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ORIGINAL ARTICLE

Polypyrimidine tract binding protein 1 exacerbates cardiac fibrosis by regulating fatty acid-binding protein 5

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Abstract

Aims The activation of cardiac fibroblasts (CFs) leads to overproduction of collagens and subsequently cardiac fibrosis. However, the regulatory mechanism of CF function in the process of cardiac fibrosis remains unclear. This work investigated the function of polypyrimidine tract binding protein 1 (PTBP1)/nuclear receptor NR4A1 (Nur77)/fatty acid-binding protein 5 (FABP5) axis in myocardial fibrosis.

Methods and results Cardiac fibrosis was induced in mice suffered left anterior descending ligation. In parallel, neonatal mouse CFs were isolated and stimulated with transforming growth factor-β1 (TGF-β1). Cardiac fibrosis was evaluated by Masson's trichrome staining. Expression of PTBP1, Nur77, FABP5, collagen I, and collagen III was measured by quantitative real-time PCR and western blotting. Proliferation of CFs was assessed by 5-ethynyl-2′-deoxyuridine assay. Molecular interaction was validated by RNA-binding protein immunoprecipitation, chromatin immunoprecipitation, and dual luciferase reporter assay. PTBP1 was up-regulated ($P < 0.05$), whereas Nur77 ($P < 0.05$) and FABP5 ($P < 0.05$) were down-regulated in the fibrotic hearts of mice and TGF-β1-exposed CFs. PTBP1 overexpression facilitated proliferation ($P < 0.05$) and collagen I ($P < 0.05$) and collagen III ($P < 0.05$) expression of CFs after stimulation with TGF-β1. PTBP1 reduced Nur77 stability ($P < 0.05$) to inhibit Nur77 expression ($P < 0.05$) in CFs. Nur77 bound to FABP5 promoter to promote the transcription ($P < 0.05$) and expression ($P < 0.05$) of FABP5. Silencing of Nur77 or FABP5 abolished the inhibitory effect of PTBP1 knockdown on proliferation ($P < 0.05$) and collagen I ($P < 0.05$) and collagen III ($P < 0.05$) expression of CFs *in vitro*. PTBP1 depletion ameliorated cardiac fibrosis ($P < 0.05$), α-smooth muscle actin ($P < 0.05$), and collagen I ($P < 0.05$) expression in myocardial infarction mice through regulating Nur77/FABP5 pathway ($P < 0.05$) *in vivo*.

Conclusions PTBP1 contributed to cardiac fibrosis via promoting CF proliferation and collagen deposition through Nur77 mRNA decay and subsequent transcription inhibition of FABP5. Our findings suggest that PTBP1/Nur77/FABP5 axis may be potential targets for cardiac fibrosis therapy.

Keywords Cardiac fibrosis; PTBP1; Nur77; FABP5; Cardiac fibroblasts

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Introduction

Cardiac fibrosis, featured by hyperproliferation of cardiac fibroblasts (CFs) and accumulation of redundant extracellular matrix in the myocardium, occurs in a wide range of cardiovascular disorders, including myocardial infarction (MI).[1] The trans-differentiation of CFs into myofibroblasts may result in the overproduction of collagens, such as collagen I and collagen III, causing the imbalance of collagen synthesis and degradation and ultimate myocardial fibrosis.[2] At present, there is a lack of measures that can effectively control the development of cardiac fibro. Thus, the development of new anti-fibrotic interventions is of great significance to improve cardiac function for patients with heart disorders.

Polypyrimidine tract binding protein 1 (PTBP1) belongs to the heterogeneous nuclear ribonucleoprotein family.[3] PTBP1 is ubiquitously expressed in various tissues, which exerts various biological functions via influencing RNA metabolism, such as stability, splicing, transport, and degradation.[4] Notably, the fibrotic roles of PTBP1 have been documented in multiple tissues, including liver,[5] lung,[6] and heart.[7] A previous study revealed that miR-133b-mediated PTBP1 inhibition relieved cardiac apoptosis and fibrosis triggered by doxorubicin.[7] However, the detailed mechanism of PTBP1 in cardiac fibrosis remains unexploited, which deserves to be further investigated.

Nuclear receptor NR4A1 (Nur77), attached to the nuclear receptor superfamily, has been recognized to participate in a series of cellular processes, especially fibrosis. Ma *et al*. reported that Nur77 deficiency promoted transforming growth factor-β (TGF-β)/Smad2/3-mediated transcription and expression of collagen I, leading renal tubulointerstitial fibrosis.[8] Besides, Nur77 knockout aggravated myocardial fibrosis in mice through facilitating endothelial-to-mesenchymal transition.[9] Interestingly, Starbase database predicted that PTBP1 was a potential RNA-binding protein (RBP) of Nur77. So far, whether PTBP1 contributes to cardiac fibrosis via regulating Nur77 has not been clarified.

Fatty acid-binding protein 5 (FABP5), as a member of FABP family, plays crucial roles in lipid metabolism and homeostasis.[10] Mounting evidence has also demonstrated that FABP5 acted as a driver for growth, metastasis, and angiogenesis in multiple malignancies.[11,12] As documented by Gao *et al*., FABP5 down-regulation caused cardiac hypertrophy and fibrosis via inducing myofibroblast activation and mitochondrial dysfunction.[13] Bioinformatics analysis indicated that Nur77 could directly bind to the promoter of FABP5. In this context, we predicted that PTBP1 might bind to Nur77 to reduce the mRNA stability of Nur77, which subsequently inhibit FABP5 transcription, thereby accelerating cardiac fibrosis.

To verify the above hypothesis, the *in vitro* and *in vivo* models were established to explore the function of PTBP1/Nur77/FABP5 axis in hyperproliferation and collagen deposition of CFs during cardiac fibrosis.

Materials and methods

Animal model

Male C57BL/6J mice (8 weeks old, weighing 21–25 g) were purchased from Slac Jingda Laboratory Animal Co., Ltd. (Hunan, China). The mice were randomly divided into six groups: sham, MI, MI + Adv-sh-scramble, MI + Adv-sh-PTBP1, MI + Adv-sh-PTBP1 + Adv-sh-Nur77, and MI + Adv-sh-PTBP1 + Adv-sh-FABP5. Anaesthesia was administered by intraperitoneal injection of 60 mg/kg sodium pentobarbital. Then, the left lateral thoracotomy was performed, followed by permanent left anterior descending (LAD) ligation to induce MI. The sham mice were subjected to the same surgical procedure except for LAD ligation. During MI surgery, adenoviruses carrying sh-scramble, sh-PTBP1, sh-Nur77, or sh-FABP5 (GenePharma, Shanghai, China) were injected into the left ventricular anterior wall of mice (50×10^{11} PFU per mouse). The mice were euthanized at 1, 2, 3, and 4 weeks after the operation, and the hearts were collected. All procedures were approved by the Ethics Committee of Taihe Hospital, Hubei University of Medicine.

Masson's trichrome staining

The collagen fibres in heart tissues were visualized by Masson's trichrome staining. Briefly, the heart tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm slices. Subsequently, the slices were stained using the Masson's trichrome stain kit (Solarbio, Beijing, China). The photomicrographs were captured under a light microscope, and the percentage of fibrotic areas (blue) was quantified using the ImageJ software.

Quantitative real-time PCR

Total RNA was obtained using RNAzol®RT reagent (GeneCopoeia, USA) and then reversely transcribed into cDNA using the SureScript™ First-Strand cDNA Synthesis Kit (GeneCopoeia). Quantitative real-time PCR (qRT-PCR) was carried out using the BlazeTaq™ SYBR® Green qPCR Mix 2.0 (GeneCopoeia). The relative gene levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in *Table 1*.

Western blotting

The tissue or cell samples were incubated with radioimmunoprecipitation assay (RIPA) protein lysate (Camilo Biological, Nanjing, China) to isolate protein. Protein concentration was detected using the bicinchoninic acid (BCA) assay kit (Bio-Rad, USA). After denaturation, the protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with defatted milk and probed with primary antibodies against PTBP1 (#72669, 1:1000, Cell Signaling Technology, USA), collagen I (ab270993, 1:1000, Abcam, UK), collagen III (ab7778, 1:5000, Abcam), α-smooth muscle actin (α-SMA) (ab124964, 1:1000, Abcam), Nur77 (sc-365113, 1:500, Santa Cruz, USA), FABP5 (#39926,

Table 1 Oligonucleotide primer sets for qPCR

Name	Sequence (5′-3′)	Length
Mouse Ptbp1 F	GATCCGACGAGCTCTTCTC	19
Mouse Ptbp1 R	CTATCGTTTCCATTGGCTGC	20
Mouse Nur77 F	TGTCCGCTCTGGTCCTCATCAC	22
Mouse Nur77 R	TCTCCTGCCACGGTAGCCATG	21
Mouse Fabp5 F	AGAGCACAGTGAAGACGAC	19
Mouse Fabp5 R	CATGACACACTCCACGATCA	20
Mouse α-SMA	GTCCCAGACATCAGGGAGTAA	21
Mouse α-SMA	TCGGATACTTCAGCGTCAGGA	21
Mouse Postn	ATAACCAAAGTCGTGGAACC	20
Mouse Postn	TGTCTCCCTGAAGCAGTCTT	20
Mouse collagen I	CCTGGCAAAGACGGACTCAAC	21
Mouse collagen I	GCTGAAGTCATAACCGCCACTG	22
Mouse Fn1	ATCCAGTCCACAGCCATTCC	20
Mouse Fn1	GGAAGGGTAACCAGTTGGGG	20
Mouse GAPDH F	CATCTTCTTGTGCAGTGCC	19
Mouse GAPDH R	CAAATCCGTTCACACCGAC	19
Human PTBP1	AGCGCGTGAAGATCCTGTT	19
Human PTBP1	CAGGGGTGAGTTGCCGTAG	19
Human Nur77	ACCCACTTCTCCACACCTTG	20
Human Nur77	ACTTGGCGTTTTTCTGCACT	20
Human FABP5	TGGCCAAGCCAGATTGTATCA	21
Human FABP5	CTGATGCTGAACCAATGCACC	21
Human GAPDH F	ACCACAGTCCATGCCATCAC	20
Human GAPDH R	TCCACCACCCTGTTGCTGTA	20

Abbreviations: F, forward; FABP5, fatty acid-binding protein 5; Fn1, fibronectin 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Nur77, nuclear receptor NR4A1; Postn, periostin; PTBP1, polypyrimidine tract binding protein 1; qPCR, quantitative PCR; R, reverse; α-SMA, α-smooth muscle actin.

1:1000, Cell Signaling Technology), and GAPDH (ab9485, 1:2500, Abcam) overnight at 4°C. After incubation with appropriate secondary antibodies (Abcam), the protein bands were detected using an enhanced chemiluminescence (ECL) detection kit (Pierce, USA).

Primary culture of cardiac fibroblasts and treatment

To isolate primary CFs, the heart was obtained from newborn C57BL/6J mouse and sliced into approximately 5 mm pieces. After digestion in 0.08% trypsin and 0.05% collagenase II, the cells were collected by centrifugation at 1000 rpm for 10 min and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Thermo Fisher, USA) containing 15% foetal bovine serum (Thermo Fisher). After culture for 24 h, the non-adherent cells were removed and the adherent CFs in Passages 1–3 were used in this study. The primary CFs were stimulated with 20 ng/mL TGF-β1 for 24 h.

Cell culture

Human CFs were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and were maintained in a specific culture medium (Procell) at 37°C with 5% CO_2. Human CFs were exposed to 20 ng/mL TGF-β1 for 24 h.

Cell transfection

Two specific siRNAs targeting PTBP1 (si-PTBP1#1, #2), si-Nur77#1, #2, si-FABP5#1, #2, and negative control siRNA (si-NC) were obtained from GenePharma. Primary CFs were transfected with shRNAs using Lipofectamine 3000 (Thermo Fisher) for 48 h. Adenoviruses carrying overexpressing plasmid for PTBP1, Nur77, or vector (GenePharma) were transfected into CFs at a multiplicity of infection (MOI) of 10 × PFU per cell for 48 h. The silencing efficiencies of siRNAs were measured and shown in *Figure S4*. The siRNAs with a higher silencing efficiency were selected for the subsequent experiments. siRNA sequences are as follows: si-PTBP1#1: sense, 5′-CCCUCAUUGACCUGCACAATT-3′; antisense, 5′-UUGUGCA GGUCAAUGAGGGTT-3′; si-PTBP1#2: sense, 5′-GCCUCAAC GUCAAGUACAATT-3′; antisense, 5′-UUGUACUUGACGUUGAG GCTT-3′; si-Nur77#1: sense, 5′-GGAGAUGCCCUGU AUUCAAGC-3′; antisense, 5′-UUGAAUACAGGGCAUCUCCAG-3′; si-Nur77#2: sense, 5′-GCUACUGUAAAUACAGAAAGG-3′; antisense, 5′-UUUCUGUAUUUACAGUAGCGU-3′; si-FABP5#1: sense, 5′-ACGUGUUAGUGCUAAUUUAUG-3′; antisense, 5′-UAAAUUAGCACUAACACGUUU-3′; and si-FABP5#2: sense, 5′-CGACUGUGUUCUCUUGUAACC-3′; antisense, 5′-UUACAAG AGAACACAGUCGUC-3′.

5-Ethynyl-2′-deoxyuridine proliferation assay

CFs were planted onto 96-well plates at a density of 1×10^4 cells per well. After various treatments, CFs were treated with 50 μM 5-ethynyl-2′-deoxyuridine (EdU) (RiboBio, Guangzhou, China) for 4 h. Then, CFs were fixed in 4% paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI). The fluorescence was observed under a fluorescence microscope (Olympus, Japan), and the percentage of EdU-positive cells was quantified using the ImageJ software.

RNA stability

To evaluate the stability of Nur77 mRNA, CFs were exposed to 5 μg/mL actinomycin D for 0, 2, 4, 6, and 8 h. Then, the expression of Nur77 was assessed by qRT-PCR as described above.

Target prediction

The Starbase online tool (https://starbase.sysu.edu.cn/index.php) and RBPsuite database (http://www.csbio.sjtu.edu.cn/bioinf/RBPsuite/) were used to predict the RBPs of Nur77. hTFtarget database (http://bioinfo.life.hust.edu.cn/hTFtarget#!/) was adopted to predict the target gene of transcription factor Nur77.

RNA-binding protein immunoprecipitation

The interaction between PTBP1 and Nur77 mRNA was validated by RNA-binding protein immunoprecipitation (RIP) assay using the Magna RIP™ RIP Kit (Millipore). The lysates of CFs (150 μg protein) were incubated with magnetic beads conjugated with anti-PTBP1 (Cell Signaling Technology) or anti-IgG (Cell Signaling Technology) at 4°C overnight. IgG was used as a negative control. A part of CF lysates was served as the input. After treatment with proteinase K, the expression of Nur77 in immunoprecipitation complexes was assessed by qPCR.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was carried out using the ChIP assay kit (Upstate, USA) following the manufacturer's protocol. Chromatin solution was prepared from CFs by cross-linking with 1% formaldehyde, followed by sonication. A portion of chromatin solution was used as the input, and the residual chromatin was incubated with antibody against Nur77 (Santa Cruz) or IgG overnight at 4°C. IgG was used as a negative control. After purification, the recovered DNA was assayed by qPCR. The primer sequences are as follows: forward, 5′-GCCTCCAGGTGACCCCTCT-3′; reverse, 5′-GTAACCGGCGTATCGCG-3′.

Dual luciferase reporter assay

The promoter sequences of FABP5 (Chr3: 10075645-10077744) were subcloned into pGL3 luciferase reporter vector. Subsequently, the constructed plasmids together with oe-NC, oe-Nur77, si-NC, or si-Nur77 were co-transfected into the 293T cells. The luciferase activity was measured at 48 h after the transfection using the Dual-Luciferase Reporter Assay System kit (Promega, USA).

Statistical analysis

Experimental data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 7 (La Jolla, CA, USA). Student's *t*-test or one-way ANOVA followed by the Tukey test was carried out to assess differences between two groups or among more than two groups. P values < 0.05 were considered statistically significant.

Results

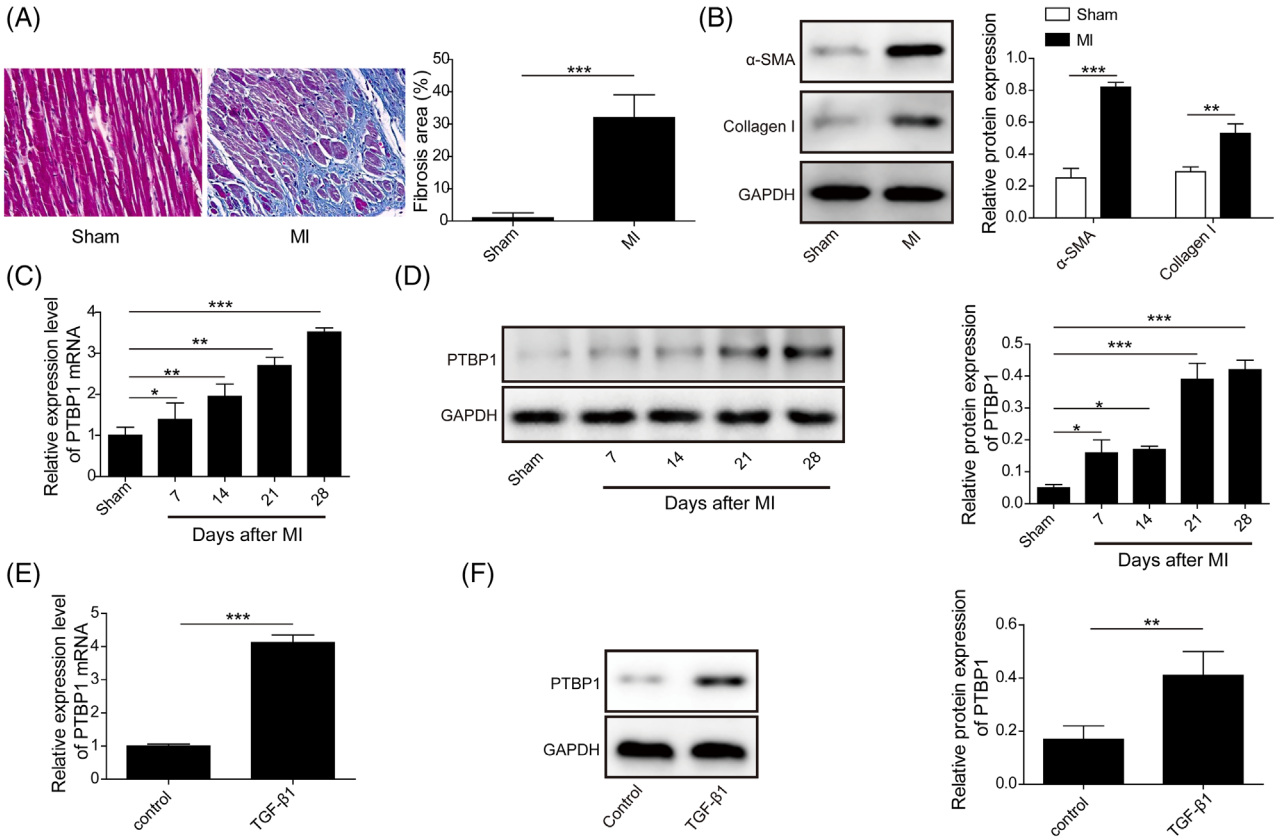
Increased level of polypyrimidine tract binding protein 1 in myocardial infarction mice and transforming growth factor-β1-exposed cardiac fibroblasts

We first examined the degree of myocardial fibrosis using Masson's trichrome staining. As presented in *Figure 1A*, the cardiac fibrosis area in MI mice remarkably elevated as compared with the sham mice at 28 days after MI. Besides, α-SMA and collagen I were up-regulated in the infracted hearts of MI mice (*Figure 1B*). Notably, the mRNA and protein levels of PTBP1 in the infracted hearts of mice were strikingly increased along with the time extension (*Figure 1C,D*). Moreover, the expression of PTBP1 was evaluated in TGF-β1-treated mouse CFs *in vitro*. Consistently, the mRNA and protein abundance of PTBP1 was enhanced in CFs after TGF-β1 exposure (*Figure 1E,F*). As shown in *Figure S5*, PTBP1 was up-regulated, whereas NUR77 and FABP5 were down-regulated in TGF-β1-stimulated human CFs. Therefore, PTBP1 was up-regulated in the *in vivo* and *in vitro* models of cardiac fibrosis.

Polypyrimidine tract binding protein 1 promoted collagen accumulation and proliferation of transforming growth factor-β1-exposed cardiac fibroblasts

To determine the biological function of PTBP1 in TGF-β1-treated CFs, PTBP1 was overexpressed or silenced by transfection with oe-PTBP1 or si-PTBP1. As illustrated in *Figure 2A*, the overexpression and silencing efficiency of PTBP1 was verified by qRT-PCR. PTBP1 overexpression or TGF-β1 stimulation evidently raised the protein levels of PTBP1, collagen I, and collagen III; however, PTBP1 depletion partly reversed TGF-β1-induced up-regulation of PTBP1, collagen I, and collagen III (*Figure 2B*). Furthermore, EdU proliferation assay indicated that the proliferative ability was enhanced in PTBP1-overexpressed or TGF-β1-stimulated CFs and that PTBP1 knockdown weakened the promotive effect of TGF-β1 on proliferation of CFs (*Figure 2C*). These results suggested that PTBP1 was involved in TGF-β1-induced collagen accumulation and proliferation of CFs.

Figure 1 Up-regulation of polypyrimidine tract binding protein 1 (PTBP1) in the *in vivo* and *in vitro* models of cardiac fibrosis. (A) Masson's trichrome staining was adopted to evaluate cardiac fibrosis in mice ($N = 5$). (B) The protein levels of α-smooth muscle actin (α-SMA) and collagen I in the infracted heart tissues were measured by western blotting ($N = 5$). (C, D) Quantitative real-time PCR and western blotting analysed PTBP1 expression in the infracted heart tissues at 1, 2, 3, and 4 weeks after operation ($N = 5$). (E, F) The levels of PTBP1 in cardiac fibroblasts treated with or without transforming growth factor-β1 (TGF-β1) were assessed by quantitative real-time PCR and western blotting ($N = 3$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MI, myocardial infarction. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



Polypyrimidine tract binding protein 1 bound to nuclear receptor NR4A1 mRNA to reduce its stability

PTBP1, as an RBP, has been demonstrated to exert its function via interaction with target RNA.[14] By overlapping Starbase and RBPsuite databases, 52 RBPs were predicted to interplay with Nur77 (*Figure S3A*). Subsequently, we determined the expression of Nur77 in CFs after transfection with siRNAs specific for these 52 RBPs, respectively. We found that four RBP siRNAs led to increase in Nur77 expression (fold change ≥ 2) and that four RBP siRNAs led to decrease in Nur77 expression (fold change ≤ 0.5) (*Figure S3B*). Because si-PTBP1 could promote Nur77 expression with the highest fold change, we selected PTBP1 as the upstream regulator of Nur77 in this study. As assessed by qRT-PCR and western blotting, Nur77 level was reduced in PTBP1-overexpressed CFs but raised in PTBP1-silenced CFs (*Figure 3A,B*). In addition, RIP assay revealed that Nur77 mRNA could be immunoprecipitated by anti-PTBP1 (*Figure 3C*). Moreover, the stability of Nur77 mRNA in CFs was increased after PTBP1 deficiency, whereas PTBP1 overexpression resulted in the opposite result (*Figure 3D*). Furthermore, the effect of Nur77 on myofibroblast differentiation was investigated. Our results indicated that si-Nur77 transfection enhanced the levels of myofibroblast markers [α-SMA and periostin (POSTN)] and extracellular matrix proteins [collagen I and fibronectin 1 (FN1)] in TGF-β-stimulated CFs (*Figure S2*). These observations indicated that Nur77 was destabilized by PTBP1 to promote myofibroblast differentiation.

Nuclear receptor NR4A1 contributed to transcription and expression of fatty acid-binding protein 5

FABP5, a member of FABP family, promoted growth, metastasis, and angiogenesis in multiple cancers.[11,12] In mouse CFs, knockdown of FABP5 could promote TGF-β1-induced

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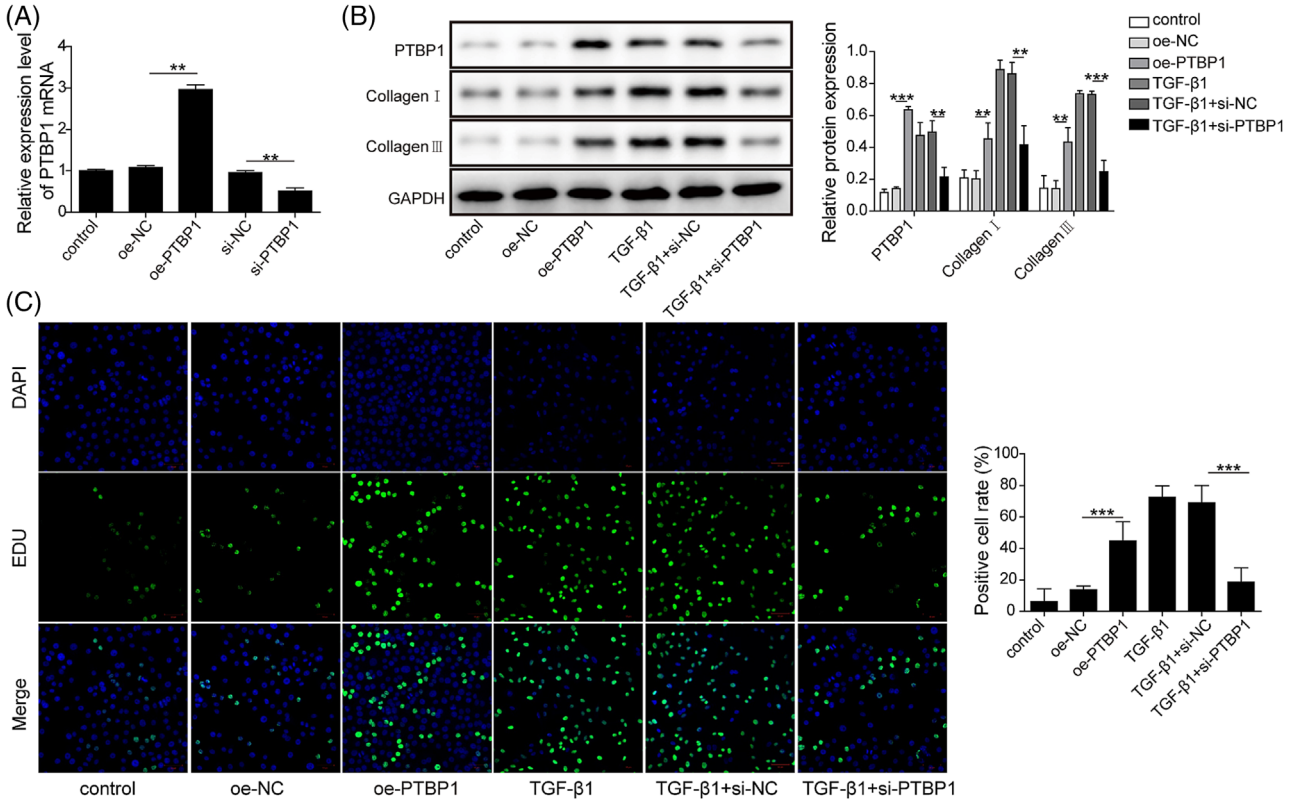
Figure 2 Polypyrimidine tract binding protein 1 (PTBP1) contributed to transforming growth factor-β1 (TGF-β1)-induced collagen accumulation and proliferation of cardiac fibroblasts (CFs). CFs were transfected with oe-PTBP1 or si-PTBP1. (A) Quantitative real-time PCR analysis of PTBP1 level in CFs with various transfections. (B) The protein abundance of PTBP1, collagen I, and collagen III was detected by western blotting. (C) 5-Ethynyl-2′-deoxyuridine (EdU) proliferation assay was utilized to determine proliferative ability of CFs ($N = 3$). DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. $^{**}P < 0.01$, $^{***}P < 0.001$.



oxidative stress, mitochondrial dysfunction, and the expression of myofibroblast activation marker genes.[13] So far, the detailed mechanism by which FABP5 is modulated in cardiac fibrosis has not been clarified. hTFtarget database predicated that Nur77 could bind to the promoter of FABP5, suggesting that FABP5 might be a target gene of Nur77. To evaluate the regulation of Nur77 in FABP5 expression, CFs were transfected with oe-Nur77 or si-Nur77. We found that overexpression of Nur77 remarkably enhanced the mRNA and protein levels of FABP5 in CFs. On the contrary, deficiency of Nur77 reduced FABP5 expression in CFs (*Figure 4A,B*). ChIP assay demonstrated that Nur77 directly bound to the promoter of FABP5 (*Figure 4C*). Besides, the luciferase activity of FABP5 was decreased by Nur77 knockout but increased by Nur77 overexpression, indicating the promotive effect of Nur77 on FABP5 transcription (*Figure 4D*). The interaction between Nur77 and PTBP1 was weakened upon TGF-β1 administration (*Figure S1*). Taken together, Nur77 bound to FABP5 promoter to facilitate its transcription in CFs.

Polypyrimidine tract binding protein 1 facilitated collagen accumulation and proliferation of cardiac fibroblasts through nuclear receptor NR4A1/fatty acid-binding protein 5 axis

Western blotting results revealed that the protein levels of Nur77 and FABP5 in the heart tissues of MI mice were gradually declined with the prolonging of postoperative time (*Figure 5A*). Additionally, the expression of Nur77 and FABP5 was reduced in TGF-β1-treated CFs, which could be reversed by PTBP1 depletion (*Figure 5B,C*). Co-transfection with si-Nur77 or si-FABP5 caused decrease in Nur77 or FABP5 expression as compared with TGF-β1 + si-PTBP1 group, and Nur77 expression was not affected by FABP5 silencing (*Figure 5B,C*). Furthermore, PTBP1 down-regulation reduced collagen I and collagen III expression in TGF-β1-exposed CFs, whereas the inhibitory effects of si-PTBP1 on collagen I and collagen III expression were counteracted by silencing of

Figure 3 Polypyrimidine tract binding protein 1 (PTBP1) reduced mRNA stability of nuclear receptor NR4A1 (Nur77) via direct interaction with Nur77 mRNA. (A, B) Expression of Nur77 in PTBP1-overexpressed or -silenced cardiac fibroblasts was measured by quantitative real-time PCR and western blotting. (C) The binding of PTBP1 to Nur77 mRNA was evaluated by RNA-binding protein immunoprecipitation assay. (D) After exposure to actinomycin D, the stability of Nur77 was assessed by quantitative real-time PCR (N = 3). GAPDH, glyceraldehyde 3-phosphate dehydrogenase. *$P < 0.05$, **$P < 0.01$.

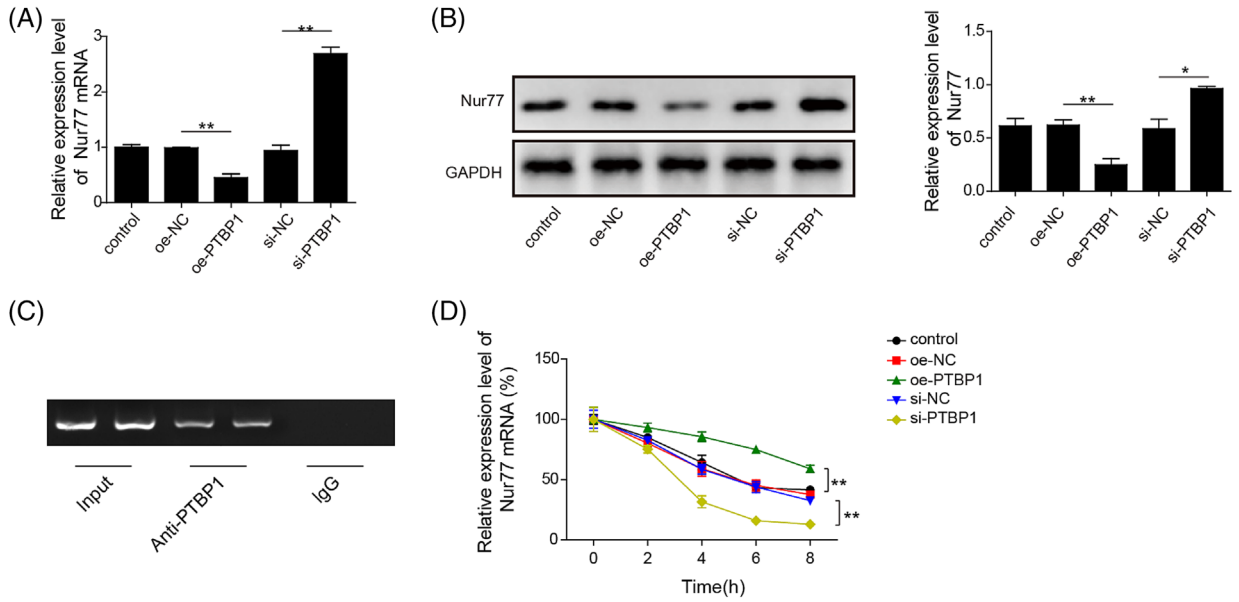
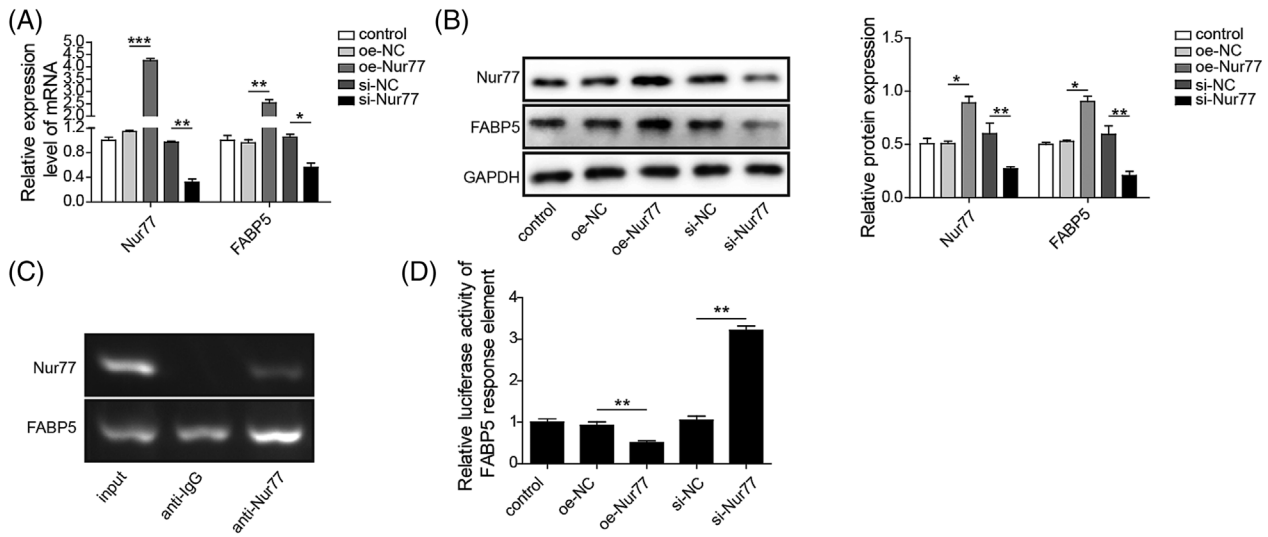


Figure 4 The transcription and expression of fatty acid-binding protein 5 (FABP5) was activated by nuclear receptor NR4A1 (Nur77). Cardiac fibroblasts were transfected with oe-Nur77 or si-Nur77. (A, B) Quantitative real-time PCR and western blotting analysis of Nur77 and FABP5 expression in cardiac fibroblasts with multiple treatments. (C) Chromatin immunoprecipitation assay validated the interaction between Nur77 and FABP5 promoters. (D) The regulation of Nur77 in FABP5 transcription was determined by dual luciferase reporter assay (N = 3). GAPDH, glyceraldehyde 3-phosphate dehydrogenase. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.



Nur77 or FABP5 (*Figure 5C*). Accordingly, si-PTBP1-mediated inhibition in proliferation of TGF-β1-treated CFs was abolished by co-transfection with si-Nur77 or si-FABP5 (*Figure 5D*). The above findings suggested that Nur77/FABP5 pathway was involved in PTBP1-mediated collagen accumulation and proliferation of CFs.

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Figure 5 Polypyrimidine tract binding protein 1 (PTBP1)/nuclear receptor NR4A1 (Nur77)/fatty acid-binding protein 5 (FABP5) axis modulated transforming growth factor-β1 (TGF-β1)-induced collagen accumulation and proliferation of cardiac fibroblasts (CFs). (A) Western blotting analysis of Nur77 and FABP5 levels in heart tissues of mice (N = 5). CFs were transfected with si-PTBP1 in combination with or without si-Nur77 or si-FABP5. (B) The mRNA levels of Nur77 and FABP5 were assessed by quantitative real-time PCR. (C) The protein levels of Nur77, FABP5, collagen I, and collagen III were detected by western blotting. (D) The proliferative capacities of CFs from different groups were evaluated by 5-ethynyl-2′-deoxyuridine (EdU) proliferation assay (N = 3). DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MI, myocardial infarction. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

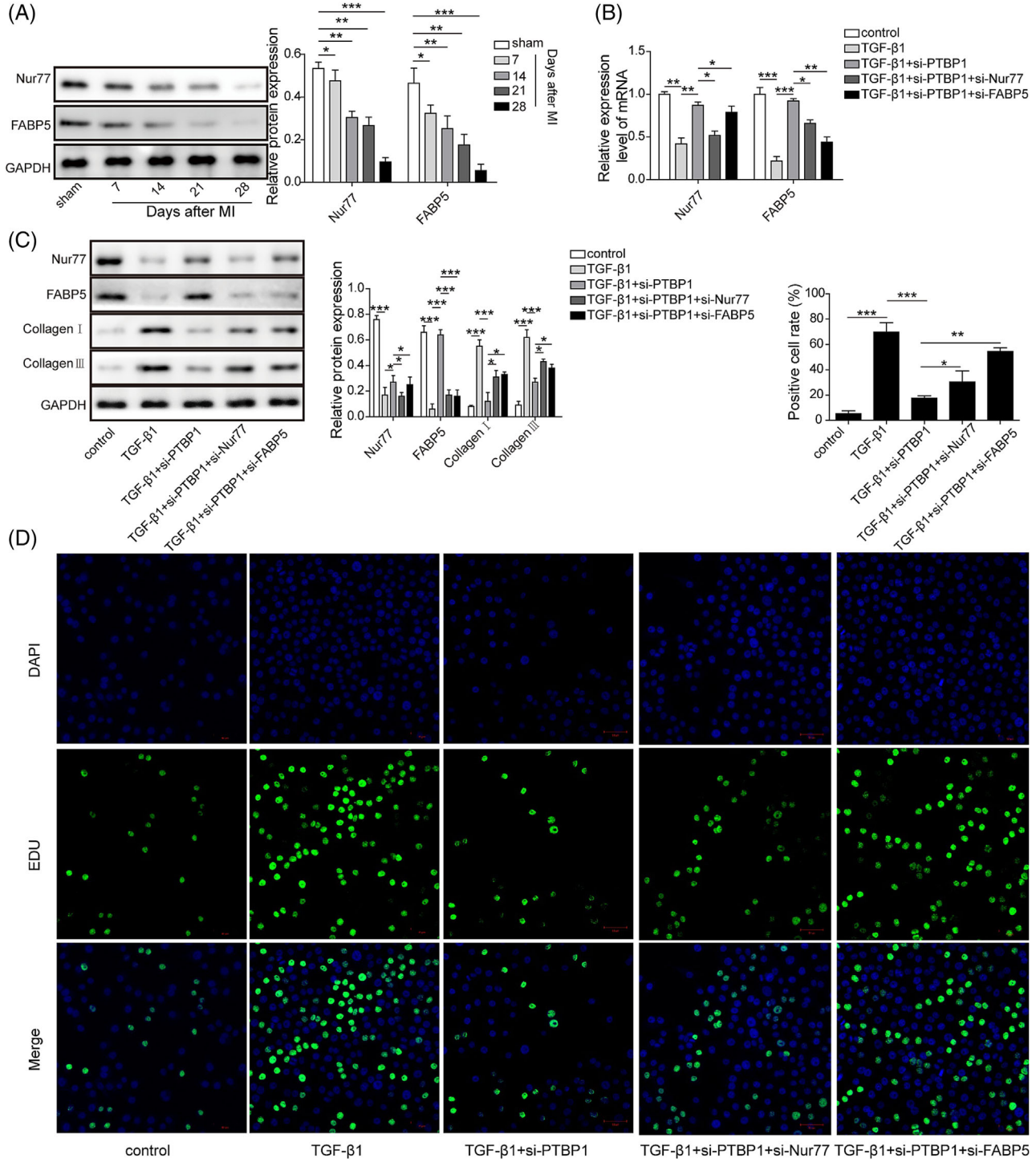

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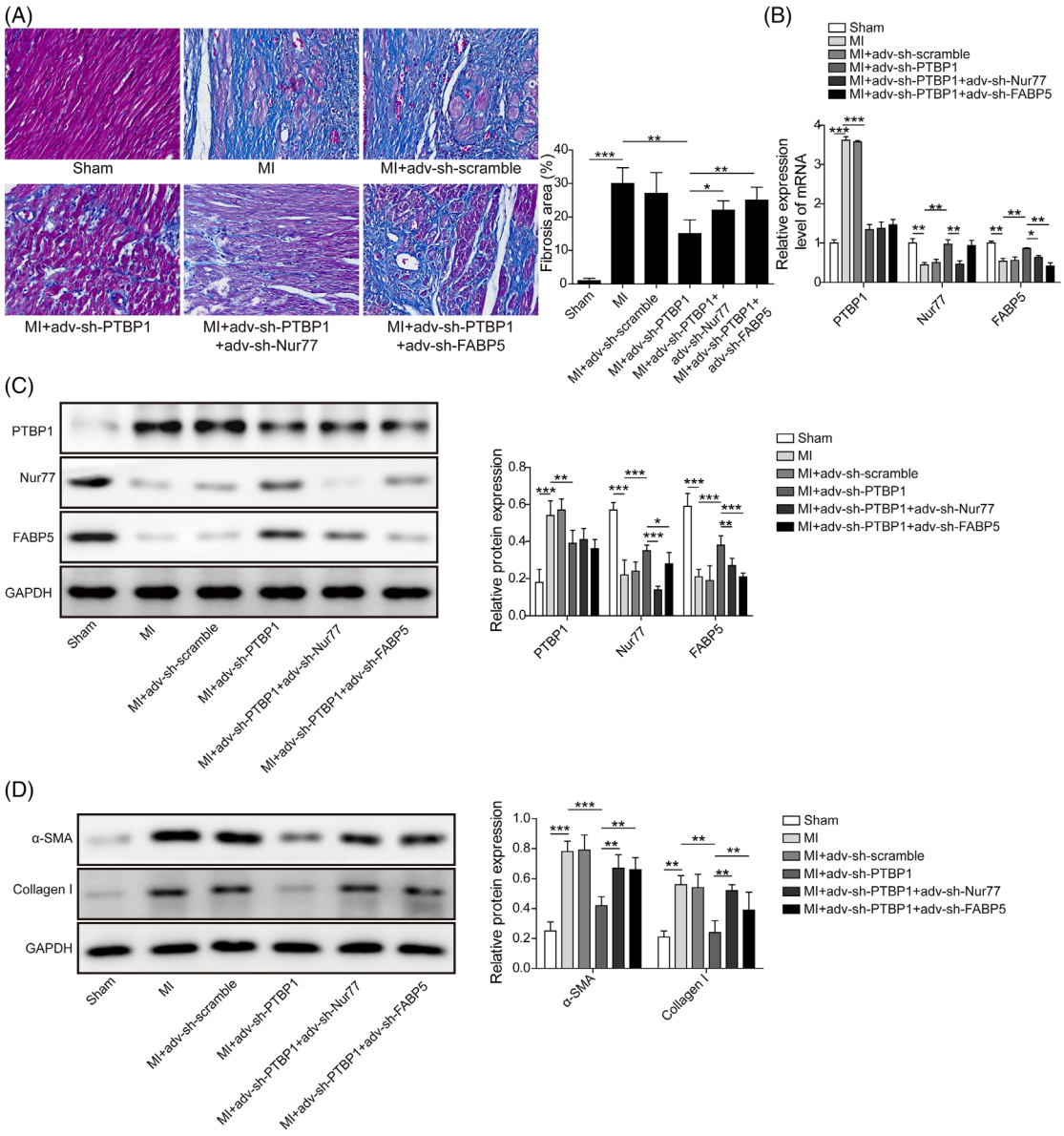
Figure 6 Polypyrimidine tract binding protein 1 (PTBP1) silencing inhibited *in vivo* cardiac fibrosis by regulating nuclear receptor NR4A1 (Nur77)/fatty acid-binding protein 5 (FABP5) pathway. (A) Cardiac fibrosis in mice was evaluated by Masson's trichrome staining at 28 days after myocardial infarction (MI) ($N = 5$). (B, C) Quantitative real-time PCR and western blotting analysis of PTBP1, Nur77, and FABP5 expression in the infracted heart tissues at 28 days after MI ($N = 5$). (D) The protein levels of α-smooth muscle actin (α-SMA) and collagen I in the infracted heart tissues were detected by western blotting at 28 days after MI ($N = 5$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



Polypyrimidine tract binding protein 1 knockdown ameliorated cardiac fibrosis in myocardial infarction mice by regulating nuclear receptor NR4A1/fatty acid-binding protein 5 pathway

To clarify the influence of PTBP1/Nur77/FABP5 axis on cardiac fibrosis *in vivo*, adenoviruses containing sh-PTBP1, sh-Nur77, or sh-FABP5 were delivered into mice following MI. Masson's trichrome staining showed that the abundant deposition of collagen in infracted hearts was attenuated by PTBP1 knockdown; however, Nur77 or FABP5 silencing abolished PTBP1 knockdown-mediated inhibition in cardiac fibrosis at 28 days after MI (*Figure 6A*). In addition, the mRNA and protein levels of PTBP1 were increased, whereas Nur77 and FABP5 levels were reduced in the infracted heart tissues, which were reversed in PTBP1-depleted group

at 28 days after MI. In sh-Nur77 or sh-FABP5 group, sh-PTBP1-mediated up-regulation of Nur77 or FABP5 was counteracted, whereas FABP5 silencing did not affect Nur77 expression (*Figure 6B,C*). Accordingly, PTBP1 deficiency down-regulated α-SMA and collagen I in the infracted hearts of MI mice, which was abrogated by knockdown of Nur77 or FABP5 at 28 days after MI (*Figure 6D*). Collectively, these findings suggested that the *in vivo* cardiac fibrosis after MI surgery was attenuated by PTBP1 knockdown through Nur77/FABP5 pathway.

Discussion

Cardiac fibrosis, the consequence of multiple myocardial disorders, is featured by CF hyperproliferation, activation, and deposition of collagens in the myocardium.[15] The formation of cardiac fibrosis ultimately results in ventricular stiffness and remodelling, myocardial dysfunction, and even heart failure.[1] In our study, we demonstrated that PTBP1 was up-regulated, whereas Nur77 and FABP5 were down-regulated in the fibrotic myocardium of mice and TGF-β1-stimulated CFs. PTBP1 contributed to TGF-β1-induced collagen accumulation and proliferation of CFs via reducing the stability of Nur77 and subsequent transcription inhibition of FABP5. Our data indicated that targeting PTBP1/Nur77/FABP5 axis might be an effective intervention for delaying cardiac fibrosis progression.

PTBP1 acts as a crucial regulator of a broad range of biological processes, such as growth, differentiation, T-cell activation, apoptosis, and embryonic development.[16] Recently, Shu *et al*. reported that PTBP1 was involved in the regulation of pyroptosis and M1 macrophage polarization, which affected liver fibrosis.[5] In addition, up-regulation of PTBP1 by lncRNA HOTTIP accelerated the progression of pulmonary fibrosis.[17] Although the contribution of PTBP1 to doxorubicin-induced cardiomyocyte fibrosis has been documented,[7] the modulation of PTBP1 in CF proliferation and deposition of collagen protein remains unclear. Our results for the first time revealed that PTBP1 facilitated the proliferation and collagen protein expression of CFs, providing novel evidence for PTBP1 as a contributor to cardiac fibrosis.

In terms of mechanism investigation, PTBP1, functioned as an RBP, has been recognized to influence pre-mRNA splicing, mRNA stability, and translation.[18] For instance, PTBP1 conferred chemoresistance of osteosarcoma cells via decreasing the mRNA stability of SLC31A1.[19] Cho *et al*. documented that PTBP1 restrained lung tumourigenesis via interaction with AXL to decrease its mRNA stability.[20] As analysed by bioinformatics, we predicted the potential binding of PTBP1 to Nur77. The cardioprotective action of Nur77 has been widely reported. A previous study showed that Nur77 protected against cardiomyocyte apoptosis by enhancing relaxin-3 transcription.[21] Furthermore, Nur77 attenuated isoproterenol-triggered cardiac hypertrophy and fibrosis through modulating neuropeptide Y (NPY)–neuropeptide Y receptor type 1 (NPY1R) pathway.[22] In this study, Nur77 silencing was demonstrated to facilitate myofibroblast differentiation in mouse CFs, which was contrary to the study by Medzikovic *et al*.[23] We will validate this result in human CFs in our future study. Moreover, we validated the direct binding of PTBP1 to Nur77 mRNA, which led to declined stability of Nur77 mRNA. Our study provided first evidence for the interplay and regulation of PTBP1 in Nur77.

Another important finding in the study was that Nur77 transcriptionally activated FABP5 in CFs. As one of the important transcriptional factors, Nur77 exerts biological roles via modulating its target gene expression.[24] For instance, Nur77 transcriptionally promoted lncRNA WFDC21P expression to inhibit hepatocarcinogenesis by the modulation of glycolysis.[25] In line with the previous observations, here, we found that Nur77 directly bound to FABP5 promoter and induced its transcription and expression. FABP5, an intracellular lipid chaperon, has been revealed to facilitate fatty acid transportation from circulation into heart.[26] Deficiency of FABP5 exacerbated myocardial dysfunction in mice with diabetic cardiomyopathy.[27] A recent study reported that impaired mitochondrial function and cardiac fibrosis were found in FABP5-deficient mice.[13] Here, we demonstrated that Nur77 and FABP5 were lowly expressed in cardiac models. Knockdown of Nur77 or FABP5 abrogated the inhibitory effect of PTBP1 silencing on CF proliferation and collagen accumulation. Therefore, Nur77/FABP5 pathway was involved in PTBP1-mediated cardiac fibrosis.

To sum up, PTBP1 aggravated TGF-β1-induced collagen accumulation and hyperproliferation of CFs. PTBP1 exerted its promotive effects on cardiac fibrosis through reducing the stability of Nur77 mRNA and thereby inhibiting the transcription of FABP5. Therefore, targeting PTBP1/Nur77/FABP5 axis may be intended to prevent cardiac fibrosis.

Conflict of interest

The authors declare that they have no conflict of interest.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The primary mouse CFs were stimulated with 20 ng/mL TGF-β1 for 24 h, and the binding or Nur77 to FABP5

promoter was evaluated by ChIP assay. (N = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S2. The primary mouse CFs transfected with si-NC or si-Nur77 were treated with 20 ng/mL TGF-β1 for 24 h, and the mRNA levels of α-SMA, POSTN, collagen I, and FN1 were detected by qRT-PCR. (N = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S3. (A) The potential RNA-binding proteins (RBPs) binding to Nur77 were predicted by Starbase and RBPsuite databases. (B) The mRNA level of Nur77 in primary mouse CFs transfected with various siRNAs was assessed by qRT-PCR. (N = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S4. The silencing efficiencies of siRNAs in primary mouse CFs were validated by qRT-PCR. (N = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure S5. The human CFs were stimulated with 20 ng/mL TGF-β1 for 24 h, and the mRNA levels of PTBP1, Nur77, FABP5 were determined by qRT-PCR. (N = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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