**MiR-142 and miR-212** **synergistically inhibits the proliferation and collagen formation of TGF-β1-induced cardiac fibroblasts by regulating c-Myc/TP53INP1**

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**Running title:** The role of miR-142/212 in CFs

**Abstract**

**Objective:** To investigate the mechanism of miR-142 and miR-212 on cardiac fibrosis in heart failure (HF) after myocardial infarction (MI).

**Methods:** The mouse model of HF after MI was established. Cardiac fibroblasts (CFs) were induced by TGF‑β1 to simulate cardiac fibrosis induced by HF *in vitro*. The molecule expressions were measured using qRT-PCR and western blot. MTT assay was used to detect CFs proliferation. Dual-luciferase reporter assay was used to measure the relationship between miR-142/miR-212 and c-Myc.

**Results:** The expressions of miR-142 and miR-212 were reduced in HF mice and TGF‑β1‑induced CFs, while c-Myc protein expression was increased and TP53INP1 protein expression was decreased. Moreover, overexpression of miR-142 and miR-212 inhibited the proliferation and collagen formation of CFs induced by TGF-β1 more significantly compared with overexpressing the two miRNAs separately. The results also showed that both miR-142 and miR-212 could target c-Myc, and overexpression of miR-142 and miR-212 diminished the effects of TGF-β1 on the proliferation and collagen formation of CFs as well as TP53INP1 expression, but overexpression of c-Myc abolished the effects.

**Conclusion:** Overexpression of miR-142 and miR-212 synergistically supressed the proliferation and collagen formation of TGF-β1-induced CFs by regulating c-Myc/TP53INP1.

**Keywords:** heart failure, cardiac fibroblasts, miR-142, miR-212, synergistically regulation

**1 Introduction**

Cardiovascular diseases (CVDs) are the leading cause of death globally [[1](#_ENREF_1)]. With the development of social economy and the change of life style in China, risk factors such as abnormal glucolipid metabolism and metabolic syndrome are increasing day by day, leading to the rapid growth of the incidence of CVD. Heart failure (HF) is a complex clinical syndrome and a manifestation of various heart diseases at advanced stage [[2](#_ENREF_2)]. Myocardial infarction (MI) is the most common cause of HF worldwide [[3](#_ENREF_3)]. Studies reported that the basic mechanism for the development of post-MI HF is ventricular remodeling, and cardiac fibrosis is a key part in the progress [[4](#_ENREF_4)]. However, the exact mechanism of cardiac fibrosis in the development of post-MI HF is not yet fully understood. Therefore, it is of great significance to elucidate the cytological and molecular mechanism of cardiac fibrosis after MI for finding new intervention targets that can regulate HF.

Tumor protein p53-induced nuclear protein 1 (TP53INP1), a stress-induced gene, plays a role in cell cycle arrest and p53-mediated apoptosis [[5](#_ENREF_5)]. Overexpression of TP53INP1 could promote apoptosis in multiple cell lines, including fibroblasts [[6](#_ENREF_6)]. He et al. [[7](#_ENREF_7)] reported that TP53INP1 inhibited the proliferation of cardiac fibroblasts (CFs), improving cardiac remodeling. However, the regulatory mechanism of TP53INP1 in CFs proliferation has not yet been fully elucidated. Weng et al. [[8](#_ENREF_8)] found that c-Myc could down-regulate TP53INP1 expression by binding to its core promoter in esophageal carcinoma. c-Myc is a proto-oncogene, which is related to the occurrence and development of multiple tumors [[9](#_ENREF_9), [10](#_ENREF_10)]. Previous studies suggested that c-Myc also involved in CVDs. For example, Hou et al. [[11](#_ENREF_11)] showed that c-Myc was up-regulated in failing hearts of human ischemic heart disease. Liu et al. [[12](#_ENREF_12)] demonstrated that activated c-Myc facilitated DNA damage and p53-mediated apoptosis as well as cell viability reduction, which was responsible for oxidative cardiac impairment of diabetic cardiomyopathy. Together, we suspected that the role of TP53INP1 in CFs proliferation might be related to c-Myc.

MicroRNAs (miRNAs) are a class of noncoding RNAs with the length between 18 and 25 nucleotides. MiRNAs can involve in various cellular biology processes by regulating specific target gene expression [[13](#_ENREF_13)]. Over the last several years, miRNAs have been considered regulator of normal cardiac function, and their abnormal expression is associated with CVDs [[14](#_ENREF_14), [15](#_ENREF_15)], including post-MI HF [[16](#_ENREF_16)]. For instance, up-regulation of miR-24 could reduce cardiac fibrosis after MI through a furin-TGF-β pathway [[17](#_ENREF_17)]; miR-223 promoted cell proliferation, migration, and differentiation in CFs by targeting RASA1, thus mediated cardiac fibrosis in post-MI HF [[18](#_ENREF_18)]. Based on the bioinformatics software (TargetScan), both miR-142 and miR-212 have binding sites with c-Myc. It has been reported that miR-142 inhibits hypoxia/reoxygenation‑induced apoptosis and fibrosis of cardiomyocytes [[19](#_ENREF_19)], and miR-212 reduces MI‑induced cardiomyocytes apoptosis [[20](#_ENREF_20)]. However, the mechanism of the two miRNAs in cardiac fibrosis in post-MI HF remains unclear.

In this study, we measured the expressions of miR-142, miR-212, c-Myc and TP53INP1 in CFs after MI, and investigated their role in cardiac fibrosis after MI, aiming to provide a novel strategy for the management of HF.

**2 Methods**

**2.1 Animals**

C57BL/6J mice (11-12 weeks old) were purchased from Guangdong Medical Laboratory Animal Center. The mice were randomly divided into sham group and HF group (n=6). All animal experimental procedures were approved by the Ethics Committee for Animal Studies of the Department of Geriatric Orthopaedics, The Third Hospital of Hebei Medical University.

The mouse model of HF after MI was established by ligation of the left anterior descending coronary artery (LAD) [[7](#_ENREF_7)]. Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection. Then, the LAD was ligated using 8-0 prolene suture. The mice in sham group did not ligation. After ligation for 14 days, the mice were euthanized, and the hearts were collected for the following experiments.

**2.2 Cardiac fibroblasts isolation**

Cardiac fibroblasts (CFs) were prepared from the above mice as described previously [[7](#_ENREF_7)]. Briefly, the heart were digested and centrifuged. Then, the ventricular fibroblasts were isolated from supernatant containing the depleted myocardial cells and passaged twice to remove endothelial cells. The CFs were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, USA), and 1% penicillin-streptomycin in 5% CO2 incubator at 37°C. The CFs were collected for qRT-PCR and western blot assays.

**2.3 Cell culture**

Primary mouse CFs were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin in 5% CO2 incubator at 37°C.

**2.4 Cell treatment**

MiR-142 mimic, miR-212 mimic, and pcDNA-c-Myc were synthesized by GenePharma (China), and transfected into primary mouse CFs using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instruction. After transfection for 24 h, cells were treated with transforming growth factor-beta 1 (TGF-β1, 5 ng/ml) for 48 h.

**2.5 Cell proliferation assay**

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to detect CFs proliferation. Briefly, CFs were added into 96-well plates at the concentration of 3×104 cells per well, and then incubated overnight in DMEM supplemented with 10% FBS. MTT (5 mg/mL, 20 μl) was added into each well, and incubated for 4 h. After removed the supernatant, 150 μl DMSO was added into each well to dissolve the resultant formazan crystals. The absorption was measured at a wavelength of 450 nm.

**2.6 Quantitative real time PCR (qRT-PCR)**

Total RNA was extracted from the cultured cells using TRIzol Reagent (Invitrogen, USA), and reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara, Japan) according to the manufacturer’s instructions. The MiScript SYBR-Green PCR kit (Qiagen, Germany) was used for real-time PCR to detect the expression of miR-142 and miR-212. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The 2-ΔΔCt method was used to analyze the data.

**2.7 Western blot**

Total protein was extracted from the cultured cells using RIPA Lysis Buffer (Beyotime, Beijing, China) for 30 min on ice, and then centrifuged at 16,000×g for 10 min (4°C). After collected the supernatant, a BCA protein assay kit (Thermo Fisher Scientific, USA) was used to calculate the protein concentration. Then, the protein was separated by 12% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA) followed by blocking with 5% skim milk for 1 h. After that, the membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies included anti-c-Myc antibody (1/500, Abcam), anti-TP53INP1 antibody (1/2000, Abcam), and anti-β-actin antibody (1/1000, Abcam). After washed in TBST for 3 times, the membranes were incubated with horseradish peroxidase-conjugate secondary antibody (1/2000, Santa Cruz) for 1 h at room temperature. Protein-antibody complexes were detected by the enhanced chemiluminescence system.

**2.8 Dual-****luciferase reporter assay**

The c-Myc 3’-UTR containing miR-142/212 binding sites was cloned downstream of a luciferase coding region in a pmirGLO vector (Promega, USA), respectively. The binding sites in the c-Myc 3’-UTR for miR-142, miR-212, or both were mutated. After co-transfection of luciferase reporter constructs and wild-type (WT) or mutated (MUT) corresponding miRNAs for 48 h, the cells were collected, and the relative luciferase activity was measured using a Dual-Glo™ Luciferase Assay System (Promega, USA).

**2.9 Statistical analysis**

All data analysis were performed using GraphPad Prism 7.0 and are presented as mean ± standard deviation (SD). Comparison of the two groups was performed by Student’s t-test. The statistical significance between different groups was evaluated using one-way ANOVA followed by the Newman-Keuls post hoc test. The significance level was set as *P* < 0.05.

**3 Results**

**3.1** **The** **expressions of miR-142 and miR-212 were reduced in HF mice and TGF‑β1‑induced CFs**

To explore the relationship between miR-142/miR-212 and HF, we first isolated CFs from HF mice, and measured the expressions of miR-142 and miR-212. As shown in Fig. 1A, miR-142 and miR-212 were low expressed in HF group compared with sham group. Meanwhile, c-Myc protein expression was increased, but TP53INP1 protein expression was decreased in HF group (Fig. 1B). To simulate cardiac fibrosis induced by HF *in vitro*, primary mouse CFs were induced by TGF‑β1. Results showed that TGF‑β1 enhanced CFs proliferation with time dependent (Fig. 1C). After 48 h, the protein expressions of Collagen I and III were up-regulated in TGF‑β1 group (Fig. 1D). In addition, TGF‑β1 could reduce the expressions of miR-142 and miR-212 as well as TP53INP1 protein expression, but increase c-Myc protein expression (Fig. 1E&F).

**3.2 Overexpression of miR-142 and miR-212** **synergistically regulated****the proliferation and collagen formation of CFs stimulated by TGF-β1**

To investigate the effects of miR-142 and miR-212 on TGF-β1-induced CFs, CFs were treated with TGF-β1 for 48 h after the transfection of miRNAs mimic. MiR-142 mimic could up-regulate miR-142 expression, and miR-212 mimic could up-regulate miR-212 expression (Fig. 2A). Results revealed that overexpression of miR-142 or miR-212 diminished the effects of TGF-β1 on cell proliferation in CFs, and overexpression of miR-142 and miR-212 inhibited the proliferation of CFs induced by TGF-β1 more significantly (Fig. 2B). Moreover, although miR-142 and miR-212 could individually suppress the protein expressions of Collagen I and III, the simultaneous introduction of both miR-142 and miR-212 showed a cooperative repression of Collagen I and III expressions (Fig. 2C).

**3.3 miR-142 and miR-212 targeted c-Myc**

Both miR-142 and miR-212 were found on mouse chromosome 11 (Fig. 3A). To identify whether c-Myc 3’UTR possessed a target site for miR-142 and miR-212, the bioinformatics tool (TargetScan) was used to predict the binding sites (Fig. 3B). Dual-luciferase reporter assay revealed that compared with the WT, the luciferase activity of MUT 1 and 2 was slightly increased when cells transfected with miR-142 mimic or/and miR-212 mimic, and the luciferase activity was returned to the negative control level in double MUT group (Fig. 3C). To explore how miR-142/miR-212 regulated c-Myc, CFs were transfected with miRNAs mimic or inhibitor. As shown in Fig. 3D, c-Myc expression was markedly reduced in CFs transfected with miR-142/212 mimic, and up-regulated in CFs transfected with miR-142/212 inhibitor.

**3.4 Overexpression of miR-142 and miR-212** **synergistically suppressed the proliferation and collagen formation of TGF-β1-induced CFs by regulating** **c-Myc/TP53INP1**

To investigate the mechanism of miR-142 and miR-212 on TGF-β1-induced CFs, CFs were treated with TGF-β1 for 48 h after the transfection of miR-142/212 mimic and pcDNA-c-Myc. As shown in Fig. 4A, miR-142 mimic and miR-212 mimic diminished the effects of TGF-β1 on c-Myc and TP53INP1 expression in CFs, and pcDNA-c-Myc changed the effects (Fig. 4A&B). Meanwhile, overexpression of miR-142 and miR-212 dramatically reversed the TGF-β1-mediated up-regulation of CFs proliferation and down-regulation of Collagen I and III expressions, but overexpression of c-Myc abolished the effects (Fig. 4C&D).

**4 Discussions**

In this study, we demonstrated for the first time that miR-142 and miR-212 could synergistically regulate the proliferation and collagen production of CFs. This might provide a new strategy for the treatment of post-MI HF.

The biological function of miR-142 in cancer has been well confirmed. For example, Wang et al. [[21](#_ENREF_21)] reported that miR-142 could inhibit lung cancer growth through targeting PIK3CA. Deng et al. [[22](#_ENREF_22)] pointed out that miR-142 suppressed the proliferation and invasion of cervical cancer cells by down-regulating FZD7. Recently, the role of miR-142 in CVDs has attracted wide attention. Zhan et al. [[23](#_ENREF_23)] suggested that miR-142 might be a potential therapeutic target for ischemic heart disease, and down-regulation of it could attenuate hypoxia-induced injury. Wang et al. [[19](#_ENREF_19)] demonstrated that miR-142 suppressed hypoxia/reoxygenation-induced myocardial injury by the direct inhibition of HMGB1 expression. Moreover, Vegter et al. [[24](#_ENREF_24)] reported that miR-142 was lowly expressed in acute and chronic HF. The similar findings have been found in the current study. We found that the expression of miR-142 was reduced in HF mice and TGF‑β1‑induced CFs, and overexpression of miR-142 could inhibit the proliferation and collagen formation of TGF-β1-induced CFs, which indicated that miR-142 might be a specific biomarker for HF.

In addition, it has been showed that miR-212 is an important regulator in multiple biological processes. Lin et al. [[25](#_ENREF_25)] found that miR-212 overexpression could reduce epithelial-mesenchymal transition of ovarian cancer cells. Xiao and his colleagues [[26](#_ENREF_26)] revealed that miR-212 was involved in non-alcoholic fatty liver disease (NAFLD), and down-regulation it could prevent NAFLD via targeting FGF-21. Chen et al. [[27](#_ENREF_27)] showed that miR-212 inhibited LPS-induced inflammatory response through targeting HMGB1 in sepsis. Moreover, miR-212 was also associated with CVDs. Previous studies demonstrated that overexpression of miR-212 could protect heart against MI via AQP9 and PI3K/Akt signaling pathway [[20](#_ENREF_20)]. Importantly, Ucar et al. [[28](#_ENREF_28)] reported that miR-212 might be related to HF. In the present study, we found a low expression of miR-212 in HF mice and TGF‑β1‑induced CFs, and the role of miR-212 in CFs was similar to miR-142. And interestingly enough, miR-142 and miR-212 could synergistically inhibit the proliferation and collagen formation of TGF-β1-induced CFs.

MiRNAs exert their role through regulating their target genes. Thus, we focused on using bioinformatics analysis and dual-luciferase reporter assay to investigate the hypothetical targets of miR-142 and miR-212. We identified c-Myc as a direct target gene of miR-142 and miR-212, and both of the two miRNAs could negatively regulate c-Myc expression. c-Myc is an important protein, and is linked to multiple cell functions including cell cycle progression, differentiation, and apoptosis [[29](#_ENREF_29)]. As early as 2002, it has been reported that c-Myc is related to the development of CVDs [[30](#_ENREF_30)]. In diabetic cardiomyopathy, up-regulation of c-myc could promote myocardial oxidative stress injury [[12](#_ENREF_12)]. In HF, the protein and mRNA levels of c-Myc were markedly increased, which made it a potential therapeutic target [[11](#_ENREF_11)]. In the current study, miR-142 and miR-212 exerted the synergistic effect in TGF-β1-induced CFs through down-regulating c-Myc, and TP53INP1 was involved in the mechanism. As we know, TP53INP1 is a key regulator for the biological process of cardiac fibroblasts [[7](#_ENREF_7)] and cardiomyocytes [[31](#_ENREF_31)]. In our study, we found that overexpression of miR-142 and miR-212 could up-regulate TP53INP1 via c-Myc, and then promote the proliferation and collagen formation of CFs stimulated by TGF-β1.

In conclusion, the present study demonstrates that overexpression of miR-142 and miR-212 inhibits TGF-β1-induced proliferation and collagen formation of CFs partly at least, by the direct inhibition of c-Myc. These findings expand our understanding of the pathogenesis of cardiac fibrosis after MI, and may provide new targets for the treatment of HF.

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**Figure legends**

**Figure 1:** The expressions of miR-142 and miR-212 in HF mice and TGF‑β1‑induced CFs. CFs were isolated from HF mice, and the molecular expressions were measured using qRT-PCR and western blot. (A) The expressions of miR-142 and miR-212. (B) The protein expression of c-Myc and TP53INP1. \**P*<0.05, *vs* sham group. Primary mouse CFs were induced by TGF‑β1 (5 ng/ml) for 48 h. (C) The proliferation of CFs induced by TGF‑β1 for 0, 24, and 48 h was detected using MTT assay. (D) The protein expression of Collagen I and III. (E) The expressions of miR-142 and miR-212. (F) The protein expression of c-Myc and TP53INP1. \**P*<0.05, *vs* control group.

**Figure 2:** The effect of miR-142 and miR-212 on the proliferation and collagen formation of CFs stimulated by TGF-β1. CFs were treated with TGF-β1 for 48 h after the transfection of miRNAs mimic. (A) The expressions of miR-142 and miR-212. (B) The proliferation of CFs induced by TGF‑β1 for 0, 24, and 48 h. (C) The protein expression of Collagen I and III. \**P*<0.05, *vs* control group; #*P*<0.05, *vs* TGF-β1+pre-NC group.

**Figure 3:** miR-142 and miR-212 targeted c-Myc. (A) Both miR-142 and miR-212 were found on mouse chromosome 11. (B) The binding sites of miR-142 and miR-212 on c-Myc. (C) The relative luciferase activity. (D) c-Myc expression in CFs transfected with miR-142/212 mimic or their inhibitor. \**P*<0.05, *vs* NC or pre-NC group.

**Figure 4:** Overexpression of miR-142 and miR-212 synergistically suppressed the proliferation and collagen formation of TGF-β1-induced CFs by regulating c-Myc/TP53INP1. CFs were treated with TGF-β1 for 48 h after the transfection of miR-142/212 mimic and pcDNA-c-Myc. (A) The protein expression of c-Myc. (B) The protein expression of TP53INP1. (C) The proliferation of CFs induced by TGF‑β1 for 0, 24, and 48 h. (D) The protein expression of Collagen I and III. \**P*<0.05, *vs* control group; #*P*<0.05, *vs* TGF-β1+pre-NC group.