Cardiac Fibroblast-Specific Activating Transcription Factor 3 Promotes Myocardial Repair after Myocardial Infarction

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Abstract

Background: Myocardial ischemia injury is one of the leading causes of death and disability worldwide. Cardiac fibroblasts (CFs) have central roles in modulating cardiac function under pathophysiological conditions. Activating transcription factor 3 (ATF3) plays a self-protective role in counteracting CF dysfunction. However, the precise function of CF-specific ATF3 during myocardial infarction (MI) injury/repair remains incompletely understood. The aim of this study was to determine whether CF-specific ATF3 affected cardiac repair after MI.

Methods: Fifteen male C57BL/6 wild-type mice were performed with MI operation to observe the expression of ATF3 at 0, 0.5, 1.0, 3.0, and 7.0 days postoperation. Model for MI was constructed in ATF3TGfl/flCol1a2-Cre+ (CF-specific ATF3 overexpression group, $n = 5$) and ATF3TGfl/flCol1a2-Cre− male mice (without CF-specific ATF3 overexpression group, $n = 5$). In addition, five mice of ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− were subjected to sham MI operation. Heart function was detected by ultrasound and left ventricular remodeling was observed by Masson staining (myocardial fibrosis area was detected by blue collagen deposition area) at the 28th day after MI surgery in ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− mice received sham or MI operation. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect cell proliferation/cell cycle-related gene expression in cardiac tissue. BrdU staining was used to detect fibroblast proliferation.

Results: After establishment of an MI model, we found that ATF3 proteins were increased in the heart of mice after MI surgery and dominantly expressed in CFs. Genetic overexpression of ATF3 in CFs (ATF3TGfl/flCol1a2-Cre+ group) resulted in an improvement in the heart function as indicated by increased cardiac ejection fraction (41.0% vs. 30.5%, $t = 8.610$, $P = 0.001$) and increased fractional shortening (26.8% vs. 18.1%, $t = 7.173$, $P = 0.002$), which was accompanied by a decrease in cardiac scar area (23.1% vs. 11.0%, $t = 8.610$, $P = 0.001$). qRT-PCR analysis of CFs isolated from ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− ischemic hearts revealed a distinct transcriptional profile in ATF3-overexpressing CFs, displaying pro-proliferation properties. BrdU-positive cells significantly increased in ATF3-overexpressing CFs than control CFs under angiotensin II stimuli (11.5% vs. 6.8%, $t = 31.599$, $P = 0.001$) or serum stimuli (31.6% vs. 20.1%, $t = 31.599$, $P = 0.001$). The 5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester assay showed that the cell numbers of the P2 and P3 generations were higher in the ATF3-overexpressing CFs at 24 h (P2: 91.6% vs. 71.8%, $t = 8.465$, $P = 0.015$) and 48 h (P3: 81.6% vs. 51.1%, $t = 9.029$, $P = 0.012$) after serum stimulation. Notably, ATF3 overexpression-induced CF proliferation was clearly increased in the heart after MI injury.

Conclusions: We identify that CF-specific ATF3 might contribute to be MI repair through upregulating the expression of cell cycle/proliferation-related genes and enhancing cell proliferation.

Key words: Activating Transcription Factor 3; Cardiac Fibroblast; Myocardial Infarction; Proliferation

INTRODUCTION

Myocardial infarction (MI) is a common cardiovascular disease with high mortality and morbidity. Growing evidence has indicated that the death of cardiomyocytes, angiogenesis of endothelial cells, activation of cardiac fibroblasts (CFs), accumulation of the extracellular matrix, and perivascular and myocardial interstitial inflammation

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are key pathological features of MI-induced injury/repair. Following MI, infarct healing is initiated, including cardiac stem cell activation, CF proliferation, and inflammatory cell infiltration. Poor myocardial reconstruction after MI is the major cause of early cardiac rupture and later heart failure. Myocardial fibrosis is an important pathological change in various heart diseases, but its pathogenesis is very complex and has not yet been fully elucidated.[1] Inflammation plays a pivotal role in cardiac remodeling, especially in myocardial fibrosis. Abnormal growth of CFs is critically involved in the pathophysiology of cardiac hypertrophy/remodeling.[2]

Transcription factors (TFs) that bind to *cis*-regulatory DNA sequences are responsible for either positively or negatively influencing the transcription of specific genes, essentially determining whether a particular gene will be turned on or off in an organism. Evidences have shown that TFs play a leading role in this regulation, with findings revealing their direct regulation of a number of genes whose expression is changed in hypertrophied myocardium,[3] fibrosis,[4] and inflammation.[5] Identifying the regulatory mechanism of potential TFs responsible for changes in gene expression under ischemic conditions could help to reveal MI-induced injury/repair mechanisms and enable the discovery of drug targets. Activating TF 3 (ATF3), a stress-inducible TF encoded by an immediate early gene, whose expression in most cell types is low or undetectable, can be induced by various extra- and intracellular signals.[6] Our previous study revealed that ATF3 modulated the function of CFs, mediates fibroblast-cardiomyocyte communication, and eventually inhibits hypertrophy and heart failure,[7] suggesting that it is a hub of the adaptive-response network in CFs. CFs are key cells that replace damaged and lost cardiomyocytes and contribute to cardiac repair.[8] However, the role of CF-specific ATF3 in MI-induced repair remains unknown. The aim of this study was to determine whether CF-specific ATF3 affected cardiac repair after MI.

METHODS

Ethical approval
All protocols were approved and all experiments were performed in accordance with the guidelines set by the Animal Care and Use Committee of Capital Medical University.

Animals and myocardial ischemia surgery
Fifteen male C57BL/6 wild-type (WT) mice were purchased from Beijing Huafukang Co., Ltd. We had established conditional CF-specific ATF3 overexpression mice (ATF3TGfl/flCol1a2-Cre+) and control mice (ATF3TGfl/flCol1a2-Cre−) as described previously,[7] and all mice were housed in a specific pathogen-free level animal house in the Beijing Institute of Heart, Lung and Blood Vessel Disease. All experiments were performed using 10- to 12-week-old male mice. In all experiments, ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− mice were

intraperitoneally treated with tamoxifen dissolved in corn oil (2 mg/kg) for 7 days at 8 weeks of age and then subjected to *in vivo* or *in vitro* stimuli.

WT and ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− mice underwent surgery to induce a MI model by occlusion of the left anterior descending coronary artery as described previously.[9,10] Fifteen WT mice were performed with MI operation. There were three mice in each group. Mice were sacrificed at 0, 0.5, 1.0, 3.0, and 7.0 days after the operation by carbon dioxide narcosis, and heart tissue was harvested. Twenty ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− male mice were performed with sham or MI operation. There were five mice in each group. After 4 weeks, the mice were received the transthoracic echocardiography test and then sacrificed.

Gene ontology enrichment analysis
Gene ontology analysis commonly begins with enrichment carried out on a short list of genes with statistically significant differential expression. In this method, gene ontology enrichment analysis is applied on the differentially expressed genes between these two groups.

Cardiac fibroblast isolation and culture
The heart was excised and washed three times using phosphate-buffered saline (PBS) until all the blood in the heart tissue had been removed. The heart tissue was cut into small pieces and transferred to a 50-ml centrifuge tube with the addition of 200 U/ml collagenase II. The tubes were agitated at 37°C for 30 min in a shaker and filled with PBS and centrifuged at 400 ×*g* for 5 min, and the supernatant was discarded. The pellet was filled with PBS with 1 U/ml dispase II, agitated, and then centrifuged. The pellet was resuspended in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS) and finally cultured in a 10 cm × 10 cm Petri dish. The cells were cultured at 37°C in a 5% CO_2 incubator and until the cells were fused into a single layer. CFs, isolated from the heart of ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− mice, were treated with starvation. After CFs had been starved and then treated with PBS, angiotensin II (Ang II), or FBS, CF proliferation was evaluated with BrdU assay and 5(6)-carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) assay.

Proliferation assay
BrdU assay
ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− mice were injected with BrdU (200 μg/g) at the day before MI surgery. After 0, 1.0, and 3.0 days, the heart tissues were harvested and sectioned into 5-μm thick slices. Tissue sections were fixed for 15 min using 4% formaldehyde, followed by three washes for 3 min each. Next, the sections were incubated with 0.3% Triton X-100 for 15 min to allow membrane penetration, followed by washing with PBS; then, the sections were incubated with 2 mol/LHCl for 30 min, followed by washing; and the sections were then incubated with sodium tetrafluoroborate. The sections were blocked by serum at room temperature for 30 min and probed using diluted antibodies at

4°C overnight. The sections were incubated with secondary antibodies for 30 min at room temperature, and diamidino-phenyl indole (DAPI) was used to stain cell nuclei, and the sections were observed using a Nikon ECLIPSE 90i microscope (Thermo Fisher Scientific, Waltham, MA, USA) at ×200 field of view.

5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester assay

Fibroblast was starvation by serum deprivation for 24 h. Fibroblasts were harvested and washed two times with PBS, resuspended at a final concentration of 1×10^6 cells/ml, then stained with 5 μmol/L CFSE (Invitrogen, Carlsbad, CA, USA), and incubated at 37°C for 10 min. After incubation, the labeling was quenched with 1 ml of ice-cold (4°C) RPMI 1640 containing 2 mm of L-glutamine, 50 mg/L of gentamicin sulfate, and 2 mg/L of amphotericin B and supplemented with 10% FBS (both Cultilab reagents, Brazil). Residual CFSE-labeled fibroblasts were washed the labled fibroblalsts were cultured and harvested at 0.5,1.0,3.0, and 7.0 days and analyzed by BD FACSCalibur analyzer (BD Biosciences,San Jose, CA, USA) and BD CellQuest software (BD Biosciences, San Jose, CA, USA) to determine fbroblast proliferation. With cell division and proliferation, the CFSE staining was expected to be diluted and the fluorescence strength shifted closer to the control cells.

Western-blotting analysis

Western blotting was performed as previously described.[11] Briefly, total protein from heart homogenate was extracted by sodium dodecyl sulfate (SDS) lysis buffer containing a protease inhibitor cocktail and phosphatase inhibitors (Roche, Mannheim, Germany). The protein content was measured using a bicinchoninic acid assay. The equal amount of protein samples were separated using 10% SDS-polyacrylamide gel electrophoresis and then were transferred to nitrocellulose membranes which were incubated with the ATF3cc antibodies (1:500, Santa Cruz) and glyceraldehyde phosphate dehydrogenase (GAPDH) Abs (1:1000, Santa Cruz) at 4°C overnight and then with infrared dye-conjugated secondary antibodies (1:10,000; Rockland Immunochemicals) in Tris-buffered saline with Tween for 1 h at room temperature. Images were quantified using the Odyssey Infrared Imaging System.

Quantitative real-time polymerase chain reaction analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously described.[12] Heart tissues were extracted by the Trizol reagent method (Invitrogen). Total RNA was reversed to first-strand cDNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed using of SYBR Green Master Mix (Takara, Otsu, Shiga, Japan) on the iCycler iQ system (Bio-Rad). Gene expression levels were normalized to GAPDH. All samples were run in duplicate. The primer sequences were as follows: *Ccna1*, Forward, 5'-TGATGCTTGTCAAATGCTCAGC-3', Reverse, 5'-AGGTCCTCCTGTACTGC-3'; *Ccnb1*, Forward, 5'-GGAGACGTGAGACGAGTCCAT-3', Reverse, 5'-GCAATAAACATGGCCGTTACACC-3'; *Ccnd1*, Forward, 5'-GCGTACCCTGACACCAATCTC-3';Reverse, 5'-CTCCTCTTCGCACTTCTGCTC-3'; *Cdc45*, Forward, 5'-GATTTCCGCAAGGAGTTCTACG-3', Reverse, 5'-TACTGGACGTGGTCACACTGA-3'; *Cdk1*, Forward, 5'-AGAAGGTACTTACGGTGTGGT-3', Reverse, 5'-GAGAGATTTCCCGAATTGCAGT-3'; *Mcm2*, Forward, 5'-ATCCACCACCGCTTCAAGAAC-3', Reverse, 5'-TACCACCAAACTCTCACGGTT-3'; *Mcm3*, Forward, 5'-AGCGCAGAGAGACTACTTGGA-3', Reverse, 5'-CAGCCGATACTGGTTGTCACT-3'; *Mcm4*, Forward, 5'-GAGGAAAGCAGGTCGTCACC-3', Reverse, 5'-AGGGCTGGAAAACAAGGCATT-3'; *Mcm5*, Forward, 5'-CAGAGGCGATTCAAGGAGTTC-3', Reverse, 5'-CGATCCAGTATTCACCCAGGT-3'; *Mcm6*, Forward, 5'-ACCAACCCAAGGTTTGGAGG-3', Reverse, 5'-TAATGCTCTCAGCGGTCTGTT-3'; *Mcm7*, Forward, 5'-AGTATGGGACCCAGTTGGTTC-3', Reverse, 5'-GCATTCTCGCAAATTGAGTCG-3'; *Pcna*, Forward, 5'-TTTGAGGCACGCCTGATCC-3', Reverse, 5'-GGAGACGTGAGACGAGTCCAT-3'.

Histopathology

After MI, mice were euthanized by an overdose of pentobarbital (100 mg/kg), and heart tissues were fixed in 4% paraformaldehyde and sectioned (5 μm). Masson trichrome staining was performed as described previously.[13] Cardiac fibrosis was calculated by the proportion of collagen-stained areas to the total myocardial area. For immunofluorescence, heart sections were stained with anti-ATF3 (1:100) and anti-discoidin domain receptor 2 (DDR2) (1:50) and then incubated with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate-conjugated secondary Abs. Images were captured by Nikon Eclipse TE2000-S microscope (Nikon, Japan). Quantification was used by Image-Pro Plus 3.0 (Nikon). Images analyzed by a person blinded to treatment.

Transthoracic echocardiography

On the 28th day after surgery, the cardiac function was evaluated using the Vevo 770 high-resolution microimaging system (VisualSonics, Toronto, Canada) as previously described.[10] The ultrasonic probe frequency was 30 HZ with an ultrasound measurement under the guidance of a two-dimensional image. All test images were used the same ultrasound parameters, and each image was measured at least five consecutive cycles.

Statistical analysis

Data are presented as means ± standard error (SE). Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 13.0 (SPSS Inc., Chicago, IL, USA) software. Differences were evaluated using the unpaired Student's *t*-test between two groups. A $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Activating transcription factor 3 upregulated in cardiac fibroblasts after myocardial infarction

WT mice underwent surgery to induce MI. Heart tissues were harvested at 0, 0.5, 1.0, 3.0, and 7.0 days after surgery. Western-blotting analysis revealed that ATF3 expression started to increase by 1.5-fold at 0.5 day after MI, compared with that in sham-operated mice, and it was still higher at 7 days after surgery [Figure 1a and 1b]. Double immunofluorescence staining for ATF3 and DDR2 (specific markers of CFs) revealed that ATF3 was dominantly
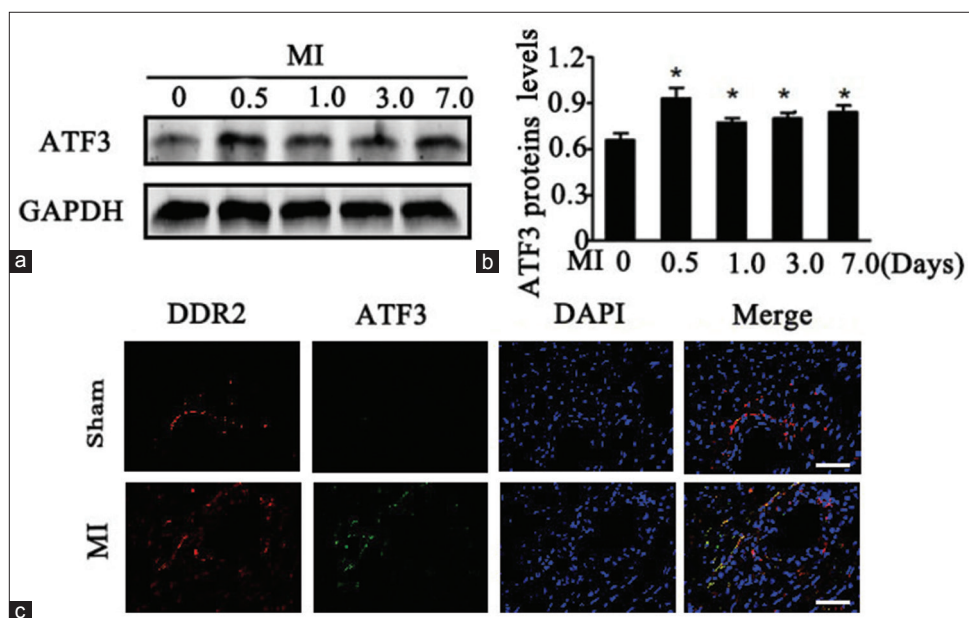
Figure 1: ATF3 expression was increased in cardiac fibroblasts after myocardial infarction (MI). (a and b) ATF3 protein expression was evaluated by western blotting in hearts after MI ($P = 0.001$ vs. 0 day, $n = 3$ per group). (c) Immunofluorescence analysis of ATF3 expression in cardiac fibroblasts 1 day after sham or MI surgery (scale bar = 100 μm). ATF3: Activating transcription factor 3; GAPDH: Glyceraldehyde phosphate dehydrogenase; DDR2: Anti-discoidin domain receptor 2; DAPI: Diamidino-phenyl indole.
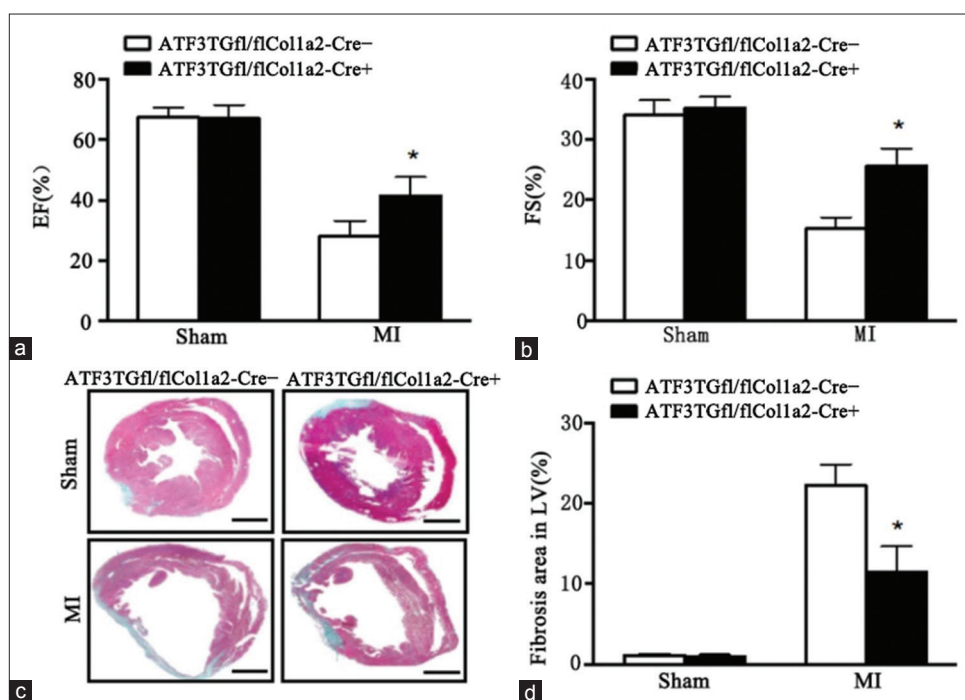
Figure 2: Cardiac fibroblast-specific ATF3 promotes myocardial repair. (a and b) M-mode echocardiography was performed on mice at 4 weeks after sham or MI surgery. Analysis of EF and FS of hearts subjected to MI ($n = 5$ per group). *$P = 0.001$ for EF or $P = 0.002$ for FS compared with ATF3TGfl/flCol1a2-Cre+ mice. (c) Representative cardiac Masson trichrome staining and quantification (scale bar = 500 μm). (d) Statistical results of the proportion of fibrosis-positive areas in LV ($n = 5$ per group). *$P = 0.001$ compared with ATF3TGfl/flCol1a2-Cre+ mice after MI. EF: Ejection fraction; FS: Fractional shortening; LV: Left ventricular.

expressed in CFs [Figure 1c]. These findings suggested that ATF3 was upregulated in response to MI injury and was mainly localized in CFs.

Upregulation of activating transcription factor 3 expression in cardiac fibroblasts protecting against cardiac scar formation and heart failure after myocardial infarction

To identify the role of ATF3 upregulation in CFs, we previously established a mouse model with conditional CF-specific ATF3 overexpression (ATF3TGfl/flCol1a2-Cre+) and an equivalent control (ATF3TGfl/flCol1a2-Cre−).[5] The mice underwent surgery to induce MI, and then, their heart function was assessed using ultrasound and pathological assessments 28 days after MI surgery. Transthoracic echocardiography showed a significant improvement of the ejection fraction (EF) and fractional shortening (FS) in ATF3TGfl/flCol1a2-Cre+ mice compared with those of control mice (EF: 41.0% vs. 30.5%, $t = 8.610$, $P = 0.001$; FS: 26.8% vs. 18.1%, $t = 7.173$, $P = 0.002$) [Figure 2a and 2b]. Moreover, histopathological analysis showed that left ventricular scar formation in ATF3TGfl/flCol1a2-Cre+ mice was significantly decreased compared with that in control mice (11.0% vs. 23.1%, $t = 8.610$, $P = 0.001$) [Figure 2c and 2d]. These results demonstrated that ATF3 overexpression in CFs protected against MI-induced heart failure and promoted myocardial repair.

Activating transcription factor 3 promotes cardiac fibroblast proliferation after myocardial infarction

qRT-PCR analysis confirmed that the mRNA levels of cyclin family members (*ccna1*, *ccnb1*, *ccnd1*, and *cdk1*), minichromosome maintenance proteins (*cdc45*, *mcm2*, *mcm3*, *mcm4*, *mcm5*, *mcm6*, and *mcm7*), and proliferation cell nuclear antigen (*PCNA*) were significantly upregulated in ATF3-overexpressing CFs [Figure 3a]. We performed a BrdU assay to examine the ATF3-induced proliferation of CFs *in vivo*. There were more BrdU-positive cells in ATF3TGfl/flCol1a2-Cre+ heart at 1 and 3 days after MI, and all of the BrdU-positive cells were also positive for DDR2, indicating that the proliferation of CFs was indeed increased in ATF3TGfl/flCol1a2-Cre+ mice [Figure 3b and 3c].

Activating transcription factor 3 overexpression promoting cardiac fibroblast proliferation *in vitro*

We further confirmed the role of ATF3 in promoting fibroblast proliferation *in vitro*. CFs, isolated from the heart of ATF3TGfl/flCol1a2-Cre+ and ATF3TGfl/flCol1a2-Cre− mice, were treated with Ang II or serum stimulation after starvation. BrdU staining showed that the BrdU-positive cells were present at a higher rate among ATF3-overexpressing CFs than among control cells under Ang II stimuli (11.5% vs. 6.8%, $t = 31.599$, $P = 0.001$) or serum stimuli (31.6% vs. 20.1%, $t = 31.599$, $P = 0.001$) [Figure 4a and 4b]. The CFSE assay also showed that the proliferation of ATF3-overexpressing fibroblasts was accelerated compared with that in control cells. The cell numbers of the P2 and P3 generations were higher in the ATF3-overexpressing CFs at 24 h (P2: 91.6% vs. 71.8%, $t = 8.465$, $P = 0.015$) and 48 h (P3: 81.6% vs.

51.1%, $t = 9.029$, $P = 0.012$) after serum stimulation [Figure 4c]. Taken together, these results showed that ATF3 promoted CF proliferation after MI injury.

DISCUSSION

We explored the role of CF-specific ATF3 in modulating MI injury/repair in this study. The obtained results revealed that ATF3 was highly expressed in the hearts of mice after MI surgery and predominantly expressed in CFs. We found that CF-specific ATF3 overexpression could promote cardiac repair and protect against heart dysfunction. In addition, both *in vivo* and *in vitro* studies indicated that the overexpression of ATF3 accelerated the proliferation of CFs.

ATF3 is a member of the family of Cyalic Adenosine monophsphate (cAMP) response element-binding protein TFs and has a basic leucine zipper-rich structure.[15] ATF3 helps cells adapt to homeostatic disturbances.[16] Many pathological insults have been shown to induce ATF3 expression, including pressure overload, β-adrenergic receptor agonists, and Ang II.[17,18] In this study, we found that ATF3 immediately increased in CFs after MI. Moreover, CF-specific ATF3 overexpression promoted cardiac repair and protected against heart dysfunction.

This study demonstrated that ATF3 promoted myocardial repair by accelerating the proliferation of CFs. We found that ATF3 had an important effect on CFs, based on the following results: (1) a pro-proliferation transcriptional profile was displayed in ATF3-overexpressing CFs, (2) ATF3 directly promoted CF proliferation under multiple stimuli, and (3) the proliferation of CFs increased in hearts of ATF3-overexpressing mice after MI. ATF3 plays a vital role in cardiac fibrosis, apoptosis, and autophagy response by regulating MEK-ERK1/2 and the JNK, NF-κB, and Beclin-1 pathways.[19] Changes in ATF3 expression are related to the cell-protective response that can induce resting cells to transition into the cell cycle and also protect cells and prevent toxins from inducing apoptosis. For example, ATF3 can induce cyclin D1 expression and then activate hepatocytes to transition into the cell cycle.[20] Furthermore, a previous study suggested that the ectopic expression of ATF3 promoted c-myc-deficient cell proliferation, predominantly by attenuating G1-stage cell inhibition.[21] ATF3 promotes cell proliferation by downregulating the expression of apoptosis-promoting genes.[22] ATF3 can also regulate RANKL-induced cell proliferation in osteoclast precursors.[23] ATF3 was linked to CF proliferation through upregulation of cell cycle-associated genes, which consisted with previous observation that transcriptional of cycle 2 (CDC2) and cyclin E2 (CCNE2), which control the cell transition from G1 phase to S phase, were downregulated in ATF3-KO cells.[22] ATF3 promotes *cyclin D1* mRNA expression though modulating activator protein-1-dependent transcription.[23] Moreover, previous study found that ATF3 promotes cancer cell proliferation through inhibiting P53 expression and then activating STAT3 phosphorylation.[24] This and other studies imply that ATF3 plays an important role in the regulation of cell proliferation.
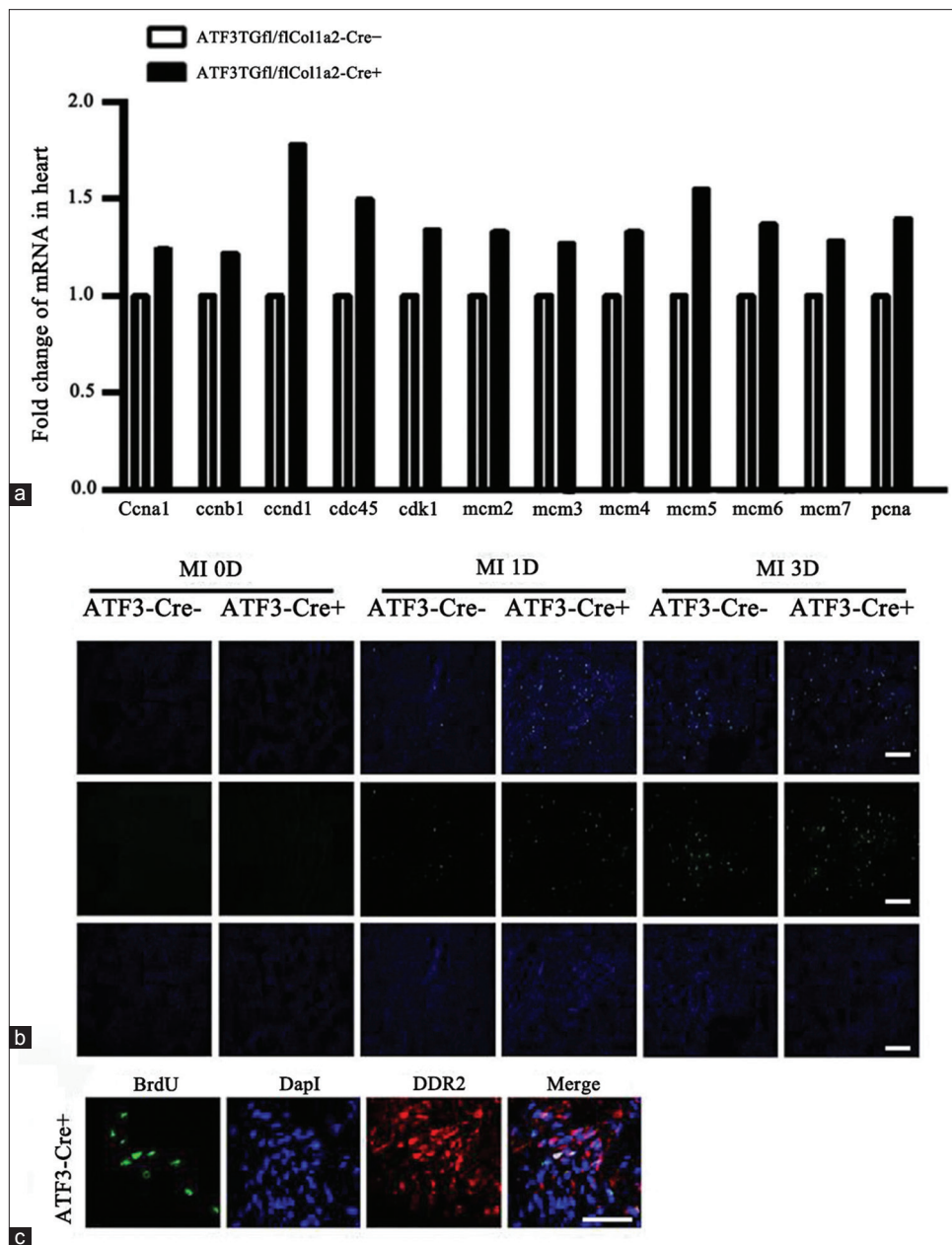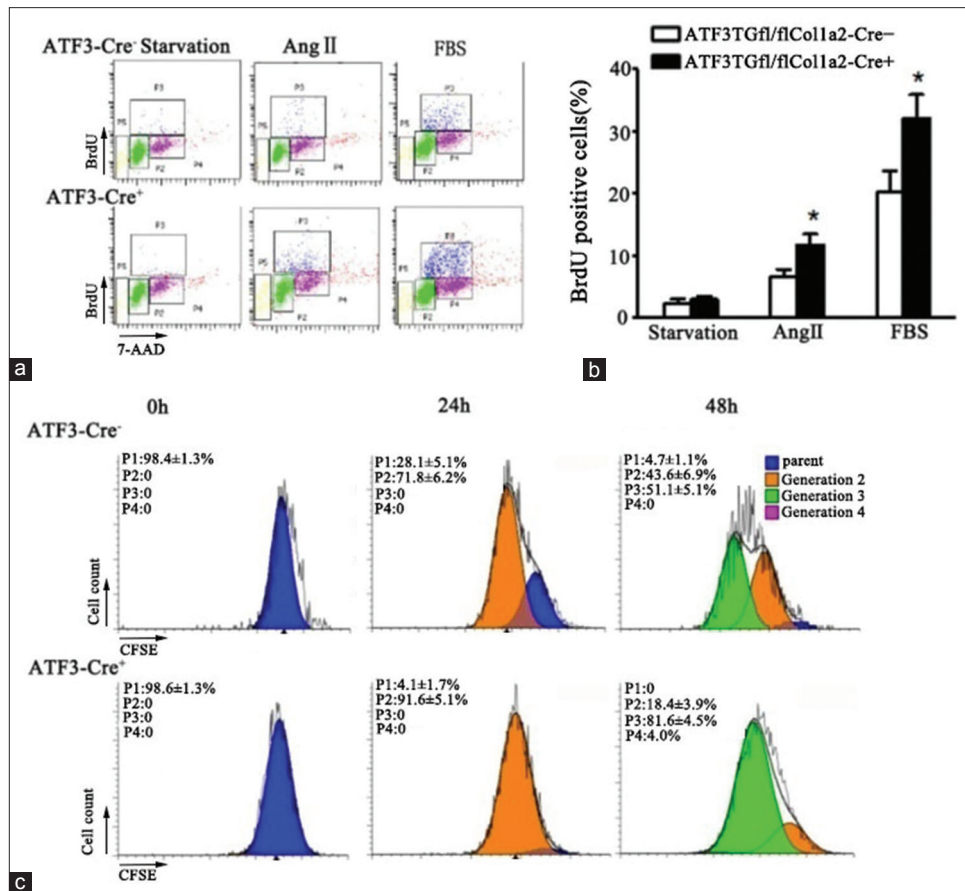
Figure 3: Fibroblast-specific ATF3 overexpression promotes cardiac fibroblast proliferation after myocardial infarction. (a) CFs were isolated from the hearts of ATF3TGfl/flCol1a2-Cre− and ATF3fl/flTGCol1a2-Cre+ mice at 1 day after MI. The expression of cell proliferation/cell cycle-related genes was evaluated in CFs using qRT-PCR analysis, ATF3TGfl/flCol1a2-Cre+ compared with ATF3TGfl/flCol1a2-Cre− CFs. Data represent the average of three independent experiments. (b) BrdU-positive cells among proliferative cells in the hearts from both types of mice at 0, 1.0, and 3.0 days after MI (scale bar = 40 μm). (c) Double immunofluorescence staining with BrdU (green) and DDR2 (red) in injured heart (at day 1st after injury) of mice; nuclei were stained with DAPI (blue; scale bar = 50 μm). ATF3: Activating transcription factor 3; CFs: Cardiac fibroblasts; qRT-PCR: Quantitative real-time polymerase chain reaction; DDR2: Anti-discoidin domain receptor 2; DAPI: Diamidino-phenyl indole.

The heart is mainly composed of cardiomyocytes, fibroblasts, and endothelial cells. CFs form the majority, accounting for 60–70% of cardiac cells. Fibroblast cells spread all over the heart tissue, wrap around myocardial cells, and connect cardiac intercellular substances. They also participate in the synthesis and deposition of extracellular matrix and are closely related to heart development, structure, the cardiac cell signaling system, and metabolism. Some studies have shown that heart failure group showed obvious hyperplasia of fibrotic tissue.[25] In addition, fibroblasts can interact with cardiomyocytes and even endothelial cells.[15] In normal heart, fibroblasts have no contractility and can only secrete a small amount of the extracellular matrix. Upon exposure to pathological stimuli, fibroblasts are activated to form myofibroblasts, which produce extracellular matrix proteins, such as collagen fibers, fibronectin, and laminin, to replace cardiomyocyte loss. Fibroblasts can also secrete growth factors or angiogenic

Figure 4: ATF3 overexpression promotes cardiac fibroblast proliferation *in vitro*. (a) CFs isolated from the hearts of ATF3fl/flTGCol1a2-Cre− and ATF3fl/flTGCol1a2-Cre+ mice were starved and treated with PBS, Ang II, and FBS addition. CF proliferation was evaluated with BrdU assay. (b) Statistical results of the BrdU-positive dells. *P = 0.001 for Ang II stimuli and P = 0.001 for FBS stimuli compared with ATF3TGfl/flCol1a2-Cre− CFs. Data represent the average of three independent experiments. (c) CFs isolated from the hearts of ATF3fl/flTGCol1a2-Cre− and ATF3fl/flTGCol1a2-Cre+ mice were starved and treated with FBS addition for 0, 24, and 48 h. At 0, 24, and 48 h, CF proliferation were evaluated with CFSE assay. PBS: Phosphate-buffered saline; FBS: Fetal bovine serum; Ang II: Angiotensin II; CF: Cardiac fibroblasts; CFSE: 5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester.

factors, which promote cardiac myocyte survival and angiogenesis. In the early phase of MI, CF proliferation prevents cardiac rupture, improves the survival rate, and promotes cardiac repair.[26,27] Some studies have shown that ATF3 deficiency was associated with more fibrosis, which was higher in the ATF3-null mice. Overall, ATFE may serve a largely maladaptive role during heart injury.[28] Emerging data indicate that upon I/R, ATF3 play essential roles in signaling during antiapoptotic, antimigration, and anti-inflammatory processes. Accumulating data suggest that ATF3 may be a potential target in I/R- or inflammation-induced organ dysfuction.[29] Thus, our finding that ATF3 promotes myocardial repair could potentially be mediated by accelerating the proliferation of fibroblasts.

To explore the clinical values of ATF3, our previous study has established a mi-RNA-aided ATF3-carrying lentivirus system preventing cardiomyocyte ATF3 overexpression, demonstrating CF-specific ATF3 overexpression.[7] Nowadays, it is well recognized that adeno-associated virus (AAV) is safety for clinical treatment. Hence, we hope to establish a mi-RNA-acded ATF3-carrying AAV system to enhance ATF3

expression in CF and improve cardiac scar formation and heart failure in patients with myocardial infarction in the future.

In summary, we have demonstrated an important function of ATF3 in CFs. We have shown that ATF3 is a potent endogenous protector against MI-induced injury, which acts by promoting CFs proliferation.

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Conflicts of interest

There are no conflicts of interest.

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ATF3通过促进成纤维细胞增殖抑制心肌梗死后心肌损伤

摘要

目的：心肌梗死是全球心血管死亡的首要病因。成纤维细胞调节生理和病理刺激下心脏功能变化。ATF3是保护性转录因子参与成纤维细胞的功能调控。但是成纤维细胞特异性ATF3在心梗损伤/修复中的机制还不清楚。

方法：15只雄性C57BL/6野生小鼠分别行冠脉左前降支结扎手术观察ATF3的表达和定位情况，选取我们前期构建的成纤维细胞特异性ATF3过表达小鼠（ATF3TGfl/flCol1a2-Cre+）及对照组小鼠（ATF3TGfl/flCol1a2-Cre-）各10只，对其进行心肌梗死手术及假手术处理，在术后第28 天，采用超声检测心功能、Masson染色观察左心室重塑情况。采用qRT-PCR检测心脏组织中细胞增殖/细胞周期相关基因表达及采用BrdU染色检测心脏中细胞增殖。

结果：与术前相比，心肌梗死手术后ATF3表达升高,并且高表达在成纤维细胞。与ATF3TGfl/flCol1a2-Cre-对比，在心肌梗死术后28d, ATF3TGfl/flCol1a2-Cre+的心功能明显改善（EF: 41 % vs. 30.5%, t=8.610, P=0.001; FS 26.8% vs. 18.1%, t=7.173, P=0.002），左心室纤维化面积明显降低（左心室瘢痕面积: 23.1% vs.11.0 %, t=8.610, P=0.001）。RT-PCR检测显示小鼠心脏组织中细胞增殖/细胞周期相关基因表达增加。BrdU染色检测显示ATF3过表达成纤维细胞的增殖在血管紧张素II刺激(11.5% vs 6.8%, t=31.599, P=0.001)和血清刺激(31.6% vs. 20.1%, t = 31.599, P=0.001) 明显高于野生型成纤维细胞。CFSE检测显示ATF3过表达成纤维细胞增殖能力在血清刺激后24小时（P2: 91.6% vs. 71.8%, t=8.645, P=0.015）和48小时(P3: 81.6% vs. 51.1%, t=9.029, P=0.012)都明显高于野生成纤维细胞。成纤维细胞特异性过表达ATF3小鼠心梗后心脏中成纤维细胞的增殖高于对照小鼠。

结论：ATF3可能通过促进成纤维细胞增殖抑制心肌梗死后心肌损伤。