

Original Research

# PDE-5-Inhibited BMSCs Alleviate High Glucose-Induced Myocardial Fibrosis and Cardiomyocyte Apoptosis by Activating the cGMP/PKG Pathway

Qinghua Huang<sup>1,2</sup>, Jiangbo Ma<sup>1</sup>, Hui Wu<sup>1</sup>, Jieru Zhou<sup>3</sup>, Lei Jiang<sup>4</sup>, Xianming Fei<sup>4,\*</sup><sup>1</sup>Geriatric Medicine Center, Department of Endocrinology, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, 310014 Hangzhou, Zhejiang, China<sup>2</sup>Key Laboratory of Endocrine Gland Diseases of Zhejiang Province, 310014 Hangzhou, Zhejiang, China<sup>3</sup>Jinzhou Medical University, 121001 Jinzhou, Liaoning, China<sup>4</sup>Laboratory Medicine Center, Department of Clinical Laboratory, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, 310014 Hangzhou, Zhejiang, China\*Correspondence: [feixianming@hmc.edu.cn](mailto:feixianming@hmc.edu.cn) (Xianming Fei)

Academic Editor: Rebecca Piccarducci

Submitted: 4 November 2022 Revised: 10 January 2023 Accepted: 12 January 2023 Published: 28 July 2023

## Abstract

**Background:** Phosphodiesterase-5 (PDE-5) inhibitors have been found to play an important cardio-protective role. This study aimed to clarify the inhibitory effects of PDE-5-silenced bone marrow mesenchymal stem cells (BMSCs) on high glucose-induced myocardial fibrosis and cardiomyocyte apoptosis. **Methods:** Cardiomyocytes and fibroblasts of neonatal rats were treated with high glucose (HG), and co-cultured with PDE-5-overexpressed or -knocked down BMSCs. The viability and apoptosis as well as the levels of cytokines, Cardiac troponin I and Vimentin of cardiomyocytes and fibroblasts were studied. The expressions of PDE-5, cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG), in both cells were evaluated. **Results:** BMSCs that silenced PDE-5 facilitated the viability of cardiomyocytes, decreased the viability of fibroblasts, and inhibited the apoptosis of cardiomyocytes and fibroblasts. The contents of collagen-I, collagen-III, tissue inhibitor of metalloproteinase (TIMP)-1 and Dermin in fibroblasts were decreased by the PDE-5 inhibitor, but the levels of matrix metalloproteinase (MMP)-1 in fibroblasts and troponin-I in cardiomyocytes were increased by the PDE-5 inhibitor. PDE-5 inhibitor also suppressed the expression of PDE-5 but up-regulated cGMP and PKG expression in cardiomyocytes and fibroblasts. **Conclusions:** PDE-5-inhibited BMSCs can decrease HG-induced myocardial fibrosis and cardiomyocyte apoptosis by activating the cGMP/PKG pathway, and may play a role in the prevention and treatment of diabetic cardiomyopathy.

**Keywords:** phosphodiesterase-5; BMSCs; diabetic cardiomyopathy; cGMP/PKG pathway; myocardial fibrosis; cardiomyocytes apoptosis

## 1. Introduction

The morbidity and mortality of Diabetes Mellitus (DM) are increasing annually [1]. DM is an important risk factor for the development of cardiovascular diseases. Epidemiological studies have found that the incidence of cardiovascular diseases in DM patients is 2–3 times higher than that in non-DM patients [2]. The structure and function of myocardial tissue in DM patients is affected by hyperglycemia, which can lead to diabetic cardiomyopathy (DC) [3]. The pathological characteristics of DC include myocardial hypertrophy, myocardial collagen proliferation, increased glycogen and lipid droplet deposition, and myocardial interstitial fibrosis. These pathological changes will lead to ventricular remodeling and ultimately decreased cardiac function [4]. Myocardial fibrosis is considered to be one of the main causes of cardiac dysfunction in DC patients [5]. Therefore, inhibition of the development of myocardial fibrosis plays an important role in preventing DC in DM patients.

Studies have found that the cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG) pathway is an important mediator of cardiac protection [6]. cGMP is a ubiquitous second messenger in cells, and its role is mainly mediated by PKG. The activated PKG can act on its downstream targets and is involved in the regulation of vascular tension, myocardial contractility, myocardial hypertrophy, interstitial fibrosis and cardiac remodeling [7]. Phosphodiesterase-5 (PDE-5) is the key enzyme to degrade intracellular cGMP, and PDE-5 inhibitors have been found to play a cardioprotective role by increasing cGMP activity and activating PKG [8]. Therefore, we hypothesized that PDE-5 inhibitors may improve myocardial fibrosis through the cGMP/PKG pathway, and prevent DC.

Mesenchymal stem cells (MSCs) are ideal target cells for cell replacement therapy and gene therapy, especially bone marrow-derived MSCs (BMSCs). BMSCs have many advantages, including easy access to materials, the absence of ethical issues, easy transfection and stable expression of exogenous genes, and the potential for repair of myocar-



**Table 1. Antibodies used in this study.**

Name	Catalog	Manufacturer
Cardiac troponin I	sc-365446	Santa cruz, Dallas, TX, USA
Vimentin	ab8069	abcam, Cambridge, UK
$\alpha$ -SMA Antibody	AF1032	Affinity, Chicago, IL, USA
Nkx2.5 Antibody	DF2699	Affinity, Chicago, IL, USA
Goat anti rabbit (Alexa Fluor® 488)	ab150077	abcam, Cambridge, UK
PDE-5	ab259945	abcam, Cambridge, UK
cGMP	sc-21727	Santa cruz, Dallas, TX, USA
PKG	13511S	Cell signaling technology, Danvers, MA, USA
$\beta$ -actin	AF7018	Affinity, Chicago, IL, USA
Goat anti rabbit	ab205718	abcam, Cambridge, UK
Goat anti mouse	ab6789	abcam, Cambridge, UK

dial damage [9,10]. Several studies have shown that transplanted BMSCs can protect the myocardium and improve cardiac function through the mechanisms of antioxidation, antifibrosis, antiapoptosis, and promoting angiogenesis and repairing cardiomyocytes [10–12]. Therefore, using BMSCs as the cell carrier of PDE-5 inhibitors to limit collagen proliferation and interstitial fibrosis of myocardial cells may be an effective method for the treatment of DC.

In this study, we investigated the effects of BMSCs that overexpressed or knocked down PDE-5 on the biological function of cardiomyocytes and fibroblasts induced by high glucose (HG) and the cGMP/PKG pathway, in order to prevent and potentially treat DC.

## 2. Materials and Methods

### 2.1 Experimental Animals

Sprague-Dawley (SD) rats (1-3-day-old, n = 3) supplied by Shanghai SLAC Laboratory Animal Co., Ltd (Certificate No. SCXK (Hu) 2017-0005, Shanghai, China) were raised under a 12-hour-light/-dark cycle with  $22 \pm 2$  °C and 45–50% humidity. Food and water that were freely supplied. The study was approved by the Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center (No. SYXK (Zhe) 2021-0003, Hangzhou, China).

### 2.2 Isolation of Neonatal Rat Primary Fibroblasts and Cardiomyocytes

The rats were euthanized using excessive pentobarbital sodium, and then the ventricular tissues of rats were collected, chopped on ice and digested with type II collagenase (1148090, Merck, Darmstadt, Germany) to obtain a cell suspension. High-and low-density Percoll (P4937, Merck, Darmstadt, Germany) were prepared in advance. Low-density Percoll (0.407 g/mL) was added slowly over high-density Percoll (1.017 g/mL), and the cell suspension was added over Percoll and centrifuged at 1700 g for 30 minutes. The cells on the upper layer of the centrifuge tube were fibroblasts, and the cells on the lower layer were

cardiomyocytes. The cardiomyocytes were cultured in rat cardiomyocytes complete medium (CM-R073, Procell, Wuhan, Hubei, China), and the fibroblasts were cultured in rat myocardial fibroblast complete medium (CM-R074, Procell, Wuhan, Hubei, China). All cells were maintained in a humidified atmosphere at 37 °C with 5% CO<sub>2</sub>. After the cells were cultured for 4 hours, 24 hours, 48 hours and 72 hours, the morphology and growth of the cells were observed using an optical microscope (AE2000, Motic, Shenzhen, Guangdong, China).

### 2.3 Identification of Primary Cardiomyocytes and Fibroblasts

The second generation of cardiomyocytes and fibroblasts were inoculated into a 6-well plate with cell slides. After 48 hours of incubation, the cells were fixed with 4% paraformaldehyde (abs9179, absin, Shanghai, China) and then reacted with 0.3% Triton-X 100 (A1009, Applygen, Beijing, China) for 3 minutes. Cells were blocked with 3% BSA (abs9157, absin, Shanghai, China) and incubated with primary antibody overnight at 4 °C and with the secondary antibody at room temperature for 1 hour the following day. Cells were then sealed with antifade mounting solution with DAPI (C1211, Applygen, Beijing, China). The final results were observed under a fluorescence microscope (Ts2-FC, Nikon, Tokyo, Japan). Information on the antibodies is shown in Table 1.

### 2.4 Gene Transfection

Rat BMSCs were purchased from Procell Life Science&Technology Co.,Ltd (CP-R131, Wuhan, Hubei, China). Rat BMSCs were cultured in a complete medium (CM-R131, Procell, Wuhan, Hubei, China) and maintained in a humidified atmosphere at 37 °C with 5% CO<sub>2</sub>.

The gene sequence of rat PDE-5 was obtained from the NCBI database and then amplified by polymerase chain reaction (PCR). The amplified gene sequence was inserted into the pRRL WS1.6 GFP vector (K4820-01, Invitrogen, Waltham, MA, USA), named overexpression

**Table 2. Primers used in this study.**

List of oligonucleotide sequences	5' → 3'
Primers for qRT-PCR	
PDE-5 Forward	GCTATGTTGCCCTTTGGAGAC
PDE-5 Reverse	TAGCGATCCGCAGAGATGAGT
cGMP Forward	TGATGAGGAGTGATGGGGCAG
cGMP Reverse	TGCAGAGAAGTGGCATCCAAG
PKG Forward	CTCCACAAATGCCAGTCGGT
PKG Reverse	AAGGTGTCTCTCTTGCACC
$\beta$ -actin Forward	AAGGTCGGTGTGAACGGATTT
$\beta$ -actin Reverse	CTTGTGACAAGAGAAGGCAGC

**Table 3. The number and percent of cardiomyocytes and fibroblasts cells expressed specific markers ( $\chi \pm s$ , n = 3).**

Markers	Total number of cells	Number of positive cells	Positive percent (%)
Cardiac troponin I	86.67 $\pm$ 5.51	84.33 $\pm$ 4.93	97.33 $\pm$ 0.48
Nkx2.5	133.00 $\pm$ 9.00	131.00 $\pm$ 9.00	98.49 $\pm$ 0.10
Vimentin	90.67 $\pm$ 10.79	88.00 $\pm$ 8.72	97.21 $\pm$ 1.87
$\alpha$ -SMA	103.33 $\pm$ 3.06	102.00 $\pm$ 2.65	98.72 $\pm$ 0.52

Data were presented as mean  $\pm$  SD ( $\chi \pm s$ ).

(oe)-PED-5, and the empty vector was regarded as a negative control (NC). The PDE-5 specific short hairpin RNA (shRNA) was synthesized by VectorBuilder (Guangzhou, Guangdong, China) with the target sequence (5'-GACAGCTCTAAAGACAAATTT-3'). BMSCs ( $5 \times 10^5$ /well) were inoculated into a six-well plate and cultured routinely for 24 hours. The vectors or shRNA were transfected into cells by lipofectamin2000 (11668-019, Invitrogen, Waltham, MA, USA). After 5 hours, the cell culture medium was replaced with the complete medium and the conventional culture was continued for 24 hours. The interference efficiency of oe-PED-5 was then detected by quantitative reverse transcription-PCR (qRT-PCR).

### 2.5 Cell Treatment

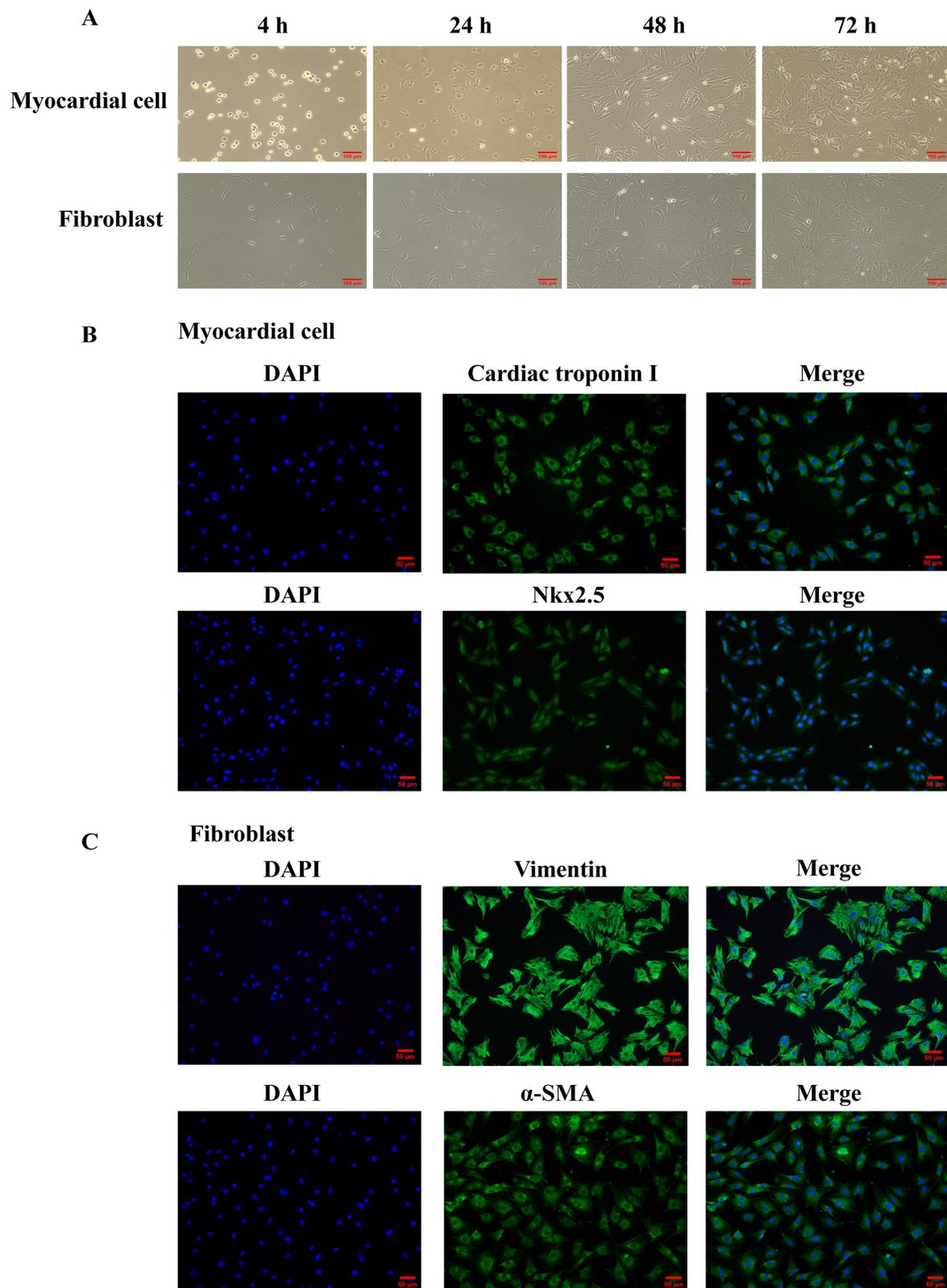
Cardiomyocytes or fibroblasts, were divided into 4 groups (control group, model group, PDE-5 OE group and PDE-5 shRNA group). Cardiomyocytes or fibroblasts ( $1 \times 10^5$  cells/well) were inoculated in a culture dish containing normal glucose (NG, 5.5 mmol/L) or HG (30 mmol/L) for 48 hours, and then transfected BMSCs ( $1 \times 10^5$  cells/well) were inoculated on a transwell insert placed above the culture dish to form a co-culture system [13]. In the control group, cardiomyocytes or fibroblasts cultured in NG were co-cultured with BMSCs transfected with NC. In the model group, cardiomyocytes or fibroblasts cultured in HG were co-cultured with BMSCs transfected with NC. In the PDE-5 OE group, cardiomyocytes or fibroblasts cultured in HG were co-cultured with BMSCs transfected with PDE-5 OE. In the PDE-5 shRNA group, cardiomyocytes or fibroblasts cultured in HG were co-cultured with BMSCs transfected with PDE-5 shRNA.

### 2.6 Cell Viability Analysis

Cell counting kit 8 (CCK8, C0005, Topscience, Shanghai, China) was used to detect the cell viability. Cardiomyocytes and fibroblasts in logarithmic phase were taken and prepared into a  $3 \times 10^4$ /mL cell suspension, which was then added into a 96-well plate at 100  $\mu$ L per well. After 24, 48 or 72 hours of cell culture, 10  $\mu$ L of CCK8 reagent was added directly to the cell culture medium and the incubation was continued for 2 hours. The absorbance at 450 nm was determined with a microplate reader (CMaxPlus, MD, San Jose, CA, USA) and cell viability was calculated according to the instructions.

### 2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Rat collagen (col)-I (ml058800, Mlbio, Shanghai, China), col-III (MM-0765R2, Meimian, Yancheng, Jiangsu, China), tissue inhibitor of metalloproteinase (TIMP)-1 (ml003040, Mlbio, Shanghai, China), desmin (MM-70789R2, Meimian, Yancheng, Jiangsu, China), Matrix metalloproteinase (MMP)-1 (ml002968, Mlbio, Shanghai, China) and troponin (Tn)-I (ml003202, Mlbio, Shanghai, China) ELISA kits were used to determine the cytokine levels in rat cardiomyocytes and fibroblasts. Briefly, the cells were centrifuged at 1000 g for 10 minutes to obtain a supernatant. The standards and cell supernatants were diluted and added to the ELISA plates for incubation for 30 minutes. The biotin-labeled antibody was used to unbind the antigen in the cell supernatant, followed by binding to biotin by affinity streptavidin-horseradish Peroxidase (HRP). After the color was developed by the substrate developing solution, the reaction was stopped. The absorbance at 450 nm was determined with a microplate reader and the cytokines levels were calculated according to the instructions.



**Fig. 1. Isolation and identification of neonatal rat primary cardiomyocytes and fibroblasts.** (A) The morphology of rat primary cardiomyocytes and fibroblasts cultured for 4 hours, 24 hours, 48 hours and 72 hours was observed under inverted microscope (magnification, 100  $\times$ ). (B) The expression of Cardiac troponin I and Nkx2.5 in rat myocardial cells (magnification, 200  $\times$ ). (C) The expression of Vimentin and  $\alpha$ -SMA in rat fibroblasts (magnification, 200  $\times$ ).

## 2.8 Apoptosis Detection

The Annexin V-FITC/PI Apoptosis Detection Kit (556547, BD, Franklin Lakes, NJ, USA) was used to determine the apoptosis of rat cardiomyocytes and fibroblasts. The cells were collected and the cell concentration was adjusted to  $1 \times 10^6$  cells/mL. The cell suspension was mixed with binding buffer, and then stained with Annexin V-FITC and PI at room temperature in the dark. Then apoptosis was detected by flow cytometry (C6, BD, Franklin Lakes, NJ, USA).

## 2.9 Western Blot Analysis

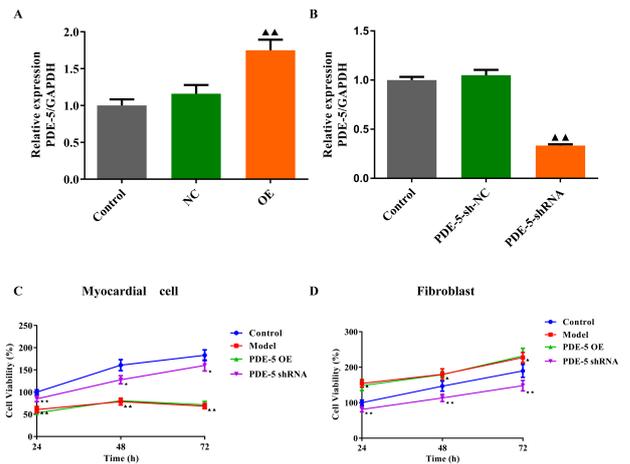
Total protein was extracted from rat cardiomyocytes and fibroblasts by the Protein Extraction Kit (G2002, Servicebio, Wuhan, Hubei, China). Then the protein was quantified by the BCA kit (T14514, Topscience, Shanghai, China) and separated by SDS-PAGE gel and electroblotted to the PVDF membrane (WJ001, Epizyme, Cambridge, MA, USA). After blocking, the membrane was probed with the primary antibody and the secondary antibody which are shown in Table 1. Finally, the protein signals were detected by the ECL luminescence reagent (SQ202, Epizyme, Cambridge, MA, USA) using the Imaging System (610020-9Q, Qinxiang, Shanghai, China).  $\beta$ -actin was used as the internal control.

## 2.10 RT-PCR Detection

Total RNA was extracted from rat cardiomyocytes and fibroblasts by the Total RNA Purification Kit (EZB-RN4, HiFunBio, Shanghai, China). Then the reverse transcription and qPCR was conducted by the cDNA Synthesis Kit (EZB-RT2, HiFunBio, Shanghai, China) and SYBR Green qPCR Mix (A0001, HiFunBio, Shanghai, China) on a Real-Time PCR system (CFX Connect, Bio-rad, Hercules, CA, USA). The mRNA levels were normalized to  $\beta$ -actin by the  $2^{-\Delta\Delta CT}$  approach [14]. The thermal cycling conditions were as follows: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s. The sequences of the primers are listed in Table 2.

## 2.11 Statistical Analysis

Data are presented as mean  $\pm$  SD. One-way analysis of variance (ANOVA) was used for the comparison among multiple groups, and pairwise comparison between groups was conducted by the Turkey test. Data with normal distribution and homogeneity test of variance were analyzed by the SNK test. Dunnett's T3 test or independent sample *T* test was used for heterogeneity of variance. Kruskal-Wallis H test was used for the measurement data that did not conform to normal distribution. All statistical analyses were performed with SPSS 16.0 (IBM Corp., Armonk, NY, USA). A  $p < 0.05$  was considered statistically significant.



**Fig. 2. The effects of PDE-5 on the viability of rat fibroblasts and cardiomyocytes.** The transfection efficiency of PDE-5 overexpression plasmid (A) and the PDE-5 sh-RNA (B) in rat BMSCs (mean  $\pm$  SD,  $n = 3$ ). ▲▲ $p < 0.01$ , ▲ indicates vs. NC group. CCK-8 was used to detect the activity of cardiomyocytes (C) and fibroblasts (D) in each group at 24 hours, 48 hours and 72 hours. ▲ $p < 0.05$ , ▲▲ $p < 0.01$ ; \* $p < 0.05$ , \*\* $p < 0.01$  (▲ indicates vs. control group; \* indicates vs. model group).

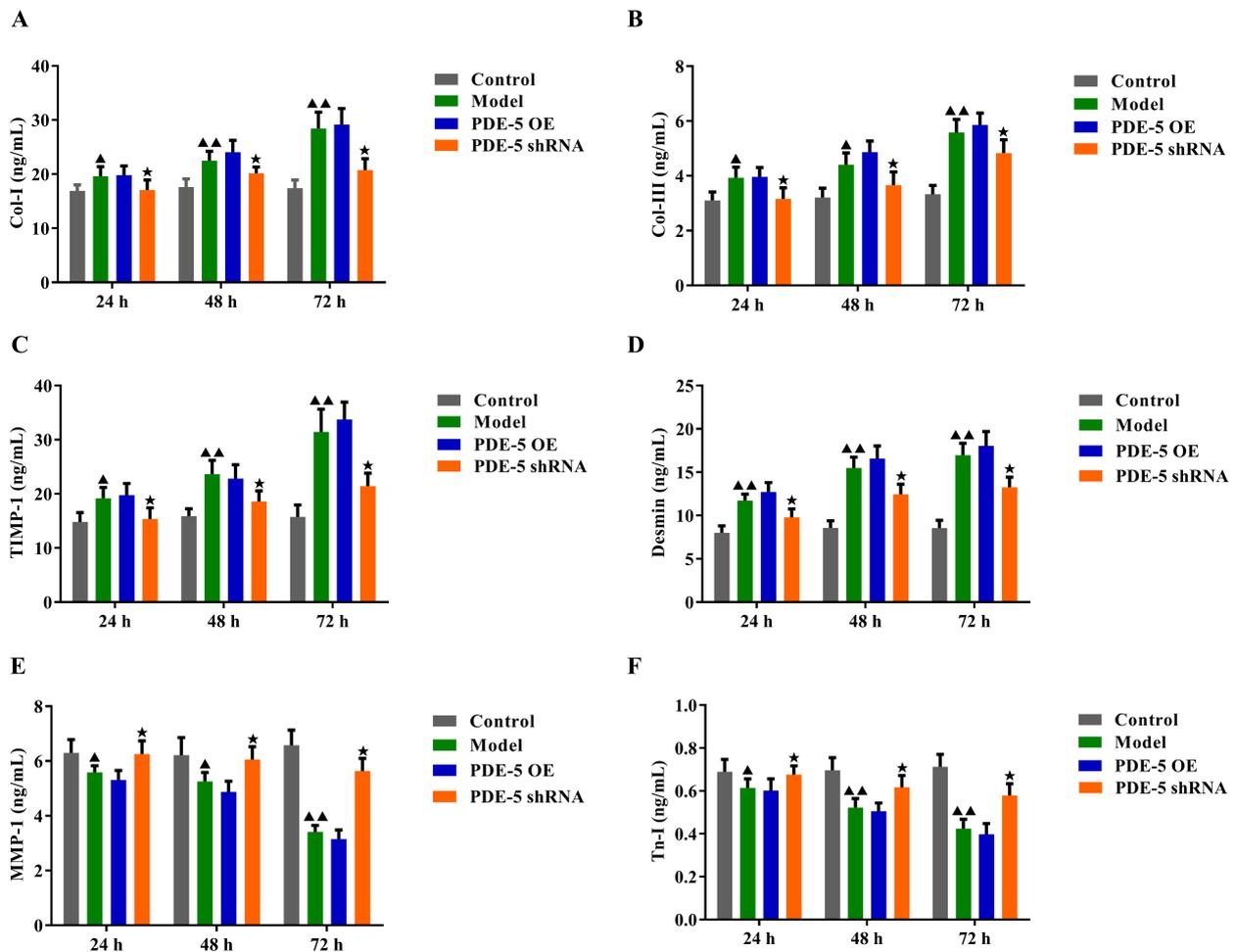
## 3. Results

### 3.1 Identification of Neonatal Rat Primary Fibroblasts and Cardiomyocytes

As shown in Fig. 1A, cardiomyocytes and fibroblasts began to adhere to the wall after 4 hours of culture, and their shapes gradually changed from round to spindle. After 24 hours of culture, cardiomyocytes began to extend out from pseudopodia, showing a triangular or polygonal shape. After 72 hours of culture, cardiomyocytes were interconnected and interwoven into a network, forming single-layer cardiomyocytes or cell clusters. After 24 hours and 48 hours of culture, fibroblasts also showed fusiform or polygonal shape, and scattered growth. After 72 hours of culture, cells proliferated faster, grew and gathered, and showed spiral and radial shapes. Cardiomyocytes specifically express Cardiac troponin I, Nkx2.5 and fibroblasts specifically express Vimentin,  $\alpha$ -SMA both of which are localized in the cytoplasm. The cells have been identified by immunofluorescence with a purity of over 90% which was listed in Table 3. We were able to successfully isolate rat cardiomyocytes and fibroblasts as shown in the results in Fig. 1B,C.

### 3.2 The Effects of PDE-5 on the Viability of Rat Fibroblasts and Cardiomyocytes

We determined the overexpression efficiency of PDE-5 by qRT-PCR and showed that the expression of PDE-5 significantly increased after BMSCs were transfected with PDE-5 overexpression plasmid (Fig. 2A,  $p < 0.01$ ). The PDE-5 gene expression levels are reduced in BMSCs after transfection with PDE-5 shRNA (Fig. 2B,  $p < 0.01$ ). We



**Fig. 3.** The effects of PDE-5-modified MSCs on the contents of Col-I, Col-III, Desmin, MMP-1, TIMP-1 and Tn-I. (A–E) ELISA was used to detect the contents of Col-I, Col-III, Desmin, MMP-1, and TIMP-1 in the supernatant of rat fibroblasts. (F) ELISA was used to detect the contents of Tn-I in the supernatant of rat cardiomyocytes (mean  $\pm$  SD,  $n = 6$ ).  $\blacktriangle p < 0.05$ ,  $\blacktriangle\blacktriangle p < 0.01$ ;  $*p < 0.05$  ( $\blacktriangle$  indicates vs. control group;  $*$  indicates vs. model group).

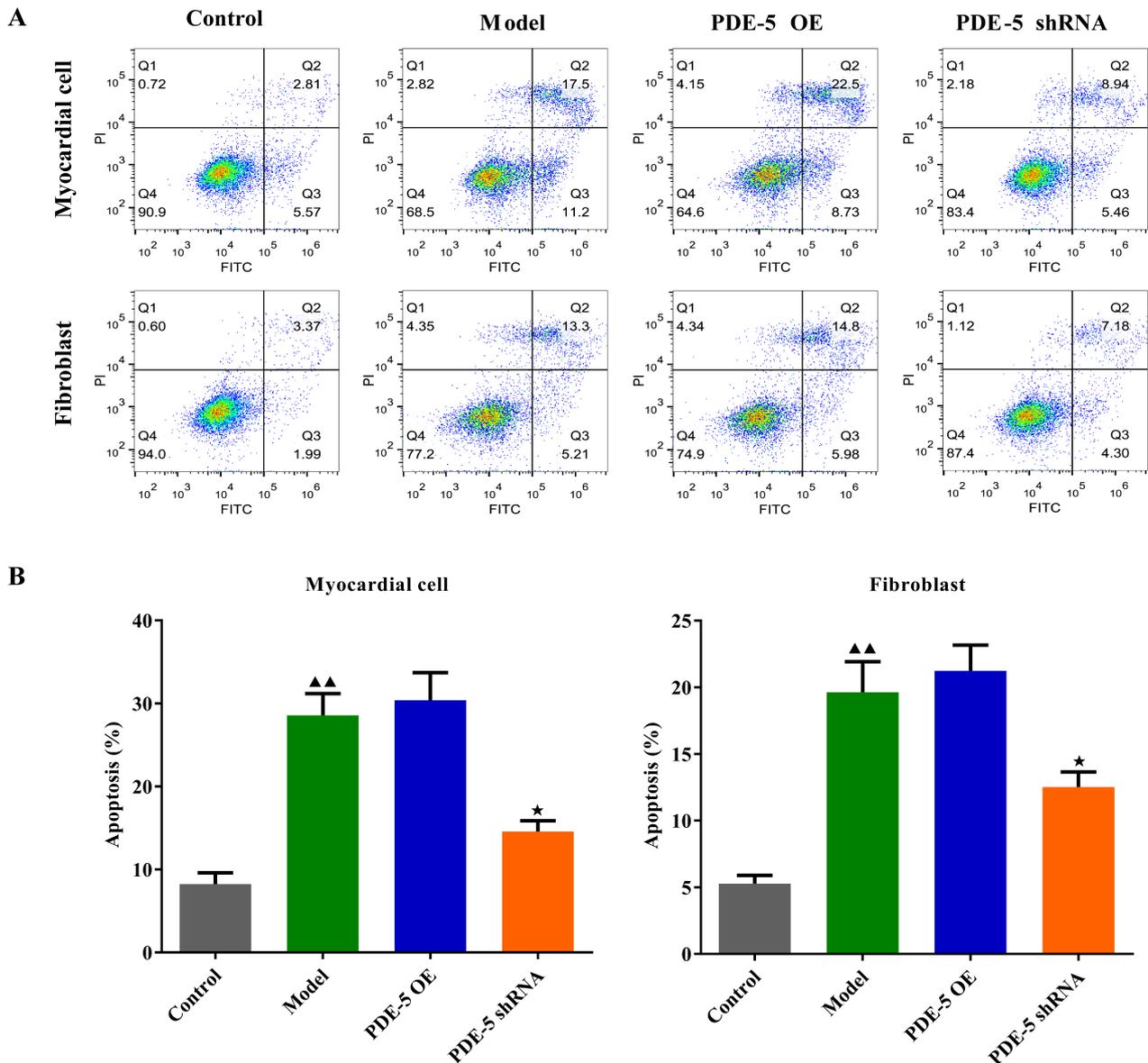
then determined the effects of BMSCs overexpressing or silencing PDE-5 on the biological behavior of cardiomyocytes and fibroblasts through a co-culture system. Compared with the control group, the viability of cardiomyocytes in the model group, the PDE-5 OE group, and the PDE-5 shRNA group was significantly lower at 24 hours, 48 hours and 72 hours (Fig. 2C,  $p < 0.05$  or  $p < 0.01$ ), while the viability of fibroblasts in the model group and the PDE-5 OE group was facilitated at 24 hours, 48 hours and 72 hours (Fig. 2D,  $p < 0.05$ ). However, the viability of fibroblasts in the PDE-5 shRNA group was decreased at 24 hours, 48 hours and 72 hours ( $p < 0.05$ ). Compared with the model group, PDE-5 silencing promoted the viability of cardiomyocytes, but inhibited the viability of fibroblasts ( $p < 0.05$  or  $p < 0.01$ ), while PDE-5 OE did not affect the viability of fibroblasts or cardiomyocytes ( $p > 0.05$ ).

### 3.3 The Effects of PDE-5 on the Release of Cytokines of Rat Fibroblasts and Cardiomyocytes

After HG culture, the contents of Col-I, Col-III, TIMP-1 and Desmin secreted by fibroblasts gradually increased, while MMP-1 secreted by fibroblasts and Tn-I secreted by cardiomyocytes decreased in a time-dependent manner (Fig. 3A–F,  $p < 0.05$  or  $p < 0.01$ ). Of interest, PDE-5 shRNA suppressed the secretion of Col-I, Col-III, TIMP-1 and Desmin in fibroblasts and elevated the secretion of MMP-1 in fibroblasts and Tn-I in cardiomyocytes (Fig. 3A–F,  $p < 0.05$ ), while PDE-5 OE did not affect the levels of these cytokines ( $p > 0.05$ ).

### 3.4 PDE-5 Inhibition Suppressed the Apoptosis of Rat Fibroblasts and Cardiomyocytes

HG culture increased the apoptosis of rat fibroblasts and cardiomyocytes compared to the normal group cells (Fig. 4A,B,  $p < 0.01$ ), while these trends were reversed by PDE-5 shRNA (Fig. 4A,B,  $p < 0.05$ ). However, PDE-5 OE



**Fig. 4. The effects of PDE-5-modified MSCs on apoptosis of fibroblasts and cardiomyocytes.** (A,B) The apoptosis of fibroblasts and cardiomyocytes in each group was determined by flow cytometry (mean  $\pm$  SD,  $n = 3$ ).  $\blacktriangle\blacktriangle p < 0.01$ ;  $\star p < 0.05$  ( $\blacktriangle$  indicates vs. control group;  $\star$  indicates vs. model group).

did not affect the apoptosis of HG-induced rat fibroblasts and cardiomyocytes ( $p > 0.05$ ).

### 3.5 The Effect of PDE-5 on the cGMP/PKG Signaling in Rat Fibroblasts and Cardiomyocytes

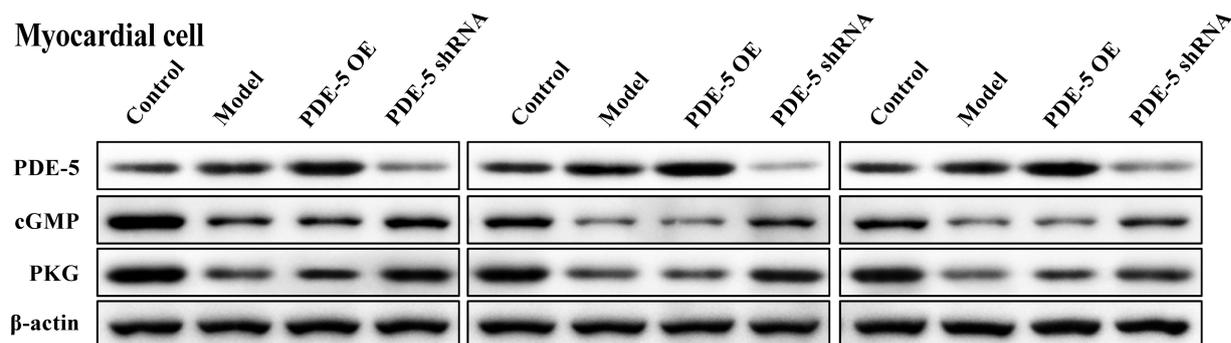
As shown in Figs. 5A–D,6A,B, we found that the protein and mRNA levels of PDE-5 were increased ( $p < 0.05$ ), while the protein and mRNA levels of cGMP and PKG were inhibited in untransfected cardiomyocytes and fibroblasts after HG culture ( $p < 0.05$  or  $p < 0.01$ ). However, PDE-5 shRNA resulted in down-regulation of PDE-5 levels and up-regulation of cGMP and PKG levels in HG-induced fibroblasts and cardiomyocytes (Figs. 5A–D,6A,B,  $p < 0.05$  or  $p < 0.01$ ). PDE-5 OE only elevated the PDE-5 levels

in HG-induced fibroblasts and cardiomyocytes (Figs. 5A–D,6A,B,  $p < 0.05$ ).

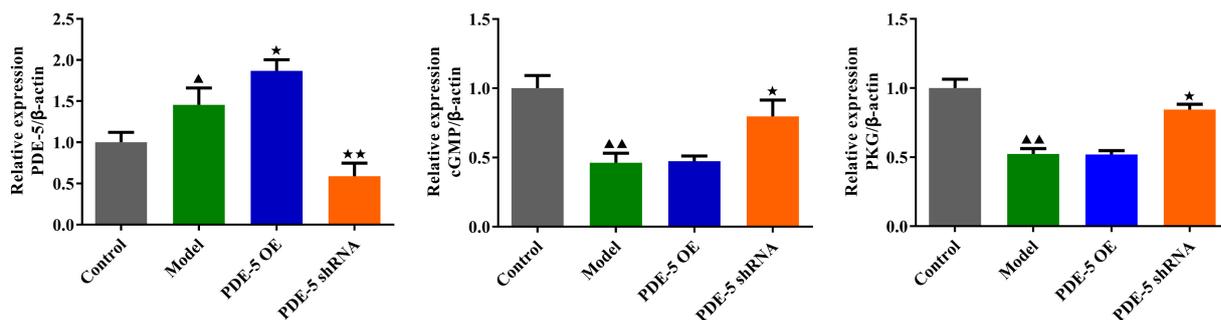
## 4. Discussion

Cardiac fibrosis, the hallmark of DC, contributes to the structural remodeling of the heart, culminating in decreased cardiac function, heart failure, and arrhythmias. It is reported that the process of cardiac fibrosis is mainly mediated by the activation of cardiac fibroblasts. Several studies have shown that there is crosstalk between cardiac fibroblasts and cardiomyocytes, which promotes the development of DC [15,16]. Therefore, in this study, fibroblasts and cardiomyocytes were cultured separately, in order to further explore the mechanism of myocardial fibrosis in DC.

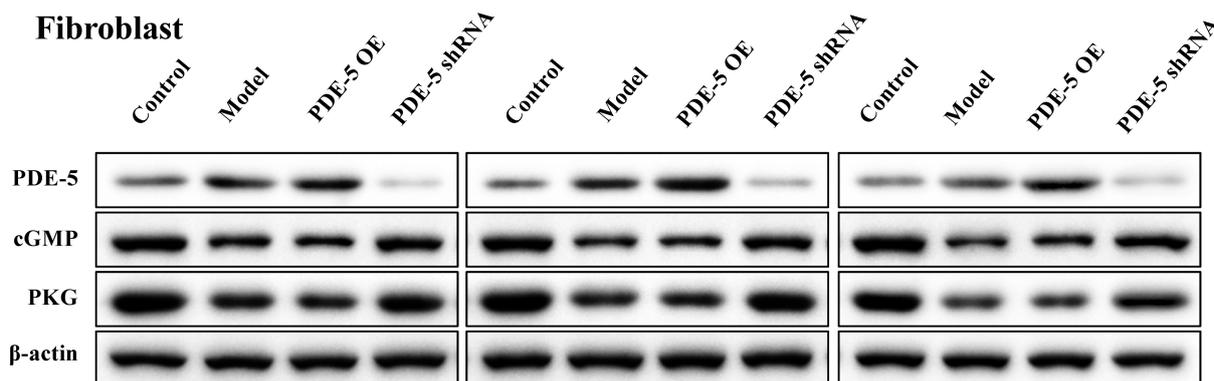
## A Myocardial cell



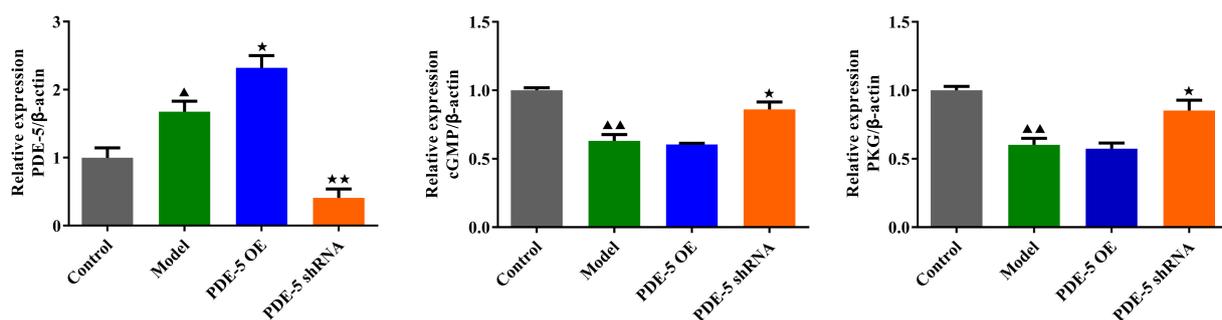
## B



## C Fibroblast



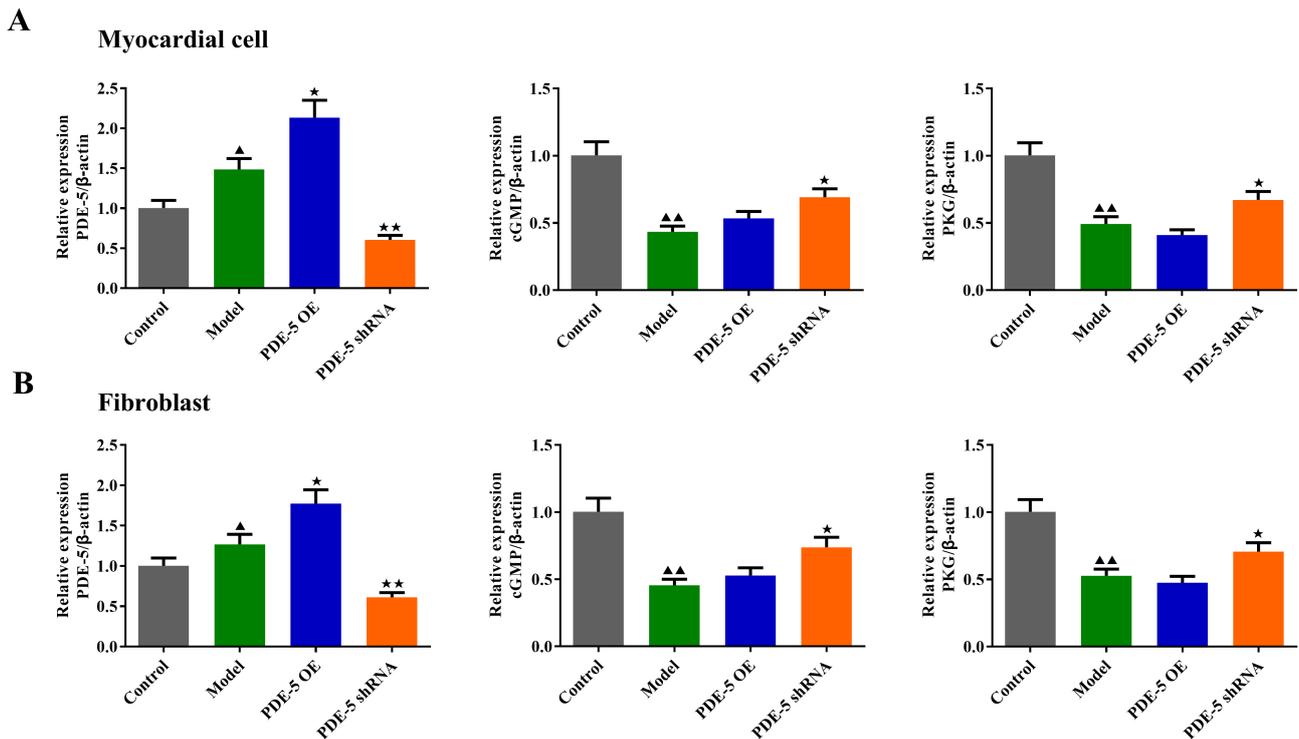
## D



**Fig. 5.** The effects of PDE-5-modified MSCs on the protein levels of PDE-5, cGMP and PKG in fibroblasts and cardiomyocytes. Western blot was used to detect the protein levels of PDE-5, cGMP and PKG in rat cardiomyocytes (A,B) and fibroblasts (C,D).  $\beta$ -actin was used as internal control (mean  $\pm$  SD,  $n = 3$ ).  $\blacktriangle p < 0.05$ ,  $\blacktriangle\blacktriangle p < 0.01$ ;  $* p < 0.05$ ,  $** p < 0.01$  ( $\blacktriangle$  indicates vs. control group;  $*$  indicates vs. model group).

It is well known that fibroblasts and cardiomyocytes can be regulated by HG. HG can improve the activity of fibroblasts and activate fibroblasts, and at the same time,

it can induce cardiomyocyte apoptosis [17,18]. Activated fibroblasts can affect cardiac function through direct and indirect effects on cardiomyocytes. Under the induction of



**Fig. 6. The effects of PDE-5-modified MSCs on the mRNA levels of PDE-5, cGMP and PKG in fibroblasts and cardiomyocytes.** qRT-PCR was used to detect the mRNA levels of PDE-5, cGMP and PKG in rat cardiomyocytes (A) and fibroblasts (B).  $\beta$ -actin was used as internal control (mean  $\pm$  SD,  $n = 3$ ).  $\blacktriangle p < 0.05$ ,  $\blacktriangle\blacktriangle p < 0.01$ ;  $* p < 0.05$ ,  $** p < 0.01$  ( $\blacktriangle$  indicates vs. control group;  $*$  indicates vs. model group).

HG, fibroblasts will secrete more fibrous collagen, such as Col-I and III, which are responsible for the changes of cardiac function [19]. MMP-1 can mediate the degradation of Col-I, while TIMP-1 is the inhibitor of MMP-1. Therefore, HG-induced fibroblasts will secrete more TIMP-1 and reduce the release of MMP-1, thus promoting the production of Col-I [20]. Desmin is the protein of mesenchymal cells, promoting extracellular matrix remodeling and mediating collagen degradation [21]. Tn-I is a specific biomarker of myocardial cells, and the down-regulation of Tn-I expression induced by HG indicates myocardial cell necrosis [22]. Consistent with a previous study [21], in our study, HG-induced fibroblasts secreted more Desmin, which indicates that fibroblasts are more likely to change to the myofibroblast phenotype. HG induction led to fibroblast activation, collagen deposition and phenotype, as well as cardiomyocyte apoptosis, which indicates that protecting cardiomyocytes by limiting fibrosis might be an important strategy for the treatment of DC.

Previous studies have shown that BMSCs can treat DM and heart disease [23]. However, the effect of BMSCs on reducing cardiac fibrosis needs to be further investigated. Ke Meng *et al.* [13] suggested that transplantation of adiponectin-modified BMSCs into DM rats could alleviate HG-induced cardiac fibrosis. In this study, we proposed a strategy of PDE-5-silenced BMSCs to treat cardiac

fibrosis. The cardioprotective effect of PDE-5 inhibitor has been widely confirmed [24]. Hoke *et al.* [25] reported that implantation of PDE-5-silenced adipose-derived stem cells into a myocardial infarction model in mice could significantly improve cardiac function and reduce myocardial fibrosis and cardiomyocyte apoptosis. Similarly, we demonstrated the therapeutic effect of PDE-5-silenced BMSCs on DC by co-culture with HG-induced fibroblasts and cardiomyocytes, that PDE-5-silenced BMSCs significantly inhibited fibroblast activation, collagen deposition, and cardiomyocyte apoptosis.

The cardio-protective effect (improvements in the pulmonary circulation, cardiac remodeling and diastolic function) of PDE-5 inhibitors is mainly mediated by activating the cGMP/PKG pathway [26]. Saihakoka *et al.* [27] found that PDE-5 inhibitors can increase cGMP and PKG activities in the heart, thus alleviating adriamycin-induced cardiomyopathy. However, it has not been reported whether PDE-5 inhibitors can alleviate myocardial fibrosis in DC patients by activating the cGMP/PKG pathway. Wei *et al.* [28] reported that myocardial injury in DM rats could be alleviated by regulating the cGMP/PKG pathway. Qin *et al.* [29] emphasized that activating the cGMP/PKG pathway in cardiac fibroblasts can alleviate HG-induced cardiac fibrosis [28]. Consistent with previous studies, in this study, we found that HG-induced inactivation of the cGMP/PKG

pathway in cardiac fibroblasts and cardiomyocytes was reversed by PDE-5-silenced BMSCs, suggesting that PDE-5 silenced-BMSCs may alleviate HG-induced cardiac fibrosis and myocardial injury by regulating the cGMP/PKG pathway. However, there are limitations in this study, such as the effect of PDE-5 on BMSCs *in vivo*. In the future, we will study the effect of PDE-5 on myocardial fibrosis in DC rats under physiological conditions by constructing a diabetic rat model.

## 5. Conclusion

This study shows that PDE-5-silenced BMSCs can alleviate the HG-induced collagen deposition, myocardial fibrosis and cardiomyocyte apoptosis by activating the cGMP/PKG pathway, suggesting that PDE-5 inhibitor-transfected BMSCs have the potential to prevent and treat diabetic cardiomyopathy. These findings may contribute to the development of therapeutic stem cell transplantation for DC.

## Availability of Data and Materials

The relevant data to support the methods and results are available on Figshare (DOI: 10.6084/m9.figshare.21501054).

## Author Contributions

XF, QH and JM designed the research study. QH, JZ, and LJ performed the research. QH, HW and LJ analyzed the data. XF, QH, HW and JM QH interpreted the data, QH, JZ, LJ and HW wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

## Ethics Approval and Consent to Participate

The study was approved by Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center (Certificate No. SYXK (Zhe) 2021-0003, Hangzhou, China).

## Acknowledgment

The authors thank all those who supported them in animal experiments and helped them to manage the data.

## Funding

This research was funded by the Public Welfare Technology Application Research Project of Zhejiang Province, China, grant number LGD20H070002 and LGD21H020004 granted to HQ and FX. Respectively, the Research Project of Zhejaing Provincial People's Hospital, grant number ZRY2020B014 granted to HQ. The Medicine

and Health Science and Technology Project of Zhejiang Province, grant number 2022KY523, 2020KY022, and 2021KY060 granted to HQ and FX, respectively. All the funders were not involved in the manuscript writing, editing, approval, or decision to publish.

## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2807155>.

## References

- [1] Cole JB, Florez JC. Genetics of diabetes mellitus and diabetes complications. *Nature reviews nephrology*. 2020; 16: 377–390.
- [2] Ritchie RH, Abel ED. Basic Mechanisms of Diabetic Heart Disease. *Circulation Research*. 2020; 126: 1501–1525.
- [3] Park JJ. Epidemiology, Pathophysiology, Diagnosis and Treatment of Heart Failure in Diabetes. *Diabetes & Metabolism Journal*. 2021; 45: 146–157.
- [4] Dillmann WH. Diabetic cardiomyopathy: what is it and can it be fixed? *Circulation Research*. 2019; 124: 1160–1162.
- [5] Frangiannis NG. Cardiac fibrosis. *Cardiovascular Research*. 2021; 117: 1450–1488.
- [6] Inverte J, Garcia-Dorado D. The cGMP/PKG pathway as a common mediator of cardioprotection: translatability and mechanism. *British Journal of Pharmacology*. 2015; 172: 1996–2009.
- [7] Nakamura T, Tsujita D. Current trends and future perspectives for heart failure treatment leveraging cGMP modifiers and the practical effector PKG. *Journal of Cardiology*. 2021; 78: 261–268.
- [8] Elhwuegi A. The wonders of phosphodiesterase-5 inhibitors: a majestic history. *Annals of Medical and Health Sciences Research*. 2016; 6: 139–145.
- [9] Mishra VK, Shih HH, Parveen F, Lenzen D, Ito E, Chan TF, *et al*. Identifying the Therapeutic Significance of Mesenchymal Stem Cells. *Cells*, 2020; 9: 1145.
- [10] Chu DT, Phuong TNT, Tien NLB, Tran DK, Thanh VV, Quang TL, *et al*. An Update on the Progress of Isolation, Culture, Storage, and Clinical Application of Human Bone Marrow Mesenchymal Stem/Stromal Cells. *International journal of molecular sciences*. 2020; 21: 708.
- [11] Cai M, Shen R, Song L, Lu M, Wang J, Zhao S, *et al*. Bone Marrow Mesenchymal Stem Cells (BM-MSCs) Improve Heart Function in Swine Myocardial Infarction Model through Paracrine Effects. *Scientific reports*. 2016; 6: 1–12.
- [12] Hare JM, DiFede DL, Rieger AC, Florea V, Landin AM, El-Khorazaty J, *et al*. Randomized Comparison of Allogeneic Versus Autologous Mesenchymal Stem Cells for Nonischemic Dilated Cardiomyopathy: POSEIDON-DCM Trial. *Journal of the American College of Cardiology*. 2017; 69: 526–537.
- [13] Roy S, Kloner RA, Salloum FN, Jovin IS. Cardiac Effects of Phosphodiesterase-5 Inhibitors: Efficacy and Safety. *Cardiovascular Drugs and Therapy*. 2021; 1–14.
- [14] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25: 402–408.
- [15] Hall C, Gehmlich K, Denning C, Pavlovic D. Complex Relationship between Cardiac Fibroblasts and Cardiomyocytes in Health and Disease. *Journal of the American Heart Association*. 2021; 10: e019338.

- [16] Tao A, Song J, Lan T, Xu X, Kvietys P, Kao R, *et al.* Cardiomyocyte-fibroblast interaction contributes to diabetic cardiomyopathy in mice: Role of HMGB1/TLR4/IL-33 axis. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*. 2015; 1852: 2075–2085.
- [17] Lin H, Guan L, Meng L, Uzui H, Guo H. SGLT1 Knockdown Attenuates Cardiac Fibroblast Activation in Diabetic Cardiac Fibrosis. *Frontiers in Pharmacology*. 2021; 12: 1–10.
- [18] Ma L, Cao Y, Zhang L, Li K, Yan L, Pan Y, *et al.* Celastrol mitigates high glucose-induced inflammation and apoptosis in rat H9c2 cardiomyocytes via miR-345-5p/growth arrest-specific 6. *the Journal of Gene Medicine*. 2020; 9: e3201.
- [19] Levick SP, Widiapradja A. The Diabetic Cardiac Fibroblast: Mechanisms Underlying Phenotype and Function. *International Journal of Molecular Sciences*. 2020; 21: 970.
- [20] Young DA, Barter MJ, Wilkinson DJ. Recent advances in understanding the regulation of metalloproteinases. *F1000Research*. 2019; 8: 195.
- [21] Parreau S, Vedrenne N, Regent A, Richard L, Sindou P, Mouthon L, *et al.* An immunohistochemical analysis of fibroblasts in giant cell arteritis. *Annals of Diagnostic Pathology*. 2021; 52: 151728.
- [22] Twerenbold R, Reichlin T, Reiter M, Mueller C. High-sensitive cardiac troponin: friend or foe? *Swiss Medical Weekly*. 2011; 141: w13202.
- [23] Liu B, Duan CY, Luo CF, Ou CW, Sun K, Wu ZY, *et al.* Effectiveness and safety of selected bone marrow stem cells on left ventricular function in patients with acute myocardial infarction: a meta-analysis of randomized controlled trials. *International journal of cardiology*. 2014; 177: 764–770.
- [24] Meng K, Cai H, Cai S, Hong Y, Zhang X. Adiponectin Modified BMSCs Alleviate Heart Fibrosis via Inhibition TGF-beta1/Smad in Diabetic Rats. *Frontiers in Cell and Developmental Biology*. 2021; 9: 644160.
- [25] Hoke NN, Salloum FN, Kass DA, Das A, Kukreja RC. Preconditioning by phosphodiesterase-5 inhibition improves therapeutic efficacy of adipose-derived stem cells following myocardial infarction in mice. *Stem Cells*. 2012; 30: 326–335.
- [26] Tzoumas N, Farrah TE, Dhaun N, Webb DJ. Established and emerging therapeutic uses of PDE type 5 inhibitors in cardiovascular disease. *British Journal of Pharmacology*. 2020; 177: 5467–5488.
- [27] Koka S, Das A, Zhu S, Durrant D, Xi L, Kukreja RC. Long-Acting Phosphodiesterase-5 Inhibitor Tadalafil Attenuates Doxorubicin-Induced Cardiomyopathy without Interfering with Chemotherapeutic Effect. *Journal of Pharmacology and Experimental Therapeutics*. 2010; 334: 1023–1030.
- [28] Wei Q, Zhu T, Xiao X, Sun L, Zhang Z, Huang T. Dioscin Attenuates Myocardial Damages in Diabetic Rats maybe by Regulating NO-sGC-cGMP-PKG Pathway. *Annals of Clinical & Laboratory Science*. 2019; 49: 97–104.
- [29] Qin L, Zang M, Xu Y, Zhao R, Wang Y, Mi Y, *et al.* Chlorogenic Acid Alleviates Hyperglycemia-Induced Cardiac Fibrosis through Activation of the NO/cGMP/PKG Pathway in Cardiac Fibroblasts. *Molecular Nutrition & Food Research*. 2021; 65: e2000810.