Naringenin inhibits transforming growth factor-β1-induced cardiac fibroblast proliferation and collagen synthesis via G0/G1 arrest

MINGXIN LIU¹, XIPING XU¹, JIANHUA ZHAO¹ and YANHONG TANG²-⁴

¹Department of Cardiology, The First People’s Hospital of Yueyang, Yueyang, Hunan 414000; ²Department of Cardiology, Renmin Hospital of Wuhan University; ³Cardiovascular Research Institute, Wuhan University; ⁴Hubei Key Laboratory of Cardiology, Wuhan, Hubei 430060, P.R. China

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Correspondence to: Dr Yanhong Tang, Department of Cardiology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan, Hubei 430060, P.R. China
E-mail: wurmheart@vip.163.com

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Abstract. The Traditional Chinese Medicine naringenin (NRG) has a number of biological effects, including anti-inflammatory, anti-oxidative, anti-tumor and anti-atherosclerotic effects. However, the mechanism underlying its effects remains unclear. The aim of the present study is to investigate the role and mechanism of NRG on proliferation and collagen synthesis of cardiac fibroblasts (CFs) induced by transforming growth factor β1 (TGF-β1). Firstly, proliferation and collagen synthesis in CFs subjected to TGF-β1 was assessed subsequent to the consumption of NRG or control treatment. Additionally, the cell cycle of different groups and the roles of cyclins and cyclin-dependent kinases (CDKs) in NRG treatment of CFs were detected. In the present study, it was revealed that treatment of CFs with NRG resulted in attenuated fibroblast α-smooth muscle actin expression, deceased proliferation and collagen synthesis when compared with a TGF-β1 stimulus. Additionally, it was demonstrated that cell population of CFs treated with NRG in the S-phase became smaller whereas that of CFs in the G0/G1-phase increased when compared with the TGF-β1 group. Mechanically, the expression of cyclin D1-CDK4/6 and cyclin E2-CDK2 were inhibited in the NRG treatment group. These results illustrated that the protective effects of NRG on proliferation and collagen synthesis of CFs were at least in part due to G0/G1 arrest. Therefore, NRG may become a novel strategy for treating cardiac fibrosis in the future.

Introduction

Cardiac fibrosis refers to excessive accumulation of collagen in the normal myocardium and significantly increased collagen concentration or change of collagen composition in heart tissue. Cardiac fibrosis is the inevitable process of a variety of clinical cardiovascular diseases that have developed to the final stage, which is the main performance of cardiac remodeling (1,2). At present, it is known that cardiac fibrosis is closely associated with arrhythmia, heart failure and mortality due to sudden cardiac arrest (3-5). It is generally known that activation and proliferation of cardiac fibroblasts (CFs) and deposition of extracellular matrix (ECM) secreted by CFs are the primary features of cardiac fibrosis (1,6). Accumulation of ECM increases myocardial hardness, reduces myocardial compliance and affects the normal diastolic and systolic function of the heart (6). Therefore, cardiac fibrosis is a key factor in cardiovascular disease outcomes, and CFs are important in the process of cardiac fibrosis (6). In the stimuli of myocardial infarction, an excessive pressure load or neurohumoral factors and CFs begin with pathologic hyperplasia and convert to myofibroblasts (6,7). Under the mediation of a variety of cytokines, myofibroblasts can migrate to the damaged area and proliferate rapidly to synthesize and release a large amount of collagen I and III, which accumulates in the myocardial interstitium and perivascular spaces (3). It may also promote crosslinking between the ECM, and cause excessive deposition of collagen that leads to cardiac fibrosis and provides a pathological basis to the occurrence and development of cardiovascular diseases (6,8). Therefore, in improving the prognosis of cardiovascular diseases and inhibiting cardiac remodeling at the cellular level, CFs have become an important target to control the progression of cardiac fibrosis. Abrogation of CF transformation into myofibroblasts, and the inhibition of CF proliferation and collagen synthesis may be a strategy for suppressing cardiac fibrotic remodeling.

Naringenin (NRG) is a type of double hydrogen flavonoid that predominantly exists in rutaceae citrus plants (9). A number of studies have confirmed that NRG demonstrates numerous biological effects on oxidative stress, the inflammatory response, disorders of lipid metabolism and apoptosis (9-11).
Recently, the cardioprotective effects of NRG have been the focus of a number of studies (12,13). However, the mechanism underlying its effects remains unclear. Therefore, the aim of the present study was to investigate the effects and mechanism of NRG on the proliferation and collagen synthesis of CFs induced by transforming growth factor β1 (TGF-β1) from the cellular level to elucidate the mechanism of NRG against cardiac fibrosis.

Materials and methods

Materials. Primary antibodies including anti-cyclin D1 (cat no. 2978), anti-cyclin dependent kinase 4 (anti-CDK4; cat no. 12790), anti-CDK6 (cat no. 3136) and anti-CDK2 (cat no. 2546) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary anti-cyclin E2 antibody (cat. no. sc-22777) was purchased from Santa Cruz Biototechnology, Inc. (Dallas, TX, USA). Primary anti-GAPDH antibody (cat. no. MB001) was purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA). Cell counting kit-8 (CCK-8; cat. no. CK04-11) was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD, USA). TRIzol reagent (cat. no. 15596018) was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Transcriptor First Strand cDNA Synthesis kit (cat. no. 04896866001) was bought from Roche Diagnostics (Basel, Switzerland). Bicinchoninic acid (BCA) protein assay kit (cat. no. 23227) was from Pierce (Thermo Fisher Scientific, Inc.). NRG (cat. no. W530098) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture and treatment. A total of 70 neonatal Sprague-Dawley rats were obtained from the Animal Experimental Center of Wuhan University (Wuhan, China) and the Center for Disease Control and Prevention of Hubei Province (Wuhan, China). Primary culture of neonatal rat CFs was prepared from ventricles of 1-3 day-old rats via the differential attachment method (1,2). Briefly, following sacrifice, rat hearts were removed from the thorax and immediately placed in 4°C PBS, and ventricles were digested with 0.125% trypsin and 0.08% collagenase type II at 37°C (5 times for 5 min each). However, in order to reduce the presence of cell fragments and blood in cell samples, the first digestion solution was discarded. Cells from the last four digestions were collected, and incubated in Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 (DMEM/F12) medium (HyClone; GE Healthcare, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare) at 37°C for 1 h. Subsequently, unattached or weakly attached cells were discarded, but attached cells were incubated in fresh DMEM/F12 supplemented with 10% FBS. After the confluence of CFs reached 80%, CFs were digested using 0.25% trypsin and then passaged. Passages 2-4 were used for subsequent experiments in the present study. The cells were cultured in a 6-well plate when they were used for flow cytometry analysis. The cells were stimulated with TGF-β1 or PBS to induce CF transformation into myofibroblasts (1). In the present study, to detect the appropriate concentration of NRG, the effects of several concentrations (0, 10, 20, 30, 40 and 50 µM) of NRG on α-SMA mRNA levels were evaluated following exposure for 48 h. Next, with the appropriate concentration, the cells were treated with different times (0, 12, 24, 48 and 72 h) to choose the appropriate treatment time. Upon the appropriate concentration and time of NRG, the cells were divided into the following groups: PBS + dimethyl sulfoxide (DMSO), PBS + naringenin, TGF-β1 + DMSO and TGF-β1 + naringenin. The use of animals in the present study was approved by the Ethics Committee of The First People’s Hospital of Yueyang (Yueyang, China).

Immunofluorescence staining. In order to detect the number of CFs that expressed α-smooth muscle actin (α-SMA) protein, CFs were stained for the marker α-SMA (1). Cells were washed three times with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized in 0.2% Triton X-100 in PBS at room temperature for 20 min. Furthermore, they were blocked with 5% bovine serum albumin (cat. no. A3733; Sigma-Aldrich; Merck KGaA) in PBS at room temperature for 30 min and then stained with anti-α-SMA antibody (cat. no. ab3280; Abcam, Cambridge, MA, USA; 1:100 dilution) overnight. The cells were incubated with a green fluorescence-marked secondary antibody (cat. no. AS1112; Aspen Biological Co., Ltd., Wuhan, China; 1:100 dilution) for 1 h at room temperature and then counterstained with DAPI for 8 min at room temperature. Finally, the cells were covered with mounting medium and kept in the dark at 4°C.

Cell proliferation assay. Cell proliferation assay was performed according to the manufacturer’s instructions of the CCK-8. Initially, the cell suspension was inoculated in a 96-well plate (100 µl/well) and incubated until the confluence of CFs was 60-70%. Following incubation with 30 µM NRG at 37°C for 48 h, CFs were then incubated with CCK-8 solution (10 µl/well) at 37°C for 2 h and the optical density of each well was measured at 450 nm using a microplate reader (Tecan Infinite M200; Tecan Group Ltd., Mannedorf, Switzerland). CFs with culture medium and CCK8, without PBS/TGF-h1 or NRG/DMSO treatment, were used as the control.

Flow cytometry analysis. Each group of ~1x10⁶ CFs, including PBS + DMSO, PBS + NRG, TGF-β1 + DMSO and TGF-β1 + NRG, was cultivated in a 6-well plate, collected, fixed in 70% ice-cold ethanol and, maintained at 4°C overnight. CFs were washed with PBS and centrifuged for 2 min at 716 x g at room temperature, and the supernatant was discarded. The cell pellet was vortexed in 500 µl PBS left behind to avoid clumping of cells. 500 µl RNase (cat. no. 10109142001; Sigma-Aldrich; Merck KGaA) was added and the cells were subsequently stained with propidium iodide (PI) (cat. no. P3566; Invitrogen; Thermo Fisher Scientific, Inc.) in the dark for 45 min. To avoid clumps, the cells were transferred through meshed blue-capped falcon tubes prior to analysis using a FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA).

Reverse transcription-quantitative polymerase chain reaction assay. CFs were collected and the total mRNA was extracted using TRIzol reagent according to the manufacturer’s instructions. cDNA synthesis was performed using the Transcriptor First Strand cDNA synthesis kit (cat. no. 04896866001;
Roche Diagnostics), according to the manufacturer's protocol. Gene differences were detected using SYBR green (cat. no. 0491385001; Roche Diagnostics) and GAPDH gene expression was used as an internal control. The reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec, then 95°C for 15 min and 60°C for 1 min. The expression levels of each gene were quantified through the analysis of the Cq value and standard curve (14). The sequences of the primer pairs were exhibited as follows: ki67: 5'-TAGAGATCTGCTGGCTTCTC-3' (forward) and 5'-TGCTCCTTGGTTGTTCCCTC-3' (reverse); proliferating cell nuclear antigen (PCNA): 5'-CAACCTTGGATCCAGAACAGGAG-3' (forward) and 5'-TAAGGTCCCGGCATATACGTGC-3' (reverse); α-SMA: 5'-GCTATTCAGGCTGTGCTGTC-3' (forward) and 5'-GTAATCGGTGTAAGATCTCGG-3' (reverse); connective tissue growth factor (CTGF): 5'-GGAAGACACATTTGGCCCTG-3' (forward) and 5'-GCAATTTAGGGCTCCGGAT-3' (reverse); collagen I: 5'-GAGCGGAGAATCTGGATCGA-3' (forward) and 5'-CTGACCCTGTCCTAGTGACA-3' (reverse); collagen III: 5'-TGCCTTGGGAGGATGGA-3' (forward) and 5'-GAAGACATTCTTCGGGCTG-3' (reverse); prolactin: 5'-GGGATGCGTTGCAGATGTAG-3' (forward) and 5'-CATGGACGAGGATGTGTT-3' (reverse).

Western blot analysis. Proteins were extracted from CFs using radioimmunoprecipitation assay lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Haimen, China), and the protein concentration was determined using a BCA protein assay kit (cat. no. 23227; Pierce; Thermo Fisher Scientific, Inc.). Proteins were loaded (10 µg/well) and separated using 10% SDS-PAGE at 120 V for 120 min. Subsequently, the proteins were transferred onto polyvinyl difluoride transfer membranes at 120 V for 120 min. Subsequently, membranes were blocked for 60 min with freshly prepared 5% skimmed milk in Tris-buffered saline with Tween 20 (TBST) at room temperature. Following this, the membranes were incubated with various primary antibodies, including cyclin D1 (1:1,000), CDK4 (1:1,000), CDK6 (1:1,000), cyclin E2 (1:500), CDK2 (1:1,000) and GAPDH (1:10,000) overnight at 4°C with gentle shaking. Membranes were washed three times with TBST, and incubated with either HRP-conjugated goat anti-mouse (cat. no. 074-1806; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA; 1:10,000 dilution) or goat anti-rabbit (cat. no. 074-1506; Kirkegaard and Perry Laboratories, Inc.; 1:10,000 dilution) secondary antibodies for 60 min at room temperature with shaking. Following four washes of the membranes, images were captured on films, which were placed in LumiGLO solution (cat. no. 7003; Cell Signaling Technology, Inc.) for 1 min at room temperature. Following development, the images were placed into an automatic image analyzer to determine the expression levels of the proteins as well as the reference gray scale values. GAPDH was used as a loading control and three independent experiments were performed.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical analyses were performed using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons between each group were performed using one-way analysis of variance followed by Fisher's least significant difference test. *P<0.05 was considered to indicate a statistically significant difference.

Results

Appropriate concentration and treatment time of NRG in CFs induced by TGF-β1. Initially, to detect the appropriate concentration of NRG, the effects of several concentrations of it were measured on the α-SMA mRNA level following exposure for 48 h. It was revealed that induction of α-SMA expression was significantly inhibited when NRG was added at a concentration of 30 µM, compared with that when 0, 10 or 20 µM NRG was added (Fig. 1A). Additionally, it was demonstrated that 30 µM NRG inhibited α-SMA expression in a time-dependent manner. Compared with the 12 and 24 h groups, the minimum expression of α-SMA was revealed at the 48 h time points (Fig. 1B). Therefore, 30 µM NRG with 48 h treatment time was selected for subsequent experiments.

NRG treatment inhibits transformation and proliferation of CFs. Transformation of CFs into cardiac myofibroblasts was investigated with α-SMA. The expression of α-SMA protein detected via immunofluorescence staining was markedly inhibited in the NRG-treated group, which indicated that NRG treatment for 48 h was able to reduce the number of CFs that expressed α-SMA protein induced by TGF-β1 (Fig. 2A). CF proliferation was investigated via CCK-8 assay (Fig. 2B). Subsequently, the effect of NRG on proliferation of CFs was further assessed by the mRNA levels of ki67 and PCNA (Fig. 2C and D). It was revealed that the mRNA levels of ki67

Figure 1. Appropriate concentration and treatment time of NRG in CFs induced by TGF-β1. (A) mRNA levels of α-SMA in CFs treated with 0, 10, 20, 30, 40 and 50 µM NRG and stimulated with 5 ng/ml TGF-β1 for 48 h (n=6 samples per group). #P<0.05 vs. 0 µM, *P<0.05 vs. 10 µM and &P<0.05 vs. 20 µM. (B) mRNA levels of α-SMA in CFs treated with 30 µM NRG and stimulated with 5 ng/ml TGF-β1 for 0, 12, 24, 48, and 72 h (n=6 samples per group). #P<0.05 vs. 0 h, *P<0.05 vs. 12 h and &P<0.05 vs. 24 h. NRG, naringenin; CF, cardiac fibroblast; TGF-β1, transforming growth factor β1; α-SMA, α-smooth muscle actin.

NRG treatment inhibits transformation and proliferation of CFs. Transformation of CFs into cardiac myofibroblasts was investigated with α-SMA. The expression of α-SMA protein detected via immunofluorescence staining was markedly inhibited in the NRG-treated group, which indicated that NRG treatment for 48 h was able to reduce the number of CFs that expressed α-SMA protein induced by TGF-β1 (Fig. 2A). CF proliferation was investigated via CCK-8 assay (Fig. 2B). Subsequently, the effect of NRG on proliferation of CFs was further assessed by the mRNA levels of ki67 and PCNA (Fig. 2C and D). It was revealed that the mRNA levels of ki67
and PCNA were significantly inhibited in CFs following treatment with NRG compared with the TGF-β1 group (P<0.05), which indicated that NRG treatment was able to inhibit the proliferation of CFs compared with the TGF-β1 group.

NRG treatment inhibits collagen synthesis of CFs. Collagen synthesis of cardiac myofibroblasts is perceived to be important in the development of cardiac fibrosis (15,16). Therefore, the role of NRG on collagen synthesis was detected via the mRNA levels of CTGF, collagen I and collagen III. It was revealed that NRG significantly decreased the mRNA levels of CTGF, collagen I and collagen III in CFs induced by TGF-β1 (Fig. 3).

NRG treatment induces CF cell cycle arrest in the G0/G1-phase. In order to elucidate the mechanism of CF proliferation regulated by NRG, the effect of NRG on cell cycle progression was evaluated. CFs under subconfluent culture conditions were synchronized by serum starvation, which caused cell cycle arrest at the G0/G1-phase, and then treated with the indicated stimuli, labeled with PI and analyzed by flow cytometry (17,18). It was revealed that the S-phase population was significantly increased and the G0/G1-phase was significantly decreased in the TGF-β1 + DMSO group compared with the PBS groups, whereas the G0/G1-phase of CFs in the TGF-β1 group treated with NRG was significantly increased and the S-phase population was significantly lower compared with all other groups. These results indicated that NRG treatment induces CF cell cycle arrest in the G0/G1-phase (Table I).

NRG inhibits CFs entering the S-phase by downregulating the cyclin D1-CDK4/6 and cyclin E2-CDK2 complexes. The G0/G1 to S-phase transition is primarily regulated by the cyclin D1-CDK4/6 and cyclin E2-CDK2 complexes (19,20). To investigate the status of these complexes in NRG-treated CFs induced by TGF-β1, the protein levels of individual cyclins...
Table I. NRG treatment induces cardiac fibroblast cell cycle arrest in the G0/G1-phase (%).

<table>
<thead>
<tr>
<th>Stage</th>
<th>PBS + DMSO</th>
<th>PBS + NRG</th>
<th>TGF-β1 + DMSO</th>
<th>TGF-β1 + NRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>77.32±3.11</td>
<td>76.08±3.32</td>
<td>65.64±2.90abc</td>
<td>86.53±2.64abc</td>
</tr>
<tr>
<td>S</td>
<td>15.61±1.18</td>
<td>16.31±0.93</td>
<td>25.87±1.47abc</td>
<td>10.19±0.78abc</td>
</tr>
<tr>
<td>G2/M</td>
<td>7.07±0.34</td>
<td>7.61±0.73</td>
<td>8.74±0.69a</td>
<td>3.27±0.48abc</td>
</tr>
</tbody>
</table>

DNA flow cytometry analysis revealed an increased population of the G0/G1-phase and smaller S-phase in the TGF-β1 + NRG compared with the TGF-β1 + DMSO group. *P<0.05 vs. PBS + DMSO, †P<0.05 vs. PBS + NRG and ‡P<0.05 vs. TGF-β1 + DMSO. NRG, naringenin; DMSO, dimethyl sulfoxide; TGF-β1, transforming growth factor β1.

Figure 4. NRG inhibits CFs from entering the S-phase via downregulating the cyclin D1-CDK4/6 and cyclin E2-CDK2 complexes. Representative western blot analysis and statistical results of the protein levels of cyclin D1, CDK4, CDK6, cyclin E2 and CDK2 in CFs of the indicated groups (n=3 independent experiments). *P<0.05 vs. PBS + DMSO, †P<0.05 vs. PBS + NRG and ‡P<0.05 vs. TGF-β1 + DMSO. NRG, naringenin; CF, cardiac fibroblast; DMSO, dimethyl sulfoxide; TGF-β1, transforming growth factor β1; CDK, cyclin dependent kinase.

and CDKs were measured by western blot analysis. It was revealed that the expression levels of cyclin D1, CDK4, CDK6, cyclin E2 and CDK2 decreased significantly in CFs treated with NRG and TGF-β1 compared with the TGF-β1 group, which indicated that NRG repressed the G1/S-phase transition, in part, by downregulating the cyclin D1-CDK4/6 and cyclin E2-CDK2 complexes (Fig. 4).

Discussion

In the present study, the effects and the probable mechanism of NRG on CFs stimulated with TGF-β1 in vitro were investigated. It was revealed that NRG had the following effects: i) inhibited CF transformation into cardiac myofibroblasts; ii) inhibited the proliferation of CFs; iii) inhibited the synthesis of CTGF, collagen I and III in CFs stimulated with TGF-β1; iv) induced CF cell cycle arrest in the G0/G1-phase; and v) repressed the G1/S-phase transition of CFs partly via downregulating the cyclin D1-CDK4/6 and cyclin E2-CDK2 complexes following TGF-β1 induction. Although further studies are required, to the best of our knowledge the present study is the first to link the anti-proliferative effect of NRG on CFs with the inhibition of DNA synthesis via G0/G1 arrest, indicating that NRG may be used as a therapeutic agent to regulate cardiac fibrosis in the future.

Cardiac fibrosis is associated with various physiological causes, including CF proliferation, inflammation and hypertension (4). Among these causes, the transformation of CFs into cardiac myofibroblasts and activated proliferation are known to be important events in the development of cardiac fibrosis, which may promote pathological hypertrophy and fibrosis and subsequently result in cardiac remodeling and cardiac dysfunction (6). Therefore, inhibiting CF proliferation is considered important for treating cardiovascular diseases. In the present study, the anti-proliferative effect of NRG was examined on CF proliferation. NRG significantly inhibited TGF-β1-induced CF proliferation, transformation into cardiac myofibroblasts and the synthesis of CTGF, collagen I and III of CFs. Notably, the anti-proliferative effect of NRG was associated with the inhibition of DNA synthesis via G0/G1 cell cycle arrest. The cell cycle consists of four sequential phases including G0/G1, S, G2 and M. It regulates cellular proliferation by a highly controlled process involving a complex cascade of cellular events, including regulatory factor cyclins and CDKs (21,22). The cyclin D1-CDK4/6 and cyclin E2-CDK2 complexes are important mediators of the cell cycle transition from the G0/G1 to S-phase (19,20). In the present study, NRG not only inhibited cyclin D1 and cyclin E2 expression, it also inhibited CDK2/4 and CDK6 expression. Similar to the present study, Lee et al (21) demonstrated previously that (2S)-NRG inhibited the platelet-derived growth factor (PDGF)-BB-induced proliferation of vascular smooth muscle cells via a G0/G1 arrest, did not affect the signaling pathways associated with PDGF-Rβ, protein kinase B, extracellular signal-regulated kinase 1/2 or phospholipase C-γ1 but downregulated the expression of cyclin D and E, CDK2, CDK4 and reduced retinoblastoma-protein phosphorylation. This indicated that (2S)-NRG may be valuable as a therapeutic agent for managing atherosclerosis and/or vascular restenosis.

In conclusion, the results of the present study demonstrate that NRG was able to inhibit TGF-β1-induced proliferation of CFs via G0/G1 arrest, resulting in the downregulated expression...
of cyclin D1-CDK4/6 and cyclin E2-CDK2. Therefore, the results indicated that NRG may be used as a novel treatment strategy due to its anti-fibrotic effect in CFs by regulating proliferation, transformation and collagen synthesis of CFs in response to pathological stress. However, the detailed mechanism of how NRG regulates cardiac remodeling remains to be demonstrated in animal models. Additionally, detailed regulatory mechanisms of cyclins and CDKs or other signaling pathways, which are associated with the process of NRG affecting cardiac fibrosis, require further study.

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