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Bcl6 Suppresses Cardiac Fibroblast Activation and Function via Directly Binding to Smad4

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Summary: Bcl6, a critical pro-oncogene of human B-cell lymphomas, can promote tumor progress. Previous studies have found that Bcl6 participates in hypoxia injury in cardiomyocytes. However, the effect of Bcl6 on cardiac fibroblasts is still unclear. The aim of this study was to elucidate the functional role of Bcl6 in cardiac fibroblast activation and function. The neonatal rat cardiac fibroblasts were isolated and cultured. First, transforming growth factor β1 (TGFβ1) was used to stimulate fibroblast activation. A decreased expression level of Bcl6 was observed in fibroblasts after stimulation with TGFβ1. Then, cells were transfected with adenovirus Bcl6 to overexpress Bcl6. The results showed that Bcl6 overexpression induced decreased proliferation and reduced activation of fibroblasts which were stimulated with TGFβ1. It was found that activated smad2 and smad3 were not changed by overexpressing Bcl6, but smad4 was decreased. Furthermore, co-immunoprecipitation results showed that Bcl6 directly bound to smad4, and induced down-regulation of smad4. At last, smad4 activator could counteract the anti-fibroblast effects of Bcl6. In conclusion, Bcl6 may negatively regulate cardiac fibroblast activation and function by directly binding to smad4.
Key words: Bcl6; cardiac fibroblast; transforming growth factor β1; smad4

Cardiac fibrosis, featured by excessive deposition of extracellular matrix (ECM), can result in the formation of scar tissue and is related to cardiovascular diseases[1]. The proliferation and activation of resident fibroblasts account for the main pathology of cardiac fibrosis[2]. During the pathology process of various cardiovascular diseases, the release of many pro-inflammatory factors and growth factors leads to the activation of resident fibroblasts, resulting in the transformation to myofibroblasts[3]. These myofibroblasts express α-smooth muscle actin (α-SMA), a sign of fibroblast activation, secret amounts of pro-fibrosis factors, and synthesize much collagen[4]. The accumulation of extracellular matrix (ECM) protein increases stiffness of left ventricle, and impedes contraction and relaxation of hearts. Cardiac fibrosis also impairs mechano-electric coupling, leading to arrhythmias and cardiac dysfunction[1]. Currently, no evidence-based therapies show significant efficacy in cardiac fibrosis. Thus, finding a new therapeutic target is of great importance.

As an oncogene, Bcl6 can promote the deterioration of B-cell lymphoma by inhibiting proliferation and blocking terminal differentiation[5]. It was reported that Bcl6 can bind to and then repress hundreds of target genes[6]. Recently, many studies have focused on the effect of Bcl6 on cancer development, progress and metastasis. Bcl6 could also target miRNAs and regulate their expression levels, which are of great importance in the pathogenesis of various cancers[7]. Early in 1999, Yoshida *et al* found that loss of Bcl6 in mature cardiac myocytes caused eosinophilic myocarditis[8]. Later, Altieri *et al* found that Bcl6 cooperated with PPARδ, which protected heart against doxorubicin-induced senescence[9]. Recently, Gu *et al* observed that Bcl6 knockdown aggravated hypoxia injury in cardiomyocytes[10]. All these data suggest the importance of Bcl6 in cardiovascular diseases. However, the role of Bcl6 in fibroblast activation is unclear. The aim of this study is to explore the functional role of Bcl6 in cardiac fibroblast activation and function.

1 MATERIALS AND METHODS

1.1 Materials

Dulbecco's Modified Eagle's medium/F12

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(DEME/F12) was purchased from GIBICO (USA). The fetal bovine serum (FBS) was purchased from Hyclone (USA). The cell counting kit-8 (CCK-8) was obtained from Dojindo (Japan). TRIzol reagent was bought from Invitrogen (USA). The primary antibodies against total and phosphorylated smad2, smad3, smad4 and GAPDH were purchased from Cell Signaling Technology (USA) and the secondary antibodies were obtained from LI-COR Biosciences (USA). SRI011381 was purchased from MedChemExpress (USA). Adenovirus Bcl-6 (Ad-Bcl6) was obtained from Vigene Bioscience (China).

1.2 Isolation and Culture of Neonatal Rat Cardiac Fibroblasts

The neonatal rat cardiac fibroblasts were obtained from the Sprague-Daw rats born within three days. Briefly, hearts from neonatal rats were removed and cut into 1 mm^3 small pieces. Heart tissues were digested in 0.125% trypsin for 15 min at 37°C for 5 times. All digestive fluid was combined and centrifuged at 1000 r/min for 8 min. Then the cells were re-suspended and filtered through a 25-μm cell strainer. Cardiac fibroblasts were seeded on a 100-mm plate for 90 min till they adhered to the culture plate. Thereafter the suspended cardiomyocytes were removed. The remaining cardiac fibroblasts were cultured in DMEM/F12 containing 10% FBS at 37°C in a humidified incubator (SANYO 18 M) with 5% CO_2.

Cardiac fibroblasts were treated with transforming growth factor β1 (TGFβ1) (Protein-Tech, 10 ng/μL) or angiotensin Ⅱ (Ang Ⅱ, 1 μmol/L) for 24 h for protein extraction and immunofluorescence staining. Fibroblasts were transfected with Ad-Bcl6 or the negative control (Ad-NC) for 8 h to overexpress Bcl6 (MOI=50) and then treated with TGFβ1 for another 12, 24, 36 or 48 h for cell viability detection. Cells were transfected with Ad-Bcl6 for 8 h to overexpress Bcl6 (MOI=50) and then treated with TGFβ1 and SRI011381 (10 μmol/L) for 24 h.

1.3 Cell Proliferation

CCK-8 was used to examine the cardiac fibroblast proliferation. After treatment, 10 μL of CCK-8 reagent was added to each well for a 4-h incubation. The absorbance (*A*) was detected by a microplate reader (Bio-Tek, USA) at 450 nm.

1.4 Immunofluorescence

The expression of α-SMA was observed by immunofluorescence. Cells were fixed with 4% paraformaldehyde for 10 min, then permeabilized with 0.2% Triton X-100 (Amresco, USA) for 5 min. After blocked with 10% goat serum (Gibco, USA), cells were incubated with primary antibodies against α-SMA (Abcam, UK), Ki67 (Abcam, UK) and Bcl6 (Abcam, UK) overnight at 4°C. The second antibody IRDye® 800CW-conjugated antibody was used. 4′-6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) was used to stain cell nucleus.

1.5 Quantitative Real-time PCR

Total RNA was extracted by TRIzol reagent. The RNA was isolated and purified with a Smartspec Plus Spectrophotometer (Bio-Rad, USA). A total of 2 μg of each RNA sample was reversely transcribed into cDNA using oligo (dT) primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) in 20 μL reaction volume with a SYBR Green PCR Master Mix (Roche, Switzerland) to quantify PCR amplification. GAPDH gene expression was used as reference. Primers used in this manuscript were as follows: GAPDH (forward: ACTCCACTCACGGCAAATTC; reverse: TCTCCATGGTGGTGAAGACA); collagen Ⅰ (forward: TGGACATTAGGCGCAGGAA; reverse: GAGAGAGCATGACCGATGGATT); collagen Ⅲ (forward: TTCCTGGGAGAAATGGCGAC; reverse: GGCCACCAGTTGGACATGAT); connective tissue growth factor (CTGF) (forward: AGACACATTTGGCCCTGACC; reverse: TCTTAGAACAGGCGCTCCAC).

1.6 Western Blotting

Cardiac fibroblasts were lysed in RIPA lysis buffer. Cell lysates (40 μg) were fractionated via 10% SDS-PAGE (Invitrogen, USA). Proteins were transferred to a PVDF membrane (Millipore, USA) and incubated in specific antibodies against different antigens for 12 h. After incubation with secondary IRDye® 800CW-conjugated antibody for 60 min, the blots were scanned using the Odyssey infrared imaging system (Li-COR Biosciences, USA) and protein expression levels were normalized using GAPDH (Cell Signaling Technology, USA) as internal control in the total cell lysate.

1.7 Co-immunoprecipitation Assays

Cultured fibroblasts co-transfected with psicoR-HA-Bcl6 and psicoR-Flag-smad4 were lysed in immunoprecipitation buffer. For immunoprecipitation, 10 μL protein A/G-agarose beads and 1 μg antibody were incubated with each sample (500 μL) overnight at 4°C. After washing with immunoprecipitation buffer, the eluted proteins were immunoblotted with the indicated primary antibodies.

1.8 Statistical Analysis

All data were expressed as mean±standard deviations (SD). Spss19.0 was used for data analysis throughout the study. Differences between two groups were performed using the unpaired students' *t*-test. Differences among groups were determined by one-way ANOVA followed by a post hoc Tukey test. A value of $P<0.05$ was considered to be significant.

2 RESULTS

2.1 Expression Level of Bcl6 in TGFβ1- and Ang Ⅱ-stimulated Fibroblasts

To explore whether Bcl6 participates in the

pathology of fibroblast activation, the expression level of Bcl6 in TGFβ1- and Ang Ⅱ-stimulated fibroblasts was detected. It was found that the protein expression of Bcl6 was down-regulated in both TGFβ1- and Ang Ⅱ-stimulated fibroblasts as compared with control group (fig. 1A and 1B). Consistent with the Western blotting results, staining results revealed that Bcl6 was mainly located in cell nucleus and down-regulated in both TGFβ1- and Ang Ⅱ-stimulated fibroblasts (fig. 1C and 1D). These results indicate Bcl6 is down-regulated in activated cardiac fibroblasts, and may participate in the functional role of fibroblasts.

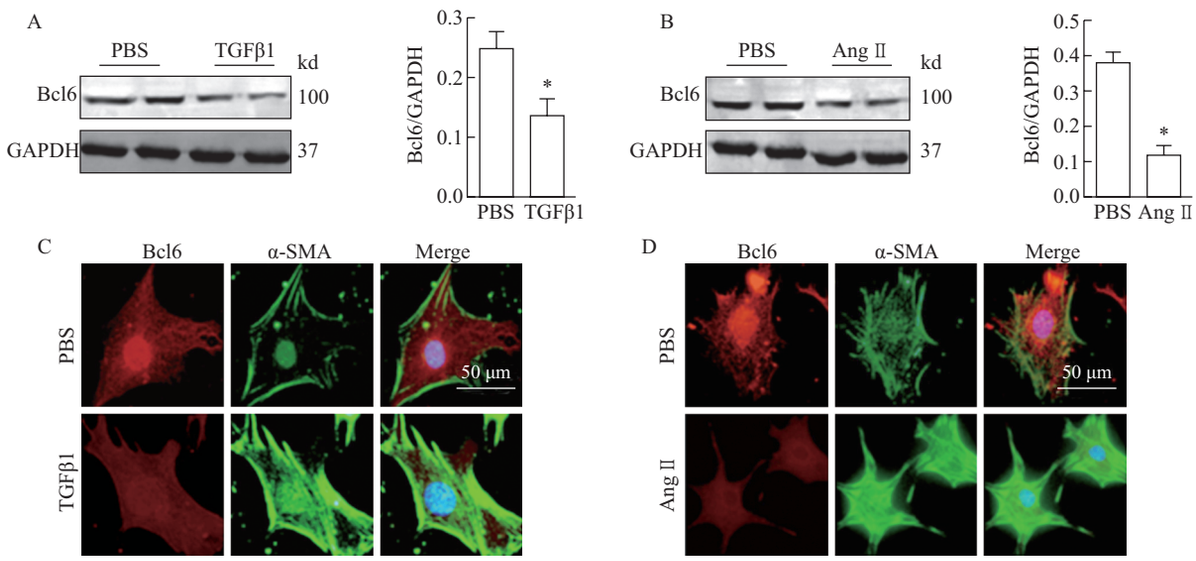

Fig. 1 The expression level of Bcl6 in TGFβ1- and Ang Ⅱ-stimulated fibroblasts
A: the expression level of Bcl6 in fibroblasts stimulated with TGFβ1 (n=6). B: the expression level of Bcl6 in fibroblasts stimulated with Ang Ⅱ (n=6). C and D: double staining of α-SMA and Bcl6 in fibroblasts stimulated with TGFβ1 (C) or Ang Ⅱ (D) (n=6). $^{*}P<0.05$ *vs.* PBS group

2.2 Bcl6 Overexpression Inhibits Fibroblast Activation and Proliferation

We then explored the role of Bcl6 in fibroblast proliferation and function. Cells were transfected with Ad-Bcl6 to overexpress Bcl6 (fig. 2A). Cell proliferation was detected by CCK-8 assay. Results showed that cell proliferation was increased in TGFβ1-stimulated fibroblasts as compared with the control group, but suppressed in Bcl6 overexpressed cells after TGFβ1 stimulation for 12, 24 and 48 h as compared with the negative control (Ad-NC) group (fig. 2B). Cells were stained with Ki67 to detect the proliferation positive cells. Consistently, the percentage of proliferation positive cells was increased in TGFβ1-stimulated group compared to the Ad-NC-PBS group, while reduced in Ad-Bcl6-TGFβ1 group compared to Ad-NC-TGFβ1 group (fig. 2C). Cell activation was further detected by α-SMA staining. Increased α-SMA expression level was observed in Ad-NC-TGFβ1 group. Bcl6 overexpression reduced α-SMA expression level in fibroblasts in response to TGFβ1 stimulation (fig. 2D). We then examined cell function by detecting the mRNA expression levels of collagen Ⅰ, collagen Ⅲ and CTGF. Increased collagen Ⅰ, collagen Ⅲ and CTGF transcription levels were observed in Ad-NC-TGFβ1 group. However, Bcl6 overexpression reduced the transcription levels of these factors in fibroblasts in response to TGFβ1 (fig. 2E).

2.3 Bcl6 Directly Binds to Smad4 and Negatively Regulates Smad4 Signaling

To explore the underlying mechanism of Bcl6 on fibroblast activation, we detected the canonical TGFβ-smad pathway. The expression levels of phosphorylated smad2, phosphorylated smad3 and smad4 were up-regulated in TGFβ1-stimulated fibroblasts, while Bcl6 overexpression merely reduced smad4 expression level without affecting smad2 and smad3 phosphorylation levels (fig. 3A and 3B). To elucidate whether Bcl6 regulates smad4 by direct binding, co-immunoprecipitation was conducted in fibroblasts. The results showed that Bcl6 could interact with smad4 in fibroblasts (fig. 3C).

2.4 Activating Smad4 Counteracts Functional Role of Bcl6

We then used a smad4 activator, SRI011381, to confirm the regulatory effect of Bcl6. Cell proliferation at 12, 24 and 48 h after TGFβ1 stimulation was reduced in Ad-Bcl6-TGFβ1 group, but increased in Ad-Bcl6-

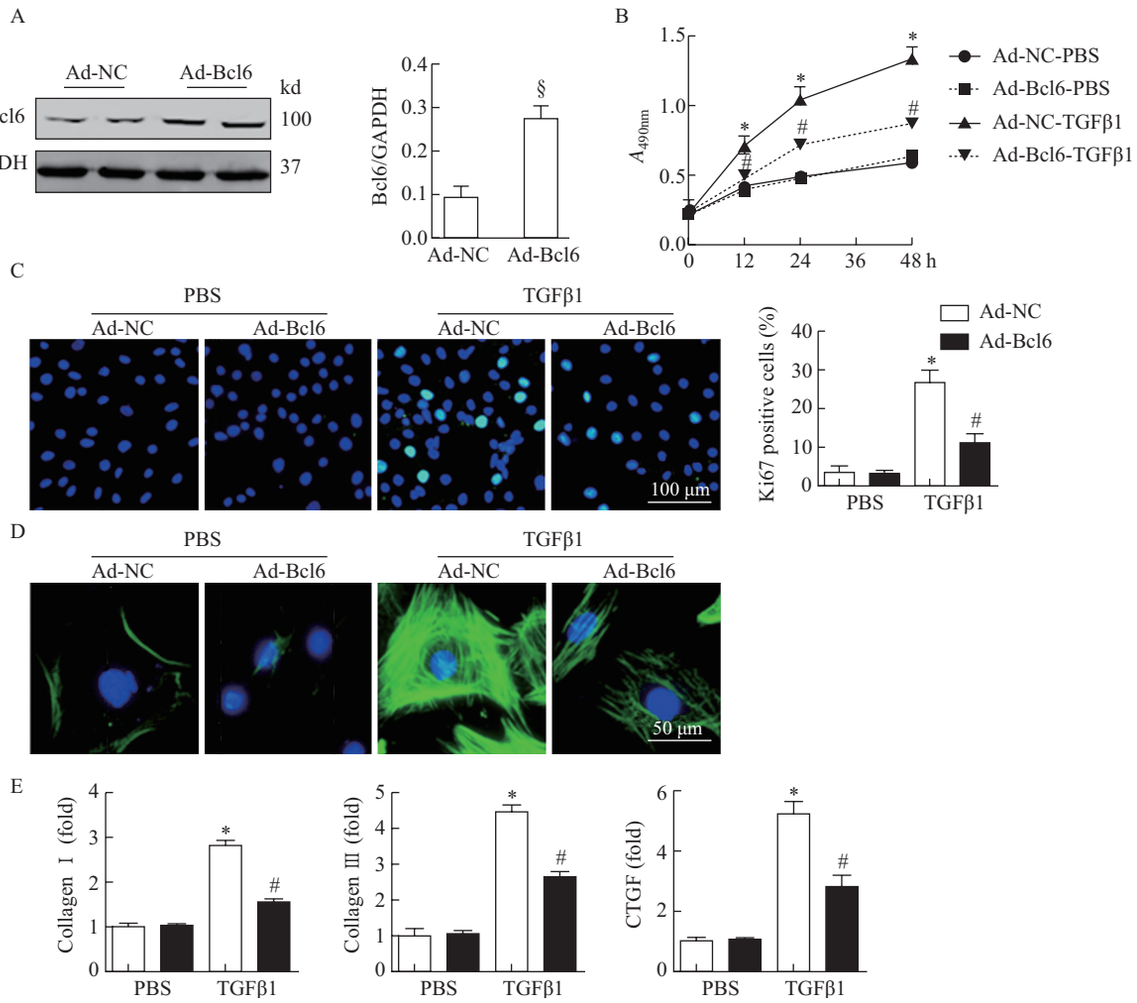

Fig. 2 Inhibitory effect of Bcl6 overexpression on fibroblast activation and proliferation

A: the expression level of Bcl6 in fibroblasts after transfected with Ad-Bcl6 (n=6). B: cell proliferation detected by CCK-8 assay (n=6). C: Ki67 staining in fibroblasts (n=5). D: α-SMA staining in fibroblasts (n=5). E: transcription level of collagen Ⅰ, collagen Ⅲ, and CTGF (n=6). $^{*}P$<0.05 *vs*. Ad-NC-PBS group, $^{\#}P$<0.05 *vs*. Ad-NC-TGFβ1 group, $^{\S}P$<0.05 *vs*. Ad-NC group

TGF1+SRI011381 group (fig. 4A). The similar phenomenon was also observed in Ki67 staining experiment. The percentage of proliferation positive cells was decreased by Bcl6 overexpression, but increased by SRI011381 (fig. 4B). As demonstrated by the increased α-SMA expression level and collagen Ⅰ, collagen Ⅲ, CTGF transcription levels, cardiac fibroblast activation and function were reduced by Bcl6 overexpression, but increased by SRI011381(fig. 4C and 4D).

3 DISCUSSION

Maladaptive cardiac fibrosis involves ECM deposition in response to long-standing stress. Unrestrained cardiac fibrosis can elicit various deleterious effects[2]. For example, interstitial fibrosis can increase myocardial stiffness, cause diastolic dysfunction, and even lead to arrhythmia and sudden cardiac death[1]. However, up to now, no targeted anti-fibrotic drugs were released despite of the widely accepted roles of fibrosis in cardiac dysfunction[11]. In this study, we found that Bcl6 was down-regulated in TGFβ1- and Ang Ⅱ-stimulated fibroblasts. Moreover, Bcl6 overexpression suppressed proliferation, activation and function of cardiac fibroblasts.

Fibroblasts are cells of mesenchymal origin that populate connective tissues and produce significant amounts of structural ECM proteins[12]. In the injury sites, fibroblasts are activated and undergo conversion to myofibroblasts which are cells that combine the presence of an extensive endoplasmic reticulum, a feature of synthetically active fibroblasts, with the expression of contractile proteins, such as α-SMA, and the de novo synthesis of matricellular proteins, such as collagen[13]. Bcl6 was reported to regulate metastasis of various cancer cells such as colorectal cancer cells and non-small cell lung cancer cells[14, 15]. Whereas, whether

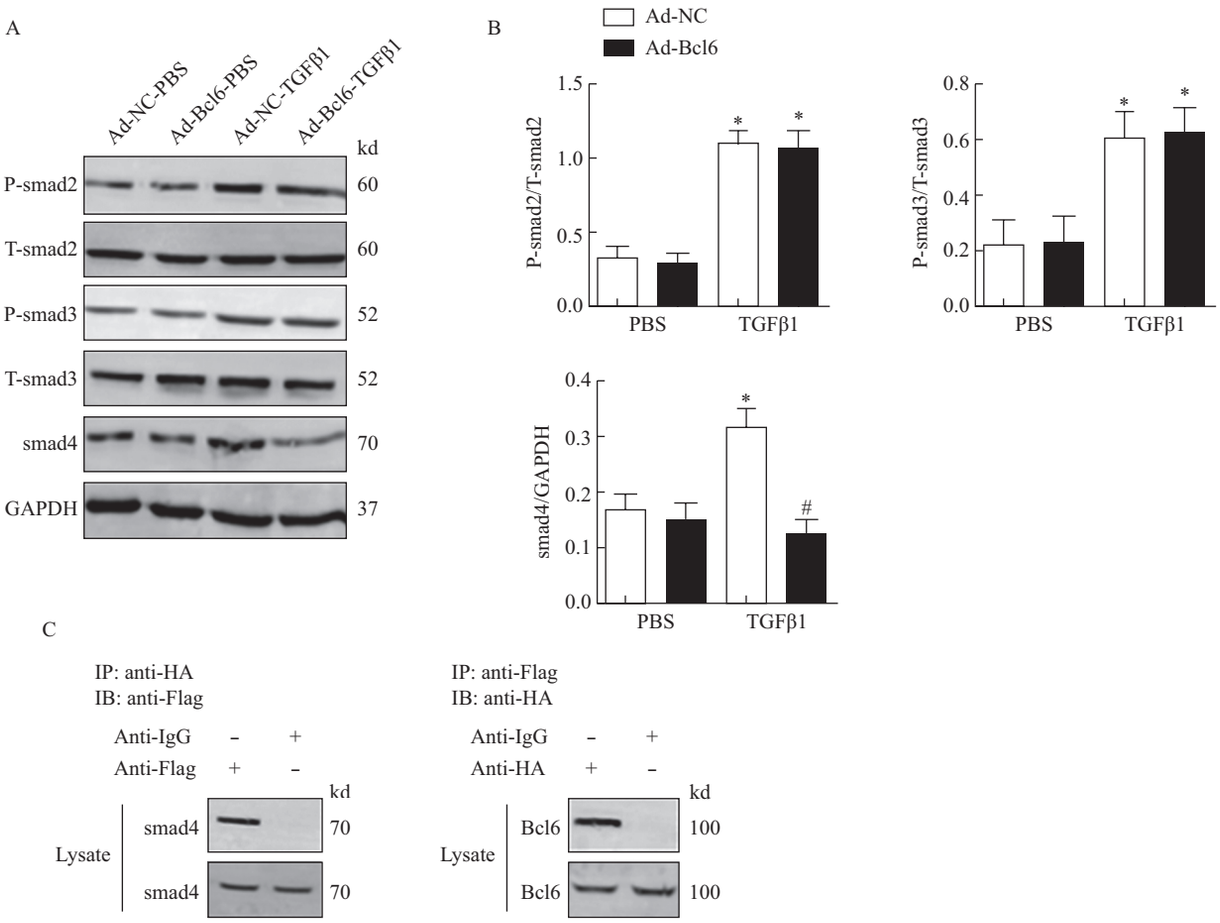

Fig. 3 The direct binding of Bcl6 to smad4 negatively regulate smad4 signaling
A and B: Western blotting (A) and quantitative results (B) of P-smad2, T-smad2, P-smad3, T-smad3 and smad4 in fibroblasts (n=6). C: co-immunoprecipitation assay for the interaction of Bcl6 and smad4. $^{*}P<0.05$ *vs.* Ad-NC-PBS group, $^{\#}P<0.05$ *vs.* Ad-NC-TGFβ1 group

Bcl6 functions on fibroblasts remains unknown. In this study, we found that Bcl6 inhibited TGFβ1-induced cell proliferation, activation and collagen synthesis, which provided an example of Bcl6 on fibrotic diseases.

Myofibroblast activation in the infarcted and remodeling myocardium requires the co-operation of growth factors and specialized matrix proteins, and signals through cell surface receptors to activate intracellular signaling pathways that lead to synthesis of contractile proteins and transcription of matrix macromolecules[16]. TGFβ also plays a crucial role in activation of fibroblasts in the remodeling myocardium by acting through pathways involving a family of intracellular effectors, the smads, or through smad-independent cascades[11]. Under stimulation, inflammatory cells, cardiomyocytes or fibroblasts release TGFβ1 which connects to the TGFβ1 receptor Ⅰ or Ⅱ on the fibroblasts surface[17]. The canonical smad2/3 pathways are activated by the signaling through TGFβR1, which causes the phosphorylation of smad2/3[18]. Activated smad2 and smad3 bind to smad4, then transfer to cell nucleus, leading to the transcriptional regulation of fibrosis associated genes[2]. A previous study has reported that Bcl6 represses smad signaling in B-cell lymphoma[19]. Bcl6 can physically interact with smad3 and smad4, disrupt the smad-p300 interaction, and repress the transcriptional activity of smad4 in B-cell lymphoma[19]. In this study, we found that instead of activating smad2 and smad3, Bcl6 down-regulated the expression of smad4. An interaction of Bcl6 and smad4 was observed, which implicated Bcl6 might interact with smad4 thus inhibiting smad signaling. These were confirmed by smad4 activator which abolished the effects of Bcl6.

It should be noted that neonatal fibroblasts were employed in this study, but the response of neonatal fibroblasts may be different from those of adult cardiac fibroblasts. Cautions should be taken when expanding our study to other applications. Then further study using adult cardiac fibroblasts should be performed to confirm the effects of Bcl6.

In summary, during the pathology of cardiac

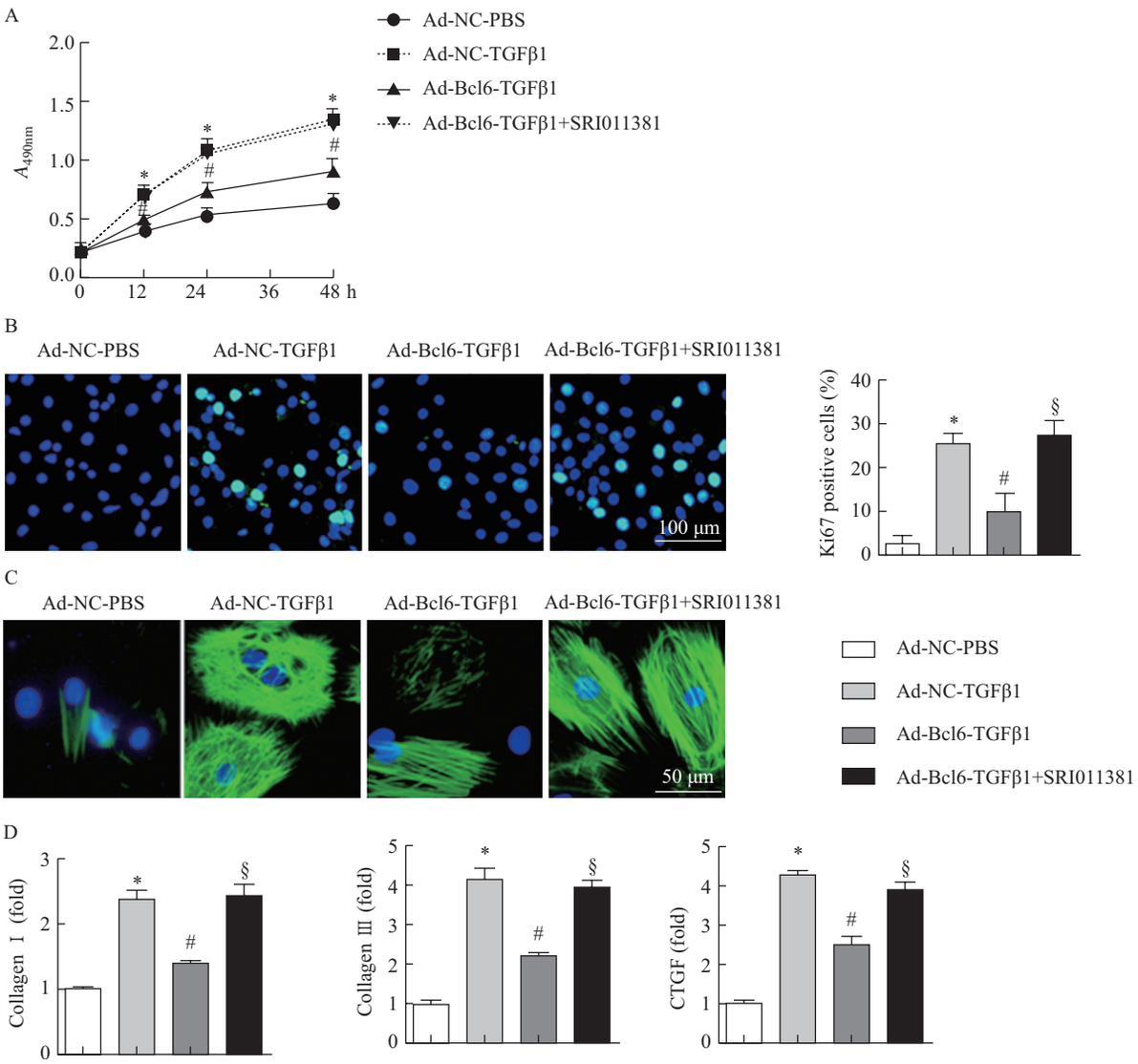

Fig. 4 Activating smad4 counteracts the functional role of Bcl6

A: cell proliferation detected by CCK-8 assay (n=6). B: Ki67 staining in fibroblasts (n=5). C: α-SMA staining in fibroblasts (n=5). D: transcription level of collagen Ⅰ, collagen Ⅲ, and CTGF (n=6). $^{*}P$<0.05 *vs*. Ad-NC-PBS group, $^{\#}P$<0.05 *vs*. Ad-NC-TGFβ1 group, $^{\S}P$<0.05 *vs*. Ad-Bcl6-TGFβ1 group

fibrosis, Bcl6 is down-regulated in cardiac fibroblasts. Overexpression of Bcl6 inhibits TGFβ1-induced fibroblast activation, proliferation and function. These effects are mediated by the interaction of Bcl6 with smad4, which subsequently inhibits the canonical TGFβ1-smad signaling. Thus, targeting on Bcl6 may be a potential new therapeutic method for the treatment of cardiac fibrosis.

Conflict of Interest Statement

The authors declare that they have no competing financial interests.

REFERENCES

1 Ma ZG, Yuan YP, Wu HM, *et al*. Cardiac fibrosis: new insights into the pathogenesis. Int J Biol Sci, 2018,14(12):1645-1657

2 Frangogiannis NG. Cardiac fibrosis: Cell biological mechanisms, molecular pathways and therapeutic opportunities. Mol Aspects Med, 2019,65:70-99

3 Li L, Zhao Q, Kong W. Extracellular matrix remodeling and cardiac fibrosis. Matrix Biol, 2018,68-69:490-506

4 Al Hattab D, Czubryt MP. A primer on current progress in cardiac fibrosis. Can J Physiol Pharmacol, 2017,95(10):1091-1099

5 Leeman-Neill RJ, Bhagat G. BCL6 as a therapeutic target for lymphoma. Expert Opin Ther Targets, 2018,22(2):143-152

6 Cardenas MG, Oswald E, Yu W, *et al*. The Expanding Role of the BCL6 Oncoprotein as a Cancer Therapeutic Target. Clin Cancer Res, 2017,23(4):885-893

7 Wei Z, Gao W, Wu Y, *et al*. Mutual interaction between BCL6 and miRNAs contributing to the pathogenesis of

various cancers. Clin Transl Oncol, 2015,17(11):841-846
8 Yoshida T, Fukuda T, Hatano M, *et al*. The role of Bcl6 in mature cardiac myocytes. Cardiovasc Res, 1999,42(3):670-679
9 Altieri P, Spallarossa P, Barisione C, *et al*. Inhibition of doxorubicin-induced senescence by PPARdelta activation agonists in cardiac muscle cells: cooperation between PPARdelta and Bcl6. PLoS One, 2012,7(9):e46126
10 Gu Y, Luo M, Li Y, *et al*. Bcl6 knockdown aggravates hypoxia injury in cardiomyocytes via the P38 pathway. Cell Biol Int, 2019,43(2):108-116
11 Bhandary B, Meng Q, James J, *et al*. Cardiac Fibrosis in Proteotoxic Cardiac Disease is Dependent Upon Myofibroblast TGF-β Signaling. J Am Heart Assoc, 2018,7(20):e010013
12 Goh KY, He L, Song J, *et al*. Mitoquinone ameliorates pressure overload-induced cardiac fibrosis and left ventricular dysfunction in mice. Redox Biol, 2019,21:101-100
13 Pattar SS, Fatehi Hassanabad A, Fedak PWM. Targeting selected extracellular matrix components to attenuate cardiac fibrosis. Ann Transl Med, 2018,6(Suppl 1):S49
14 Hu S, Cao B, Zhang M, *et al*. Epigenetic silencing BCL6B induced colorectal cancer proliferation and metastasis by inhibiting P53 signaling. Am J Cancer Res, 2015,5(2):651-662
15 Li Y, Zhang X, Yang Z, *et al*. miR-339-5p inhibits metastasis of non-small cell lung cancer by regulating the epithelial-to-mesenchymal transition. Oncol Lett, 2018,15(2):2508-2514
16 Felisbino MB, McKinsey TA. Epigenetics in Cardiac Fibrosis: Emphasis on Inflammation and Fibroblast Activation. JACC Basic Transl Sci, 2018,3(5):704-715
17 Wu QQ, Ni J, Zhang N, *et al*. Andrographolide Protects against Aortic Banding-Induced Experimental Cardiac Hypertrophy by Inhibiting MAPKs Signaling. Front Pharmacol, 2017,8:808
18 Jiang XH, Wu QQ, Xiao Y, *et al*. Evodiamine Prevents Isoproterenol-Induced Cardiac Fibrosis by Regulating Endothelial-to-Mesenchymal Transition. Planta Med, 2017,83(9):761-769
19 Wang D, Long J, Dai F, *et al*. BCL6 represses Smad signaling in transforming growth factor-beta resistance. Cancer Res, 2008,68(3):783-789

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