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Notum protects against myocardial infarction-induced heart dysfunction by alleviating cardiac fibrosis

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Abstract

Background and Objective: Cardiac fibrosis is a pathological reparative process that follows myocardial infarctionand is associated with compromised cardiac systolic and reduced cardiac compliance. The Wnt signaling pathway is closely implicated in organ fibrosis, and Notum, a highly conserved secreted inhibitor, modulates Wnt signaling. The objective of this study was to explore the role and mechanism of Notum in cardiac fibrosis. **Methods:** A mouse model of cardiac remodeling was established through left coronary artery ligation surgery, with the addition of Notum injection following myocardial infarction surgery. The protective effect of Notum on myocardial infarction was assessed by evaluating cardiac function, including survival rate, echocardiographic assessment, and cardiac contraction analyses. Inflammatory cell necrosis and infiltration were confirmed through H&E and Masson staining. The expression of fibrosis-related genes and β-catenin pathway markers was detected using Western blot quantificational RT-PCR (qRT-PCR). Additionally, EdU, wound healing, and immunofluorescence staining analyses were performed to detect the effect of Notum's in transforming growth factor beta-1 (TGF-β1) induced myofibroblast transformation. **Results:** The administration of Notum treatment resulted in enhanced survival rates, improved cardiac function, and decreased necrosis and infiltration of inflammatory cells in mice subjected to left coronary artery ligation. Furthermore, Notum effectively impeded the senescence of cardiac fibroblasts and hindered their pathological transformation into cardiac fibroblasts. Additionally, it significantly reduced collagen production and attenuated the activation of the Wnt/β-catenin pathway. Our preliminary investigations successfully demonstrated the therapeutic potential of Notum in both fibroblasts *in vitro* and in a mouse model of myocardial infarction-induced cardiac fibrosis *in vivo*. **Conclusion:** Notum inhibition of the Wnt/β-catenin signaling pathway and cardiac fibroblast senescence ultimately hampers the onset of cardiac fibrosis. Our findings suggest that Notum could represent a new therapeutic strategy for the treatment of cardiac fibrosis.

Keywords

cardiac fibrosis; Notum; Wnt/β-catenin; senescence; myocardial infarction

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1 Introduction

Cardiac fibrosis (CF), an aberrant reparative process in response to cardiomyocyte injury, is prevalent in various heart disease like hypertrophic cardiomyopathy, myocardial infarction, and dilated cardiomyopathy[1]. Studies have shown that exposure to cold increases the risk of myocardial infarction and ST-segment elevation myocardial infarction (STEMI) is more prevalent in the winter months[2]. Persistent disease states or abnormal activation of various pathways in this pathological myocardial remodeling contribute to arrhythmias, increased myocardial stiffness and ventricular dysfunction, making CF a key contributor to heart failure[3].

This condition is typified by excessive extracellular matrix (ECM) deposition and scar tissue formation. Cardiac fibroblasts, essential heart cells, are key producers of ECM components. Upon injury, these fibroblasts undergo abnormal activation, leading to enhanced migration, excessive proliferation, and a myofibroblast phenotype shift. This results in ECM accumulation, reducing tissue compliance and inducing myocardial remodeling[4]. Cardiomyocyte death or various adverse stimuli like ischemia, hypertension,

aging, or pathologies such as anthracycline exposure augment CF[5]. Therefore, inhibiting or delaying the progression of CF is crucial in preventing and treating cardiovascular diseases.

Recent studies highlight cellular effectors such as macrophages, mast cells, and lymphocytes activating profibrotic pathways through matrix protein production or mediator secretion[6]. Inflammatory signaling, the Renin-Angiotensin-Aldosterone System, transforming growth factor-β (TGF-β), and other molecular pathways also contribute significantly to fibrosis development[7]. However, the complete pathogenesis of CF warrants further investigation, and effective means to reduce or reverse this process remain limited.

The Wnt pathway plays an important role in organ development and homeostasis, including liver and heart[8]. It has been linked to organ fibrosis, such as cardiac[9] and renal fibrosis[10], by numerous studies. Studies like Wu *et al.*'s use of CGX1321 demonstrated inhibited Wnt protein secretion, reduced myocardial infarct size and fibrosis, and improved cardiac function in MI mice[11]. Qian *et al.* found that downregulation of S100A4 alleviates CF *via* the Wnt/β-catenin pathway in mice[12]. In addition, Weber and colleagues reported that Cdon deficiency causes hyperactive Wnt signaling, leading to aberrant intercellular coupling and CF[13].

Notum, an endogenous Wnt pathway feedback inhibitor, is a carboxylesterase that deacylates Wnt by removing an essential palmitoleic moiety[14]. Pentinmikko *et al.* confirmed Notum's role in senescent Paneth cells, inhibiting the regeneration of aging intestinal epithelial cells[15]. Mouse studies showed enhanced aging stem cell and tissue regeneration Notum inhibition[16]. However, Notum's role in regulating CF and its mechanism remain unconfirmed.

This study evaluated Notum's cardioprotective effect in mice with myocardial infarction (MI) and preliminarily insights into its anti-fibrotic mechanisms. These findings propose a novel strategy for the preventing and treating myocardial fibrosis.

2 Material and Methods

2.1 Animal model and treatment

All study procedures were conducted in compliance with the guidelines of Harbin Medical University and approved by the Ethical Committees of Harbin Medical University (IRB3022621).

Animal experiments adhered to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health Publication No. 85–23, revised 1996) and followed the ARRIVE guidelines. Adult male C57BL/6 mice, weighting an average of 25 g, were purchased from Changsheng Biotechnology (Liaoning, China). They were randomly divided into three groups: Sham, MI, MI+Notum. The mice were anesthetized using avertin (240 mg/kg, intraperitoneal injections), ventilated with a small animal ventilator at a tidal volume of 3 mL and underwent left coronary artery ligation surgery to induce MI. Notum (1 μg/25 g) obtained from R&D System (Bio-Techne, Minnesota, USA) was injected into the hearts of mice in the MI+Notum group immediately after MI surgery. Notum powder was diluted to 100 ng/μL with phosphate belanced phosphate buffered saline solution (PBS) administered at a dose of 1 μg/25 g body weight. A 25 g mouse required a 10 μL injection of the drug solution. Notum was intramyocardially injected into the heart at five sites (upper, lower, left, right, and center) ten days before left coronary artery ligation[17].

2.2 Cell culture

Primary cardiac fibroblasts were isolated from 2 to 3-day-old Kunming mice. Heart tissues were excised and placed in a petri dish with ice-cold dulbecco's modified eagle medium (DMEM) (Biological Industries, Israel). The tissues were cut into 1-mm cubed pieces, and digested using trypsin (0.5 mg/mL). The supernatant was centrifuged at 1300 rpm for 7 min after filtration. Next, cells were re-suspended in DMEM containing 10% fetal bovine serum (Biological Industries, Tel Aviv, Israel) and seeded onto culture plates. After 1.5 h, the non-adherent cells were removed. The cardiac fibroblasts (adherent cells) were cultured at 37°C with 5% CO_2.

Upon reaching 70%–80% density, the cells were treated with 10 ng/mL TGF-β1 (Sigma-Aldrich, St Louis, USA) and 10 ng/mL Notum (Bio-Techne, Minnesota, USA) for 24 h. In addition, cardiac fibroblasts were subjected to 100 μmol/L H_2O_2 (Sigma-Aldrich, St Louis, USA) treatment 2 h after Notum treatment for 22 h.

2.3 Western blot

Protein from heart tissues and cardiac fibroblasts were extracted with radio-immunoprecipitation assay lysis buffer (Beyotime, Jiangsu, China). Protein samples (80 or 50 μg) were separated by polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membranes (Pall Life Sciences, Ann Arbor, MI, USA), and subjected to overnight incubation at 4°C with primary antibodies. After blocking and washing, membranes were probed with secondary antibodies, and the Odyssey Infrared Imaging System was used for scanning. Western blot results were analyzed using Image Studio Ver 5.2 software.

The antibodies used in this study were: Fibronectin 1 (FN1, 15613-AP, 1∶500, Proteintech, Rosemont, USA), collagen 1 (WL0088, 1∶500, Wanlei, Shenyang, China), α-smooth muscle actin (α-SMA, ab7817, 1∶2000, Abcam, Cambridge, UK), p53 (10442-1-AP, 1∶500, Proteintech, Rosemont, USA), β-actin (60008-1-AP, 1∶1000, Proteintech, Rosemont, USA), and GAPDH (60004-1-Ig, 1∶1000, Proteintech, Rosemont, USA).

2.4 Quantificational RT-PCR (qRT-PCR)

Total RNA was extracted from heart tissues or cardiac fibroblasts following the Trizol standard protocol. The concentration and purity of the RNA samples were assessed using the Nano-Drop 8000 Spectrophotometer (Thermo, USA). Subsequently, RNA was reverse transcribed into cDNA employing the High Capacity cDNA Reverse Transcription Kit (AT341-02, Transgene, Beijing, China) as per the manufacturer's instructions. The relative mRNA levels were determined using qRT-PCR with SYBR Green I (Roche, Basel, Switzerland). The CT values were computed to assess the relative levels of mRNAs, and these values were normalized to GAPDH.

2.5 Histology analysis

The hearts were immersed in 4% paraformaldehyde for 3 days for fixation before dehydration. Subsequently, they were embedded in paraffin and sliced into 6-μm sections. Following the manufacturer's instructions, these sctions were stained with H&E and Masson's trichrome to evaluate the extent of fibrosis. The fibrotic areas were quantified using Image-Pro plus 6.0 software.

2.6 Immunohistochemistry staining

The cardiac sections were dewaxed and rehydrated using dimethylbenzene and a gradient ethanol. Subsequently, these sections underwent a 10 min treatment with 3% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval was performed using Sodium Citrate Buffer in a microwave. The sections were then incubated with a blocking solution of 50% goat serum at 37 °C for 1 h and subsequently exposed to primary antibodies against FN1 (Proteintech, Rosemont, USA), α-SMA, and β-catenin (Abcam, Cambridge, UK), as well as p16 (Wanlei, Shenyang, China). Horseradish peroxidase (HRP) conjugated secondary antibodies (ZsBio, Beijing, China) were used to combine with the primary antibodies. Then, the sections were stained with 3, 3-diaminobenzidine(DAB) (ZsBio, Beijing, China) and counterstained with hematoxylin (Solarbio, Beijing, China) to visualize the nucleus.

2.7 Immunofluorescence staining

After exposure to TGF-β1 and Notum, cardiac fibroblasts underwent three washes with PBS and were then fixed in 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were permeabilized using 0.4% Triton X-100 for 1 h at room temperature and blocked with 50% normal goat serum at 37°C for 1 h. After three additional washes, the cells were incubated with antibody against α-SMA (1∶200, Abcam, Cambridge, UK) at 4°C overnight. The following day, after another round of washing, the cells were incubated with fluorescein isothiocyanate isomer I (FITC)-conjugated goat anti-mouse antibody in the dark for 1 h. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Roche, Basel, Switzerland). Finally, immunofluorescence was visualized using a microscope (Olympus, IX73, Japan).

2.8 Proliferation assay

Primary cardiac fibroblasts were cultured and treated in 24-well plates. The Cell-Light EdU DNA Cell Proliferiation Kit (RiboBio, Guangzhou, China) was used to assess cell proliferation following the manufacturer's instructions.Fluorescent images were captured using a fluorescence microscope (Olympus, IX73, Japan).

2.9 Wound-healing Scratch assay

A scratch assay was performed to evaluate the migration of cardiac fibroblasts. Cells were seeded in 6-well plates, and wounds were generated by scratching the cell monolayer using 10-μL pipette tips. Following this, the cells were washed twice with PBS and subjected to the specified treatment for 24 h. Images were captured at 0, 12 and 24 h using the Nikon TS100 microscope (Nikon, Japan). Subsequently, Image J was used to analyze the relative cell migration.

2.10 Statistical analysis

Graph Pad Prism 8.0 was used for statistical analyses. Data are presented as mean ± standard error of mean (SEM). One way analysis of variance (ANOVA) followed by Bonferroni or Dunnett's post-hoc test was used for multiple group comparisons. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Notum improves survival and cardiac function in myocardial infarction mice

To investigate the effects of Notum on the heart, C57BL/6 mice underwent left coronary artery ligation surgery for 7 days to induce MI and concomitantly received Notum injection into the

heart (Fig. 1A). As shown in Fig. 1B, the survival rate post-MI was significantly higher in the MI+Notum group (23/25; 92%) than that in the MI group (18/25; 72%), evident up to 7 days post-surgery. Echocardiography on day 7 post-MI (Fig. 1C) revealed marked cardiac systolic dysfunction in MI mice compared to the Sham group, evidenced by decreased left ventricular ejection fraction (Sham 52.26 ± 5.25% *vs.* MI 40.38 ± 4.51%) and left ventricular short axis shortening rate (Sham 26.11 ± 3.33% *vs.* MI 19.43 ± 2.21%). Conversely, in the MI+Notum group, left ventricular ejection fraction (57.35 ± 11.81%) and left ventricular short axis shortening rate (30.05 ± 7.49%) significantly exceeded those in the MI group (Fig. 1D and 1E). Notum treatment also notably reduced LV systolic (MI 43.35 ± 17.43 μL *vs.* MI+Notum 26.54 ± 12.57 μL) and diastolic volume (MI 79.10 ± 22.08 μL *vs.* MI+Notum 59.30 ± 14.70 μL) relative to the MI group (Fig. 1F and 1G), indicating improved cardiac function and attenuated cardiac remodeling in the MI mice model with Notum treatment.

3.2 Notum mitigates MI-induced CF in mice

Histological analysis of hearts obtained 7 days post-operation revealed a disordered arrangement of myocardial tissue around the infarct region in the MI group, displaying numerous inflammatory cell necroses and infiltrations, notably improved in

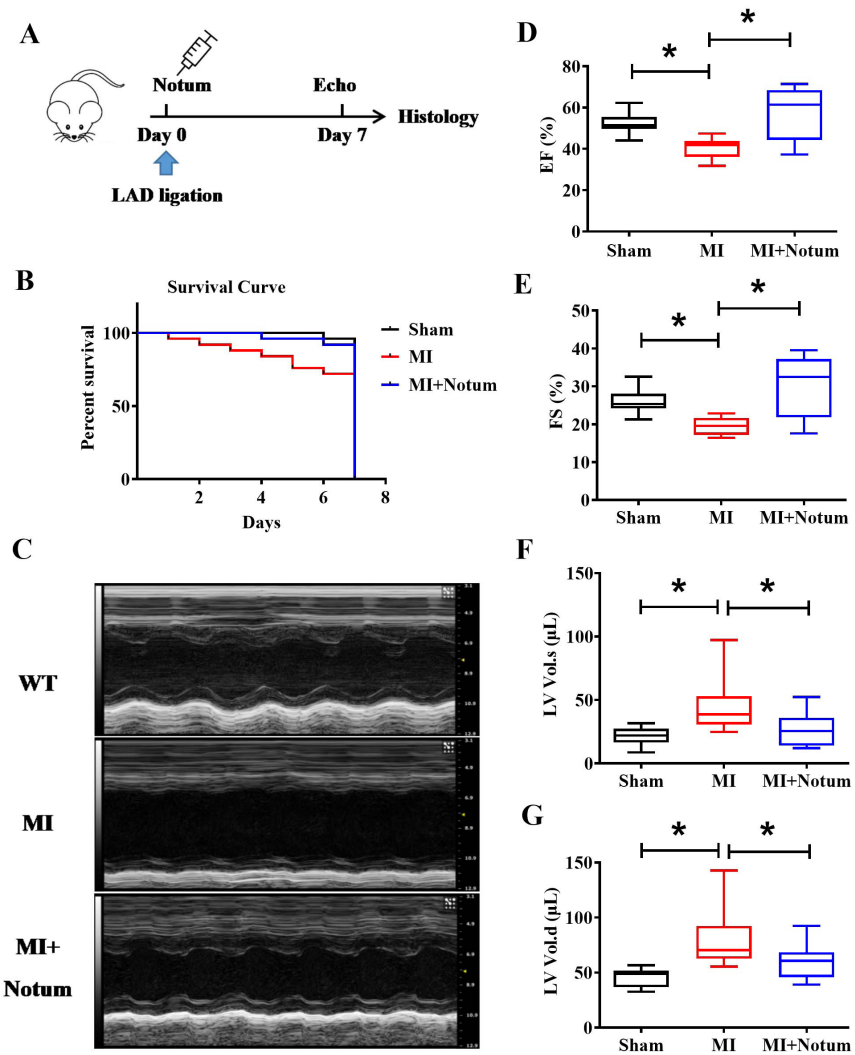

Fig. 1 Notum increases survival and improves cardiac function in myocardial injury (MI) mice. (A) Notum treatment protocol. (B) Survival curves of specified groups of mice. (C) Echocardiography was applied to assessing the heart function of MI mice after treatment with Notum ($N = 5$), as indicated by ejection fraction (EF). (D) fraction shortening (FS). (E) left ventricular systolic volume (LV.Vol.s) (F), and left ventricular diastolic volume (Lv.vol.d) (G). Data are presented as mean ± SEM. $^{*}P < 0.01$. Survival distributions were estimated by the Kaplan-Meier method and compared by log-rank test.

Notum+MI group (Fig. 2A). Similarly, Masson staining exhibited reduced fibrosis areas in the Notum-treated mice compared to the MI group (Fig. 2B).

Western blot analysis of cardiac tissue in the infarct border zone exhibited significant up-regulation of α-SMA and FN1 in MI mice, attenuated after Notum treatment (Fig. 2C). Immunohistochemistry assays confirmed Notum's inhibition of MI-induced up-regulation of α-SMA and FN1, suggesting Notum suppressed myofibroblast differentiation (Fig. 2D and 2E). Meanwhile, mRNA levels of FN1, Collagen 1α1 and Collagen 3α1 were strikingly reduced in the Notum+MI group compared to the MI group (Fig. 2F).

3.3 Notum attenuates TGF-β1-induced cardiac fibroblast activation

TGF-β1 is a classic promotes fibrogenic cytokine, which can cause the transformation of CF to MFs and lead to the deposition of ECM[18]. *In vitro*, primary cardiac fibroblasts treated with TGF-β1 and TGF-β1+Notum for 24 h showed that Notum effectively suppressed FN1 expression and collagen 1 production induced by TGF-β1. Real-time PCR demonstrated reduced mRNA levels of FN1, Collagen 1α1, Collagen 3α1 and α-SMA in the presence of Notum (Fig. 3B). Notum also mitigated TGF-β1-induced fibroblast proliferation and migration, as observed in scratch assays (Fig. 3C and 3D). Immunofluorescence revealed Notum's inhibition of TGF-β1-induced myofibroblast transformation and α-SMA overexpression of α-SMA (Fig. 3E).

3.4 Notum inhibits Wnt/β-catenin signaling activation in cardiac fibroblasts

To determine the role of the Notum in the process of CF, we explored whether Notum can inhibit the activation of Wnt/β-catenin signal pathway in cardiac fibroblasts *in vivo* and *in vitro*. As illustrated in Fig. 4A, immunohistochemistry staining illustrated reduced β-catenin expression in the infarcted border zone of MI mice after Notum injection. Correspondingly, the qRT-PCR and Western blot analyses revealed decreased β-catenin mRNA and protein levels post-Notum treatment (Fig.

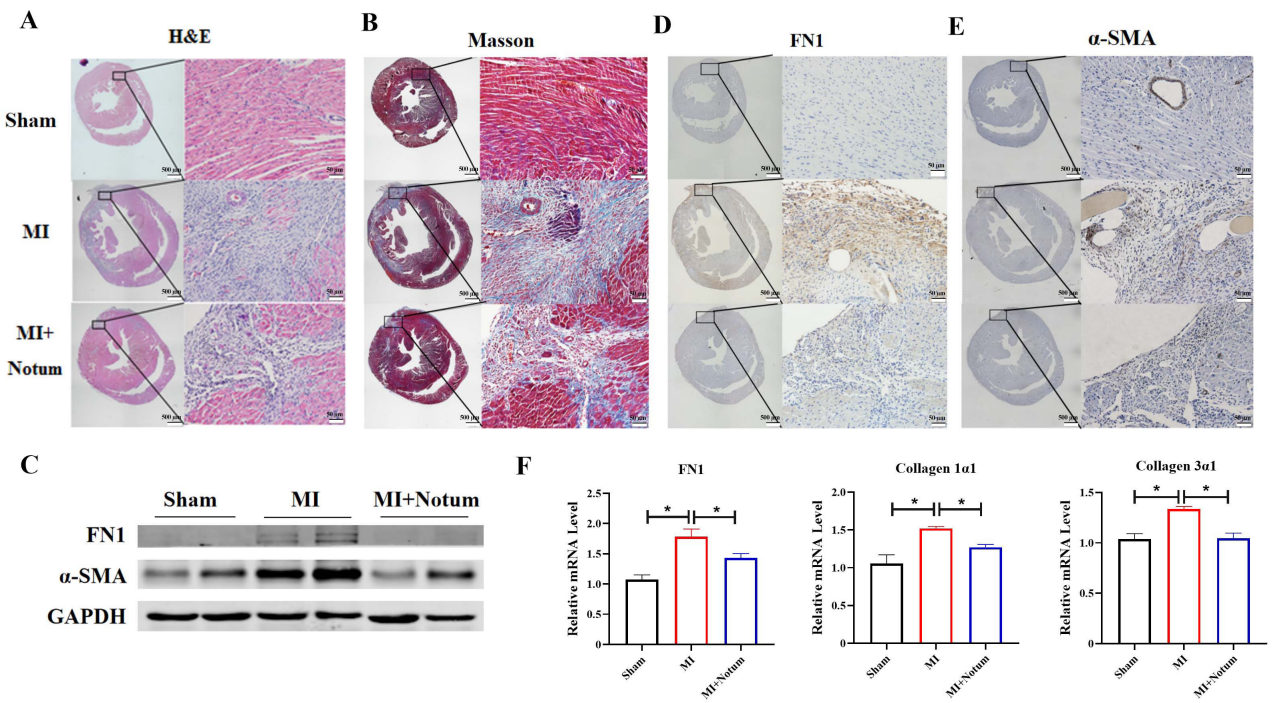

Fig. 2 Notum attenuates myocardial injury (MI)-induced cardiac fibrosis. (A-B) Representative images of H&E and Masson's trichrome-stained sections. The left panel shows the whole heart section. Scale bars: 500 μm. The right panel represents enlarged view of selected fields. Scale bars: 50 μm. *N* = 5-7. (C) Western blot analysis of fibrosis-related proteins (FN1 and α-SMA) in the hearts. *N* = 5-6. (D-E) Immunohistochemistry staining of FN1 and α-SMA in sham, MI and MI+Notum mice. The left panel is an image for the whole heart section. Scale bars: 500 μm. The right panel represents enlarged view of selected fields. Scale bars: 50 μm. *N* = 4-7. (F) Relative levels of FN1, Col1α1, Col3α1 mRNAs analyzed by qRT-PCR. *N* = 5-6, $^{*}P < 0.01$ *vs.* Sham or MI. Data are presented as mean ± SEM.

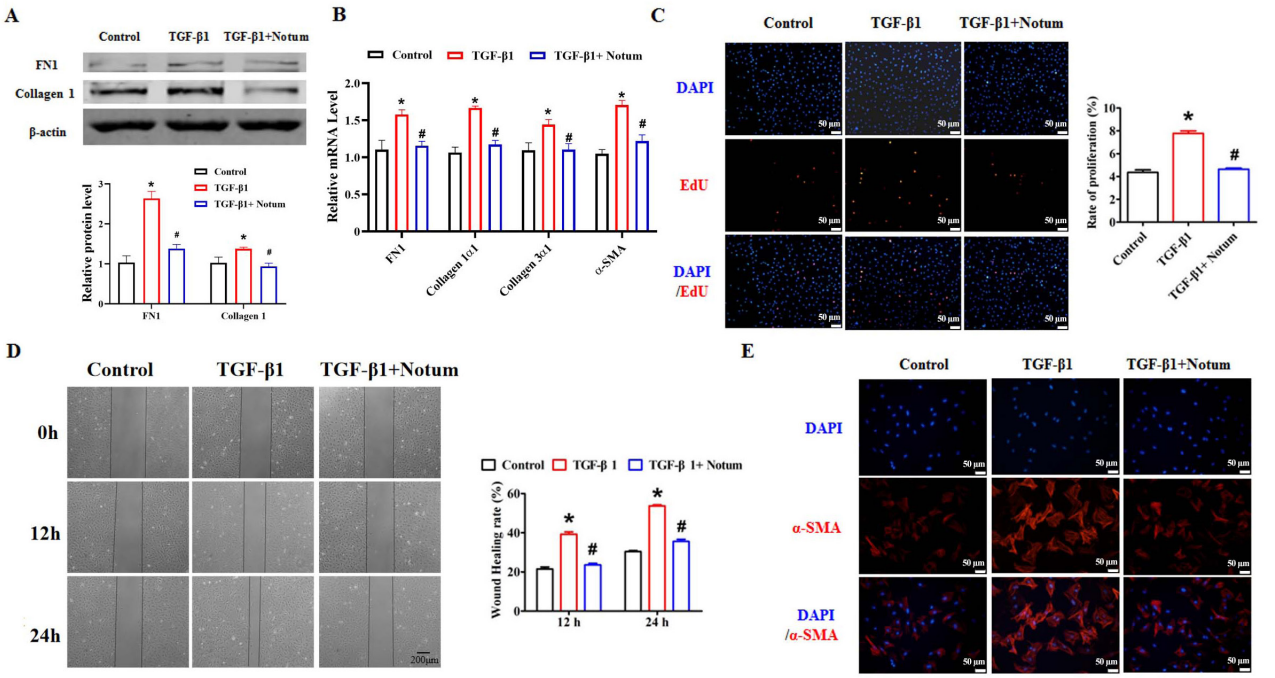

Fig. 3 Notum abrogates cardiac fibroblasts activation induced by TGF-β1. (A) Western blot analysis of FN1 and Collagen 1 in cardiac fibroblasts after 24 h treatment with TGF-β1 and Notum. $N = 6$. (B) qRT-PCR analysis of the expression of FN1, Col 1α1, Col 3α1 and α-SMA mRNAs in cardiac fibroblasts. $N = 5–6$. (C) EDU staining demonstrates the effect of Notum on TGF-β1-induced cardiac fibroblasts proliferation. Scale bars: 50 μm; $N = 8$. (D) Wound healing scratch assay evaluates the effect of Notum on TGF-β1-induced migration in cardiac fibroblasts. Scale bars: 200 μm; $N = 8$. (E) Effect of Notum on cardiac fibroblast-myofibroblast transition, as measured by immunofluorescence. Scale bars: 50 μm. Data are presented as mean ± SEM. $^{*}P < 0.01$ *vs.* CTL; $^{\#}P < 0.01$ *vs.* TGF-β1.

4B and 4C). Meanwhile, as shown in Fig. 4D, Notum treatment abolished TGF-β1-induced elevation of β-catenin and glycogen synthase kinase 3β (GSK3β, an important component of β-catenin destruction complex)[19]. Moreover, Notum reversed the mRNA upregulation of β-catenin induced by TGF-β1 in CFs (Fig. 4E).

3.5 Effect of Notum on cardiac fibroblast senescence

Aging is not a disease but a physiological and pathological process involving attenuated cellular function and weakened stress resistance[20]. The pathological senescence of myocardial fibroblasts after MI is one of the key factors inducing fibrosis. In order to further explore the mechanisms by which Notum regulates CF, primary cultured cardiac fibroblasts were treated with H_2O_2 for 2 h. Western blot results showed that H_2O_2-induced p53 upregulation, an indication of cell senescence, was essentially reversed after Notum pretreatment for 22 h, indicating attenuation of the aging pathway (Fig. 5A). Notum treatment also decreased the mRNA levels of two aging markers p21 and p16 (Fig. 5B and 5C).Furthermore, as illustrated in Fig. 5D, Notum prevented p16 upregulation in the infarcted border zone of MI mice.

4 Discussion

In this study, we investigated the pivotal role and mechanisms of action of Notum in governing CF. Our results a significant improvement in cardiac function and mouse survival within seven days post-MI after Notum intervention. Notum intervention inhibited the senescence of cardiac fibroblasts, curbing their pathological transition into cardiac myofibroblasts. Additionally, it reduced collagen production and blocked Wnt/β-catenin pathway activation. Our findings demonstrated the therapeutic potential of Notum both in fibroblasts *in vitro* and in a mouse model of CF induced by MI *in vivo*, suggesting Notum as a novel therapeutic approach for CF and its associated pathological processes (Fig. 6).

CF, arising after injury and during aging, is prevalent in almost all heart diseases. It involves excessive ECM accumulation, resulting in cardiac interstitial dilatation, decreased compliance, arrhythmias, and heart failure[21]. Currently, effective antifibrotic therapies specifically targeting cardiac fibroblasts remain

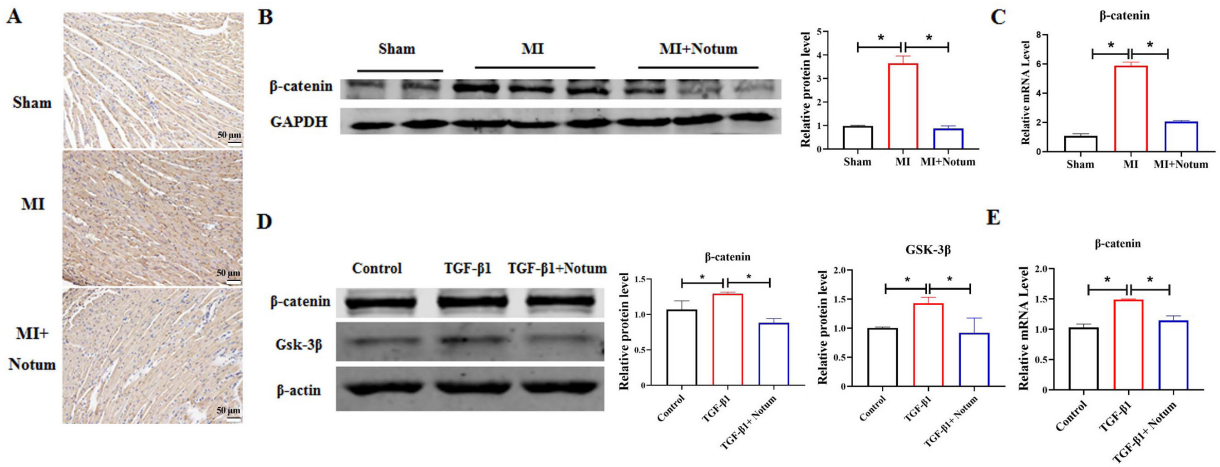

Fig. 4 Notum inhibits Wnt/β-catenin signaling activation in cardiac fibroblasts. (A) Immunohistochemistry showing decreased protein expression of β-catenin in myocardial injury (MI)+Notum-treated mice compared to MI mice. Scale bar: 50 μm; *N* = 3. (B) Western blot analysis of β-catenin in MI model after injection with Notum. *N* = 4-6, $^{*}P < 0.01$. (C) qRT-PCR analysis of the mRNA level of β-catenin in mice. *N* = 6, $^{*}P < 0.01$ *vs.* Sham or MI. (D) The protein levels of β-catenin and GSK-3β measured by Western blot in TGF-β1-induced cardiac fibrogenesis after Notum treatment. *N* = 3-5, $^{*}P < 0.01$ *vs.* Control or TGF-β1 (E) qRT-PCR analysis of the expression of β-catenin mRNA *in vitro*. *N* = 3, $^{*}P < 0.01$. Data are presented as mean ± SEM.

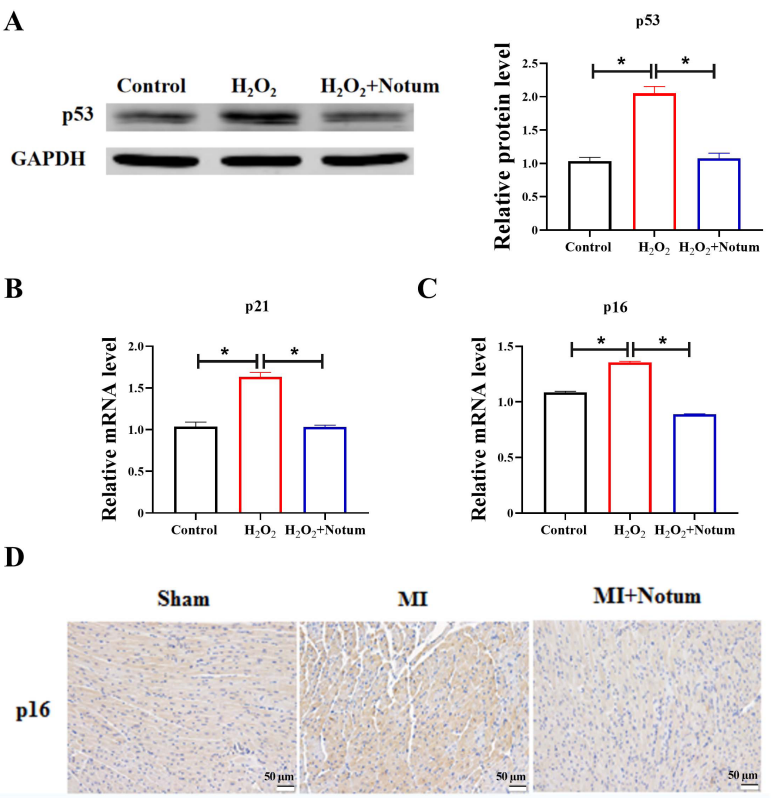

Fig. 5 The effect of Notum on cardiac fibroblasts senescence. (A) Western blot analysis of the protein level of p53 in cardiac fibroblasts treated with H_2O_2 for 2h after 22h treatment of Notum. *N* = 5, $^{*}P < 0.01$. (B) and (C) Relative mRNA levels of p21 and p16 analyzed by qRT-PCR. *N* = 3-5, $^{*}P < 0.01$. (D) Immunohistochemical staining showing the expression changes of p16 in mice. Scale bars: 50 μm; *N* = 4-5. Data are presented as mean ± SEM.

lacking[22]. Recent studies reveal that cardiomyocyte stress signals trigger inflammatory cell infiltration and cardiac fibroblast activation during cardiac pathological states like pressure overload, metabolic dysfunction, or MI. Activated cardiac fibroblasts transform into myofibroblasts, promoting proliferation, matrix deposition, cytokine expression, and fibrous scar tissue formation. Thus, targeting early fibroblast activation and later remodeling holds therapeutic promise for CF[12,23].

The Wnt signaling pathway is crucial in embryonic development and maintains tissue homeostasis, including in the heart and liver. Wnt protein, binding Frizzled receptors and co-receptors 5/6, transmit signals across the plasma membrane, ultimately stabilizing β-catenin in the nucleus to induce Wnt target gene transcription[24]. Studies, such as by Kakugawa's team, emphasize Wnt signaling pathway involvement in CF[25]. Moreover, Pentinmikko *et al* found that the Wnt/β-catenin axis promoting fibroblast activation and proliferation during CF[15].

Notum, an α/β-hydrolase superfamily member, acts as a Wnt ligand deacylase, inhibiting Wnt signaling by removing a palmitoleic acid moiety[26]. Our results indicated significant β-catenin overexpression in a mouse model of MI and TGF-β1-stimulated cardiac fibroblast activation. Crucially, Notum intervention reverse β-catenin overexpression, suggesting Notum's inhibition of the Wnt/β-catenin signaling pathway in cardiac fibroblasts as a potential anti-fibrotic mechanism.

The mouse model of MI is a standard method to induce pathological CF[27]. Pathologically, cardiac myofibroblasts secrete ECM proteins (predominantly collagen I) promoting progressive CF[28], as well as fibronectin 1 and α-SMA, markers of fibroblast-to-myofibroblast transformation[29]. Our results demonstrated intramyocardial Notum injection downregulated α-SMA and fibronectin 1 in the infarct marginal zone, inhibited myofibroblast generation, and reduced collagen I production, mitigating MI-induced CF.

TGF-β stands as a pivotal cytokine in CF regulation[30]. TGF-β1 triggers fibroblast-to-myofibroblast transformation, and regulates collagen and ECM production, promoting myocardial fibrosis[31]. *In vitro*, Notum pharmacological intervention reversed fibroblast-to-myofibroblast differentiation induced by TGF-β1, curbed fibroblast proliferation and migration, and post-transcriptionally regulated collagens 1α1 and 3α1 production. Both *in vitro* and *in vivo* results showcased Notum regulating fibroblast activation post-injury, inhibiting ECM secretion, suggesting Notum's role

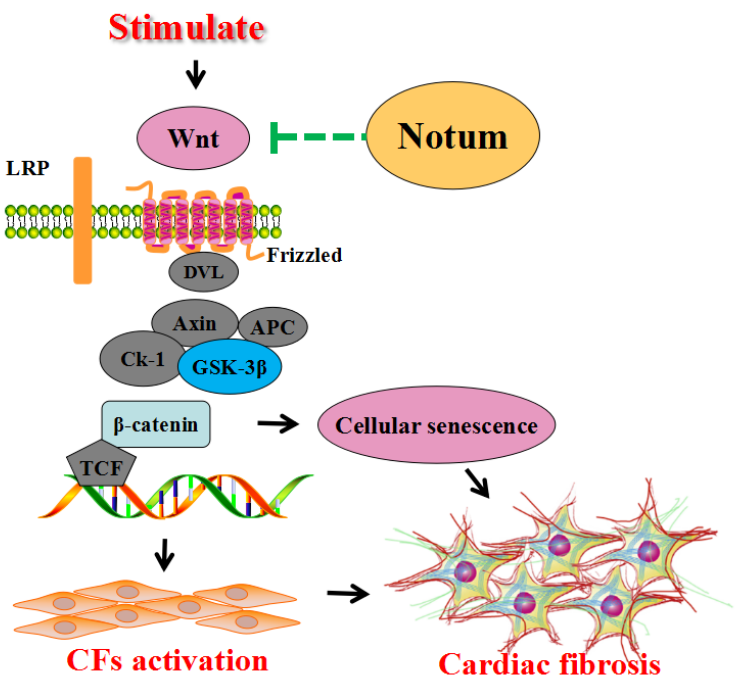

Fig. 6 Schematic diagram for the proposed mechanism for the anti-fibrotic effect of Notum in the heart. Upon receiving stimuli, cardiac fibroblasts undergo activation of the canonical WNT/β-catenin signaling pathway, promoting the fibrogenesis and senescence of cardiac fibroblasts, ultimately leading to cardiac fibrosis (CF). This triggers Notum, a carboxylesterase, to exert an anti-fibrotic effect *via* inhibiting WNT/β-catenin signaling.

beyond Wnt/β-catenin signaling modulation.

Cellular senescence, a stable cell-cycle arrest, occurs in various physiological and pathological conditions and influences tissue remodeling during development or after injury[32]. Senescent cells express specific markers, including senescence-related β-galactosidase activity and up-regulation of proteins, such as p16, p19, p21, and p53. Gourdie *et al.* demonstrated that P16 was a tumor suppressor gene[33]. Kologrivova's team found that MI could induce cardiac fibroblast senescence, inhibit the production of reparative myofibroblasts, interfere heart repair, and induce heart rupture, all of which are related to activation of the p53 signaling pathway[34]. Our study revealed Notum's inhibition of increased p53 expression in cardiac fibroblast senescence induced by H_2O_2 and mRNA level regulation of P21 and P16. Immunohistochemical experiments corroborated Notum's inhibition of P16 expression in the infarct border zone of MI mice, further elucidating Notum's protective role in CF. Notum, being an extracellular enzyme, offers diverse pharmacological intervention possibilities due to its recognized chemical structure and characteristics, rendering it a promising therapeutic agent.

In conclusion, our present work validates Notum's cardioprotective role in MI-induced CF. Notum's modulation of the Wnt/β-catenin pathway and inhibition of cardiac fibroblast senescence curbed abnormal activation and myofibroblast phenotypic differentiation, thereby inhibiting fibrosis. These findings provide propose a novel treatment avenue for CF.

Acknowledgments

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Ethical approval

All study procedures were conducted in compliance with the guidelines of Harbin Medical University and approved by the Ethical Committees of Harbin Medical University (IRB3022621).

Declaration of interests

All authors have no competing interests to declare, financial or otherwise.

Availability of Data and Materials

All data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

Liang H H and Lu Y J investigated the project application, subject construction and edited the manuscript. Jin T Z, Fang R N and Ye Z composed the manuscript, planned the experiments and data management. Fang R N, Ye Z, Li Y, and Su W performed cellular and molecular biological experiments, conducted data analysis. Shan H L and Wang Q Q performed animal studies and analyzed the data. Li T Y helped perform the analysis with constructive discussions.

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