in pulmonary, cardiac, renal, hepatic, and dermal fibrosis.

In the present study, several limitations should be acknowledged. Firstly, naturally aged mice were not utilized, which may limit the direct relevance of the findings to natural aging processes. The relationships among SAMP8, SMAR1, and SAMP8 with siRNA require further investigation to elucidate their interactions and potential synergistic effects. Furthermore, in in vivo models, the injection of siRNA may potentially impact other cells within the skeletal muscle, not just limited to NOR-10. This necessitates further validation in subsequent studies. Additionally, conditional knockout mice were not employed, which could have provided more precise insights into gene-specific functions and their roles in the studied processes. Future research should address these limitations to enhance the robustness and applicability of the findings, and explore tissue-specific IGFBP5 regulation and its interplay with other fibrogenic factors to develop precision therapies.

These findings offer new insights into understanding age-related skeletal muscle fibrosis and provide potential molecular targets for the development of therapeutic strategies aimed at skeletal muscle fibrosis. By modulating the expression or activity of IGFBP5, it may be possible to slow down or reverse skeletal muscle fibrosis, thereby improving muscle function and quality of life in the elderly.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE285869.

Ethics statement

The animal study was approved by Shanghai Chedun Experimental Animal Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LS: Writing–original draft, Writing–review and editing. ZD: Supervision, Writing–original draft, Writing–review and editing. JC: Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2025.1557703/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Images of related-IGFBP5.

SUPPLEMENTARY FIGURE S2
Western blot of IGFBP5 and the statistical analysis (compared to control group, *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001).

SUPPLEMENTARY FIGURE S3
The PCR of P16,P21,PPARG,COL-1 and IGFBP5 (compared to control group, *p < 0.05, **p < 0.01, ***p < 0.001).

SUPPLEMENTARY FIGURE S4
The immunofluorescence staining of skeletal muscle (α-SMA, IGFBP5) (CONTROL vs. D-GAL).

SUPPLEMENTARY FIGURE S5
The immunofluorescence staining of skeletal muscle (IGF-1, TGF-β) (SAMP8 vs. SAMP8+siRNA).

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