

# Induction of Substantial Myocardial Regeneration by an Extract of Chinese Herb *Rosa laevigata Michx* for Repair of Infarcted Heart

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## Abstract

Coronary Heart Disease (CHD) is the single largest killer out of all diseases in Europe and in the US. Pathologic cardiac ischemia in CHD triggers a succession of events leading to massive destruction and loss of cardiac tissues. Thus, replacement of the damaged cardiac tissues by newly regenerated myocardium would be a therapeutic ideal for pathology modifying treatment of CHD.

The aim of this study was to evaluate the ability of an active fraction isolated from Chinese herb *Rosa laevigata Michx* (aFRLM) in therapeutic cardiomyogenesis through promoting substantial regeneration of cardiac tissues in a Myocardial Infarction (MI) animal model.

Our results demonstrated that oral administration of aFRLM to MI animals could significantly improve cardiac functional performance and induce myocardial regeneration replacing the necrosed cardiac tissues in a sub-clinical MI animal model. The property of the aFRLM appears to be entirely novel and may provide a potential therapeutic alternative for MI treatment.

**Keywords:** Myocardial infarction; Therapeutic cardiomyogenesis; *Rosa laevigata Michx*; Myocardium regeneration; Echocardiography

## Introduction

Pathologic stenosis or occlusion of coronary artery triggers a succession of events leading to cardiac ischemia or massive destruction of cardiac tissues in the distribution territory of the affected coronary artery. Subsequent events of ventricle remodeling and consequent heart failure occur [1,2]. CHD is the single largest killer out of all diseases and more than 1 in 5 deaths are from CHD in Europe and in the US [3,4]. Despite therapeutic advances, replacement of the damaged cardiac tissues with regenerating myocardium remains a therapeutic ideal as it is almost impossible for adult cardiomyocytes to considerably repopulate the lost myocardium resulting from MI through the inherited intrinsic cardiac repair mechanism [5-11].

Population-based clinical studies have shown that although current advanced treatment modalities, including beta-adrenoceptor blockers, diuretics, and angiotensin-converting enzyme inhibitors, as well as surgical intervention procedures and mechanical assistance devices, are effective in improving symptoms and other outcomes in patients, these therapies restore neither the pathologic changes nor the functional performance of the damaged heart, yet with problematic complications [12,13]. Heart transplantation may be an effective treatment for end-stage heart failure, the applicability of which, however, is seriously limited by the availability of donor heart and the concurrent immunorejection upon transplantation [14,15]. Therefore, CHD remains the most prevalent cause of death and the structural and functional restoration of the damaged heart remains a formidable challenge.

Basic and clinical research accomplishments during the last two decades on cardio-protective drugs [16] or cell-transplantation mediated therapies, such as stem cells or genetically modified cells, have constituted a revolution in regenerative medicine through creating

living and functional tissues to replace damaged tissue or restore organ function lost due to aging, diseases and damages [17-20]. However, the reported severe or life threatening complications associated with the use of exogenous stem cells/progenitors, including immunorejection, the risk of tumorigenicity and the insufficient efficiency of these progenitors/stem cells to acquire cardiac lineages to reconstitute the lost cardiac tissues, hindered the clinical translation of the technology [21-23]. Therefore, an alternative strategy for cardiac regeneration and reconstitution of the damaged cardiac tissues through the inherited intrinsic repair mechanism may be more attractive since it may offer an ultimate solution for substantial treatment of damaged heart [6,24]. However, it was soon realized that the inherited intrinsic cardiac repair mechanisms including cardiogenesis through proliferation and differentiation of resident cardiac stem cells, cardiogenic differentiation of bone marrow derived circulating stem cells that migrate to the site of damage, and possible proliferation of neighboring cardiac myocytes under defined conditions, are in themselves insufficient to repopulate the lost cardiac tissues or restore cardiac function as a pump [5,6,10,11,23]. Thus, it is highly desirable to develop new strategies that can potentiate the inherited intrinsic cardiac repair mechanisms for substantial repair of damaged heart.

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*Rosa laevigata michx* (RLM), born in sunny field side of mountain of 100 to 1600 meters above sea level, is mainly distributed throughout the southeast and southwest China. RLM is known as a commonly used traditional Chinese medicine for the treatments of pelvic inflammation, diarrhea, cough, asthma, oxidative stress induced injuries, disorder of plasma blood lipid, muscular, renal or hepatic damages, and inhibition of the expression of NF- $\kappa$ B, TGF- $\beta$ 1, monocyte chemoattractant protein-1 (MCP-1) and THF- $\alpha$  [25-32]. Recent studies reported that oral administration of the extract of RLM obviously improved the survival rate and prevented ischemic reperfusion damage [33]. Further studies showed that RLM significantly decreased DNA fragmentation, up-regulated the expression of Bcl-2, and down-regulated the expressions of p53, and active Caspase-3, -9 and -8 [33]. Thus, based on the properties of RLM in anti-oxidative stress, anti-inflammation, anti-apoptosis and especially the potential action for treatment of ischemic stroke, we therefore tested the possible therapeutic effects of RLM for the treatment of ischemic myocardial infarction. The aim of this study is to work out an alternative method that can substantially potentiate the intrinsic cardiac repair mechanisms for regeneration of myocardium and repair of the damaged heart. The impact and prospects of this finding is of importance for clinical translation for repair of diseased heart.

## Materials and Methods

### Preparation of the active fraction from *Rosa laevigata Michx* (aFRLM)

A bioassay-guided fractionation was performed to isolate the active fraction from the fruit of *Rosa laevigata Michx* for repair of infarcted heart in MI animal model. Briefly, air-dried and powdered fruit of *Rosa laevigata Michx* (500 g) collected from Shanxi Province of China in September was extracted with 5 volumes of absolute ethanol at room temperature for 1 day and 10 volumes of 65% ethanol for another 3 days. The extract was concentrated in vacuum and yielded 40g of crude ethanol extract. Reverse phase column chromatography was used for further isolation of the active fraction from the extract using an Agilent 1200 series (Agilent corp. Ltd., USA). For HPLC, 50 mg extract was dissolved in 1 ml methanol and 20  $\mu$ l sample was injected into the reversed phase column (Waters, C18, 4.6 mm  $\times$  250 mm). The detection wavelength was set to 254 nm. 0.1% acetic acid-H<sub>2</sub>O (solvent A) and methanol (solvent B) were used as the mobile phase, starting with 0% B and increasing to 20% B (10 min), 30% B (10-40 min), 35% B (40-50 min), 40% B (50-60 min), 50% B (60-70 min), 70% B (70-80 min) and 100% B (80-90 min) with a solvent flow rate of 1 ml/min at 25°C.

### Preparation of bone marrow mesenchymal stem cells (MSCs) *in vitro*

4 weeks old male Sprague-Dawley (SD) rats were euthanized by CO<sub>2</sub> inhalation in compliance with AVMA panel on euthanasia guidelines. The femora and tibiae bones were aseptically removed. The bone ends were cut, and the bone marrow cavity was flushed out under aseptic conditions with alpha IMDM culture medium using a sterile 21 gauge needle [34,35]. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain single cells suspension and centrifuged at 2000 rpm for 5 min. The resulting cell pellet was resuspended in 3 ml ice-cold culture medium supplemented with 2% PBS and 1-2 mM EDTA, which was carefully put on the top of the separation medium (Ficoll-Paque solution of 1.077 g/ml density) in a 50 ml centrifuge tube and centrifuged at 445 g for 30 min. The second layer containing mononucleated cells was carefully transferred

into a tube and washed gently twice with PBS to remove Ficoll (1200 rpm for 5 min). The cell pellet was resuspended in ice-cold IMDM culture medium containing 10% heat inactivated FBS (GIBCO) and 1% penicillin/streptomycin antibiotic mixture, and cultured at 37°C, 5% CO<sub>2</sub>/95% air humidified cell culture incubator for 24 hours. The non-adherent cells in the culture were removed by washing the culture dishes with PBS, and the adherent cells were cultured by changing the culture medium every 3 days until the cells became nearly confluent after 10-15 days of culture.

### aFRLM-Induced cardiogenic differentiation of MSCs *in vitro*

The obtained MSCs were cultured with the different fractions of the ethanol extract obtained from reverse phase column chromatography respectively. The cardiogenic activity of the fractions was compared with respect to that of the crude extract. It was found that the fraction eluted from the column with 70% methanol exhibited the best comparable activity with that of the crude extract in enhancing cardiogenic differentiation of cultured MSCs. Therefore, this fraction (aFRLM) was used in the following experiments.

The obtained MSCs were cultured with aFRLM (40  $\mu$ g/ml IMDM culture medium) for 7-14 days to test the activity of aFRLM in promoting cardiogenic differentiation of the cultured MSCs. The expression of cardiac specific Myosin Heavy Chain (MHC) was assessed by immune cytochemistry at different time points [5,11]. Briefly, 4% paraformaldehyde in PBS was used to fix the cultured MSCs for 15 min and the fixed cells were permeabilized with 0.5% Triton X-100 for 15 min. Mouse monoclonal antibodies specific to MHC (1:600) (Santa Cruz) were used as the primary antibody and goat anti-mouse IgG antibodies conjugated with green Fluorophore (DyeMer<sup>TM</sup> 488/630 and 496/520) (Molecular Probe) as the secondary antibody.

### Induction of MI animal model and the treatment protocol

All protocols were conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health, and approved by the Animal Experimental Ethical Committee of the Zhangjiang High Technology Park. MI was induced in male Sprague-Dawley (SD) rats (200~250 g) by permanent ligation of left anterior descending (LAD) coronary artery as previously described [11]. In brief, the rats were anesthetized with intraperitoneal Ketamine (100 mg/kg)/Xylazine (10 mg/kg) mixture. If redosing was required, according to pain reflect test and palpebral reflex of the experimental animals, ketamine alone was injected. The animals were mechanically ventilated with room air. The incision area of the chest was shaved and cleaned with a povidone-iodine solution. The heart was exposed via a left anterolateral thoracotomy. The LAD was then ligated using a 7-0 polypropylene suture. The incision was closed in layers using resorbable 3-0 Vicryl suture. The sham-operated rats received the same thoracotomy without LAD ligation.

After surgery, the rats were allowed to recover from anesthesia in a warm environment under continuous monitoring and care until they returned to consciousness. All experimental rats were postoperatively given antibiotics (benzathine penicillin G 150,000 U/kg and procaine penicillin G 150,000 U/kg) and analgesic (buprenorphine 0.03 mg/kg) every 8 h with frequent monitoring of the signs of pain/discomfort and decreased activities.

Three days after LAD ligation, cardiac function was assessed using echocardiography. Only rats exhibiting similarly decreased values of left ventricle Ejection Fraction (EF) and fraction shortening (FS) were included for further studies. The candidate animals were randomly

divided into two groups. Eight rats in the test group were subject to aFRLM treatment (300 mg/kg weight/day in 2 ml water) through gastric gavage daily for 4 weeks. Equivalent water was orally given to the animals of vehicle-treated group (n=8). All experimental animals were sacrificed after last echocardiography measurements four weeks post treatments using CO<sub>2</sub> inhalation performed in compliance with AVMA panel on euthanasia. Briefly, the animal holding chamber was charged slowly with CO<sub>2</sub> to a concentration of 70% from a compressed CO<sub>2</sub> gas cylinder. The gas flow was maintained for at least 1.5 min after apparent clinical death was verified.

### Echocardiography assessment of the therapeutic effect of aFRLM treatment

To assess cardiac function of the experimental animals, echocardiography was performed in all the experimental animals at different time points (prior to LAD ligation, 3 days post LAD ligation, 2 and 4 weeks after aFRLM treatment) under controlled anesthesia using a Toshiba Aplio XG Echocardiography with PLT-1202 S linear array transducer [5,36]. In each experimental subject, M-mode tracing and 2-dimensional echocardiography of at least 10 consecutive cardiac cycles were recorded from the parasternal long- and short-axis views. Left ventricle systolic function was assessed by calculating Left Ventricle Ejection Fraction (LVEF) and Fractional Shortening (LVFS) with the Modified-Simpson method using conventional echocardiography [37]. Left Ventricular End Systolic Diameter (LVESD) and left ventricular end diastolic diameter (LVEDD) as well as systolic and diastolic wall thickness were measured from the M-mode tracings using the leading-edge convention of the American Society of Echocardiography. For each M-mode measurement, at least 10 consecutive cardiac cycles were sampled. Left Ventricular End Systolic Volume (LVESV) and Left Ventricular End Diastolic Volume (LVEDV) were calculated by the M-mode method [5,11,36]. All data were analyzed offline with software installed in the ultrasound system. All measurements were averaged on 10 consecutive cardiac cycles per experiment and were analyzed by an experienced investigator.

### Morphological and immunohistological assessment

The hearts of the sacrificed rats were removed and rinsed with PBS. All the specimens harvested were sectioned for histological and immunohistochemical analyses. The left ventricle of these heart specimens were cut from apex to base in 2 transverse slices and embedded in paraffin. The specimen paraffin blocks were sectioned respectively. The sections (5~20 µm) were stained with Masson's trichrome according to kit directions, which labels nucleus black, collagen blue and myocardium red. The blue stained areas (fibrous scar region) were digitized and quantified morphometrically. The volume of fibrous scar ( $\leq$  infarct volume) of a particular section was calculated based on the blue stained area and the thickness of the section. The fibrous scar volumes of all sections were added to yield the total volume of the fibrous scar [5,24]. To identify the regenerating myocardium, double immunohistochemical staining of the sections with cardiac specific Myosin Heavy Chain (MHC) and Ki-67 specific antibodies was performed according to the methods previously described [5,24]. Briefly, thin paraffin sections (5 µm) of the specimen of aFRLM- or vehicle-treated hearts were deparaffinized and stained with monoclonal rat-specific anti-Ki67 (Dako) and polyclonal anti-cardiac specific myosin heavy chain (MHC) antibodies (Santa Cruz) sequentially. Specific secondary antibody conjugated with alkaline phosphatase (Santa Cruz) was used to visualize the positively stained cells [5,11]. The newly regenerated myocytes were determined by counting cells

positively stained with both anti-Ki-67 and MHC concomitantly with the morphology of cardiac myocytes in each section.

### Statistics

All data including cardiogenic differentiation in cell culture, morphometrical analysis, and immunohistological analysis were presented as mean  $\pm$  SD. Statistical significance for comparison between two measurements from two groups is determined using the unpaired two-tailed Student's t test. Values of  $P < 0.05$  are considered to be significant.

### Results

#### Isolation of aFRLM from *Rosa laevigata Mich*

Bioassay of aFRLM indicated that this active fraction isolated from the fruit of *Rosa laevigata Mich* showed the ability to enhance cardiogenic differentiation of cultured MSCs as demonstrated by the morphological transition as well as the cardiac specific MHC expression in the cultured MSCs. The structural analysis of aFRLM by nuclear magnetic resonance spectroscopic analysis demonstrated that aFRLM mainly contained the following compounds: laevigatins, potentillin, tormentic acid, 23-hydroxytormentic acid 28-O- $\beta$ -D-glucopyranoside, 19 $\alpha$ -hydroxyasiatic acid, and 19 $\alpha$ -hydroxyasiatic acid-28-O- $\beta$ -D-glucopyranoside.

#### Induction of cardiogenic differentiation in MSCs by aFRLM

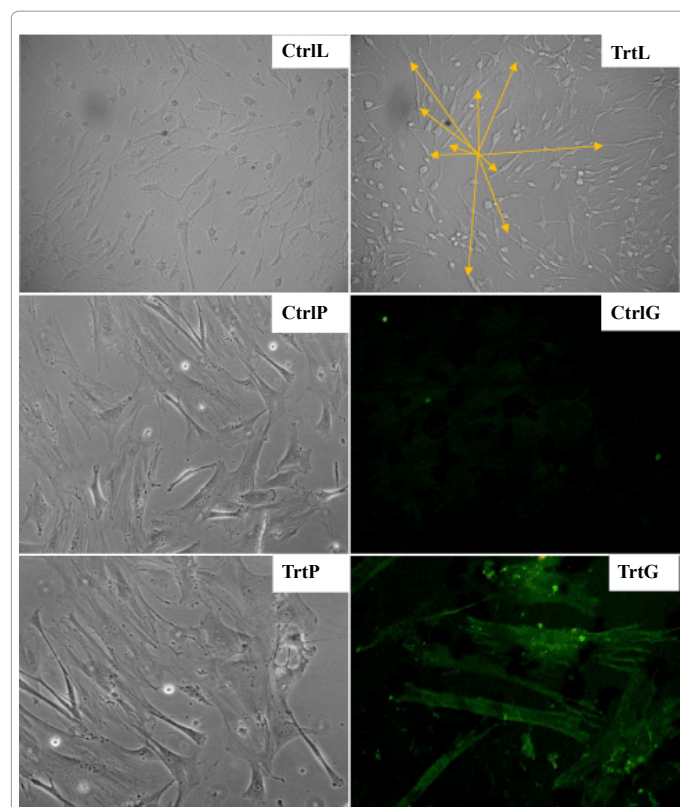
The phenotype of bone marrow derived MSCs in the anaphase appeared as flat, irregular, low refractory, triangular, and polygon morphology. The MSCs obtained from passages 5-6 were used for test of the activity of aFRLM in induction of cardiogenic differentiation. Our results showed that approximately 20% of the cultured MSCs in aFRLM-treated wells became elongated and refractive forming rod-like phenotype on day 7 compared with the flat, low refractory, irregular, and asymmetry morphology in vehicle-treated MSCs. On day 14 of the treatment, around 30% of the cultured MSCs showed the elongated myocyte-like phenotype, and more than 30% of these myocyte-like cells were positively stained with MHC specific antibodies. By contrast, the majority of vehicle-treated MSCs remained flat, irregular, asymmetry and low refractive with few MHC positively stained cells at the same time point ( $p < 0.01$ ) (Figure 1). This result indicated the potential ability of aFRLM in promoting cardiogenic differentiation of MSCs.

#### Echocardiography assessment of heart function

To evaluate the therapeutic effect of the aFRLM on MI, echocardiograph was used to measure the cardiac function of the experimental rats at different time points. Our results showed that the average baseline echocardiograms of the hearts in all experimental animals were similar ( $73.3 \pm 6.1\%$  for EF and  $52.1 \pm 5.1\%$  for FS) in all experimental animals before LAD ligation and any treatment (Figure 2). Three days post LAD ligation prior to drug treatment, the average EF ( $58.4 \pm 6.0\%$ ) and FS ( $39.8 \pm 4.8\%$ ) values decreased by approximately 20%. In vehicle-treated group, the decreased cardiac function due to LAD ligation was progressively worsened. On the other hand, cardiac function of aFRLM-treated animals was progressively improved. Compared to the vehicle-treated group, two weeks of aFRLM treatment improved EF by  $\sim 9.1\%$  ( $p < 0.05$ ) and FS by  $8.4\%$  ( $p < 0.05$ ). After four weeks of aFRLM treatment, EF and FS were increased by  $\sim 16.3\%$  ( $p < 0.01$ ) and  $\sim 15.2\%$  ( $p < 0.01$ ) respectively.

To minimize any influence of the open chest surgery on cardiac performance, sham-operated rats were examined as well.





**Figure 1: aFRLM-enhanced cardiogenic differentiation of cultured MSCs.** The majority of the vehicle-treated MSCs (14 days) under low power filed (CtrlL) and high power filed (CtrlP) phase contrast microscope remained flat, irregular, asymmetry and low refractive with few MHC positively (GFP) stained cells (CtrlG). By contrast, approximately 30% of the cultured MSCs in aFRLM-treated wells (14 days) under low power filed (TrtL) and high power filed (TrtP) became relatively elongated and high refractive myocyte-like phenotype (yellow arrow heads) with approximately 30% of these myocyte-like cells were positively (GFP) stained with MHC specific antibodies (TrtG).

Echocardiography results demonstrated that although the open chest surgery without LAD ligation in sham operated rats caused EF and FS to decrease slightly at 3 days post-surgery, the slightly decreased heart function was fully restored at week 2 and 4 post surgery (Figure 2). These results indicated that oral administration of aFRLM improved cardiac function of MI rats.

### Quantitation of infarct size

The hearts of the experimental animals sacrificed after last echocardiography measurement were removed for histological, immunohistological and computed planimetric analyses. It was found that the territory of the ligated LAD appeared flattened and slackened with pale uneven surfaces due to ischemic necrosis in the vehicle-treated animals. By contrast, the corresponding territory in aFRLM-treated hearts showed a smaller area of such appearance with relative sound tension and thickness of the ventricle wall. The infarct volume of a particular section was calculated based on the blue stained area and the thickness of the section (Figure 3A). The infarct volumes of all sections of a particular heart were added to yield the total infarct volume. It was revealed that the original infarct volumes, estimated as a percentage of the infarct volume of the total LV volume, were about similar in both aFRLM- and vehicle-treated hearts ( $n=8$  for each group) prior to treatments as estimated by the volume that the blue-stained regions occupied. However, after treatment with aFRLM, some newly

formed cardiac myocyte clusters were observed that replaced about one fourth of the original necrosed cardiac tissues making the average infarct volume ( $22.3 \pm 7.0\%$ ) in aFRLM-treated group approximately one fourth smaller than that ( $29.8 \pm 7.3\%$ ) in the vehicle-treated hearts ( $p<0.01$ ) (Figure 3B).

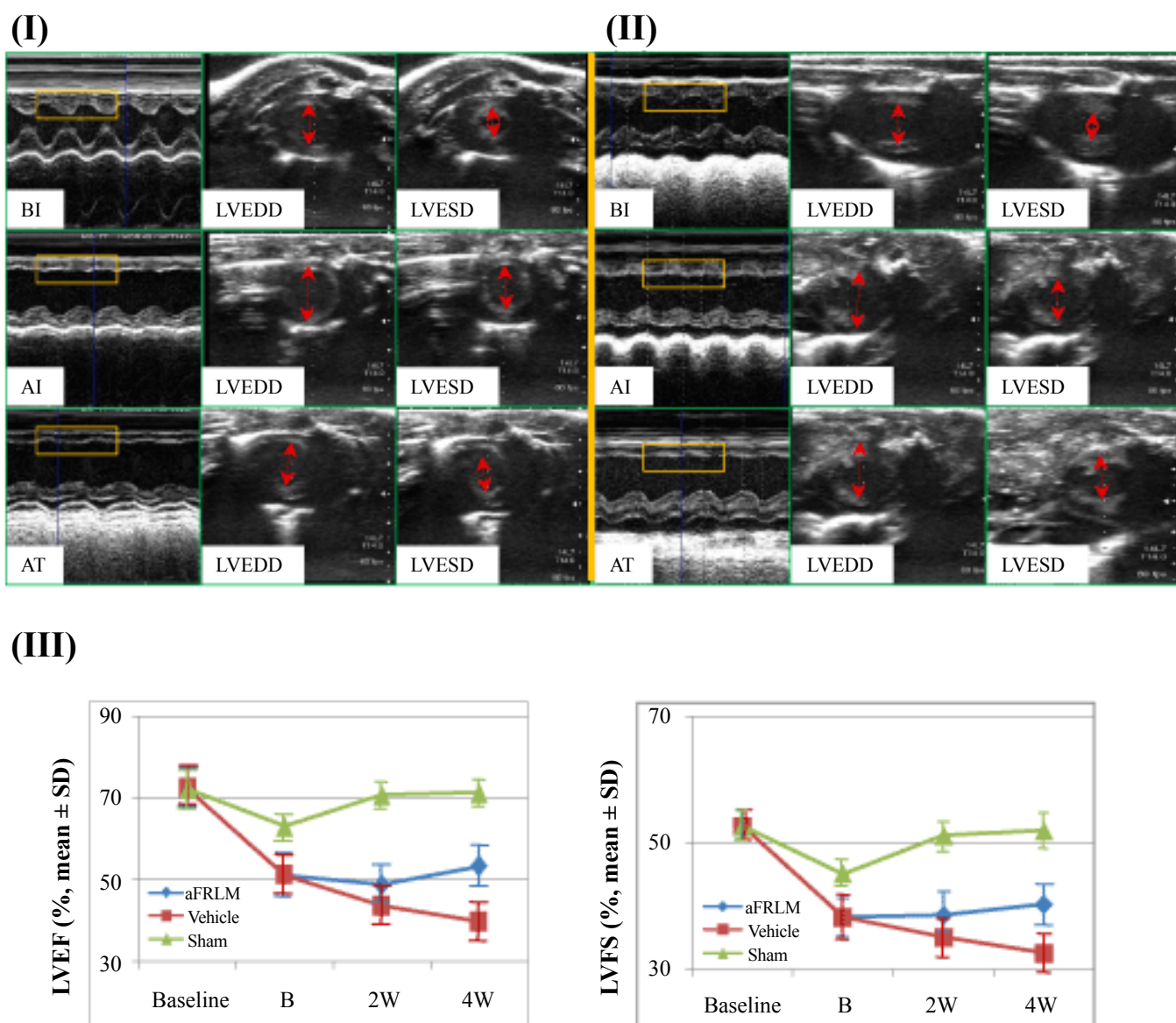
The infarcted LV wall within scar area was not only smaller but also thicker in aFRLM-treated animals than that in vehicle-treated control rats (Figure 3A). More interestingly, many myocyte-like cell clusters with good alignment were found in the central infarct region, which substantially replaced the infarcted heart tissues and thus reduced the infarct volume by approximately one fourth of the total infarct volume upon 4 weeks of aFRLM treatment. By comparison, the blue-stained fibrous scar by Masson's trichrome staining was observed throughout the whole infarct region and the red-stained myocyte-like cells, which were observed in clusters in aFRLM-treated hearts, were seldom found in the vehicle-treated MI heart (Figure 3A and 4).

In order to demonstrate whether the red stained myocyte-like cell clusters, which were found in the infarct region of aFRLM treated hearts, were newly regenerated cardiac myocytes, double immunostaining was performed using antibodies specific to MHC (myocyte marker) and Ki-67 (cell proliferation marker). The result showed that these clustered myocyte-like cells,  $\sim 1/3$  smaller than normal myocytes, were positively stained by both MHC and Ki-67 specific antibodies, implying that they were newly regenerated cardiac myocytes (Figure 4). All these results suggested that aFRLM substantially induced therapeutic myocardial regeneration and infarct repair in MI animals. The longitudinal section through the infarct region of the heart specimen showed that some of the myocyte-like cells with colocalized Ki-67 positive nuclei, MHC positive cytoplasm and striated phenotype were found in the infarct region. The sizes of these myocyte-like cells were about  $1/3$  smaller than the normal myocytes. These results confirmed that they were the newly regenerated myocytes replacing the infarcted heart tissues. By contrast, in vehicle-treated control MI heart, the fibrous scar was observed throughout the whole infarct region and regenerating myocytes were seldom identified (Figure 4).

### Discussion

First, this study demonstrated the effect of aFRLM in promoting cardiogenesis both *in vitro* and *in vivo*. Second, the ability of aFRLM-induced cardiogenesis was applied for the substantial treatment of infarcted hearts in sub-clinical MI animal model. The successful application of aFRLM for the treatment of MI would provide a convenient, safe and clinically well accepted method for MI treatment.

The therapeutic effect of aFRLM demonstrated in this study is reliable since the quality control of all important aspects in this study is high. For instance, to minimize the inaccuracies derived from the variations of experimental animals due to the individual differences in the structure of the coronary arteriolar distribution and the system error of ligation surgery, all experimental rats received baseline echocardiography prior to LAD ligation and post LAD ligation, and only the rats exhibiting similar base-line values and similarly decreased values of LVEF and LVFS after ligation were included in further studies. To assess the therapeutic effect of aFRLM, cardiac function was measured on a time lapse basis during the treatment. To reduce the inaccuracies derived from the variations of echocardiography measurement, M-mode tracing and 2-dimensional echocardiography of at least 10 consecutive cardiac cycles were recorded from the parasternal long- and short-axis views. For each M-mode measurement, at least six



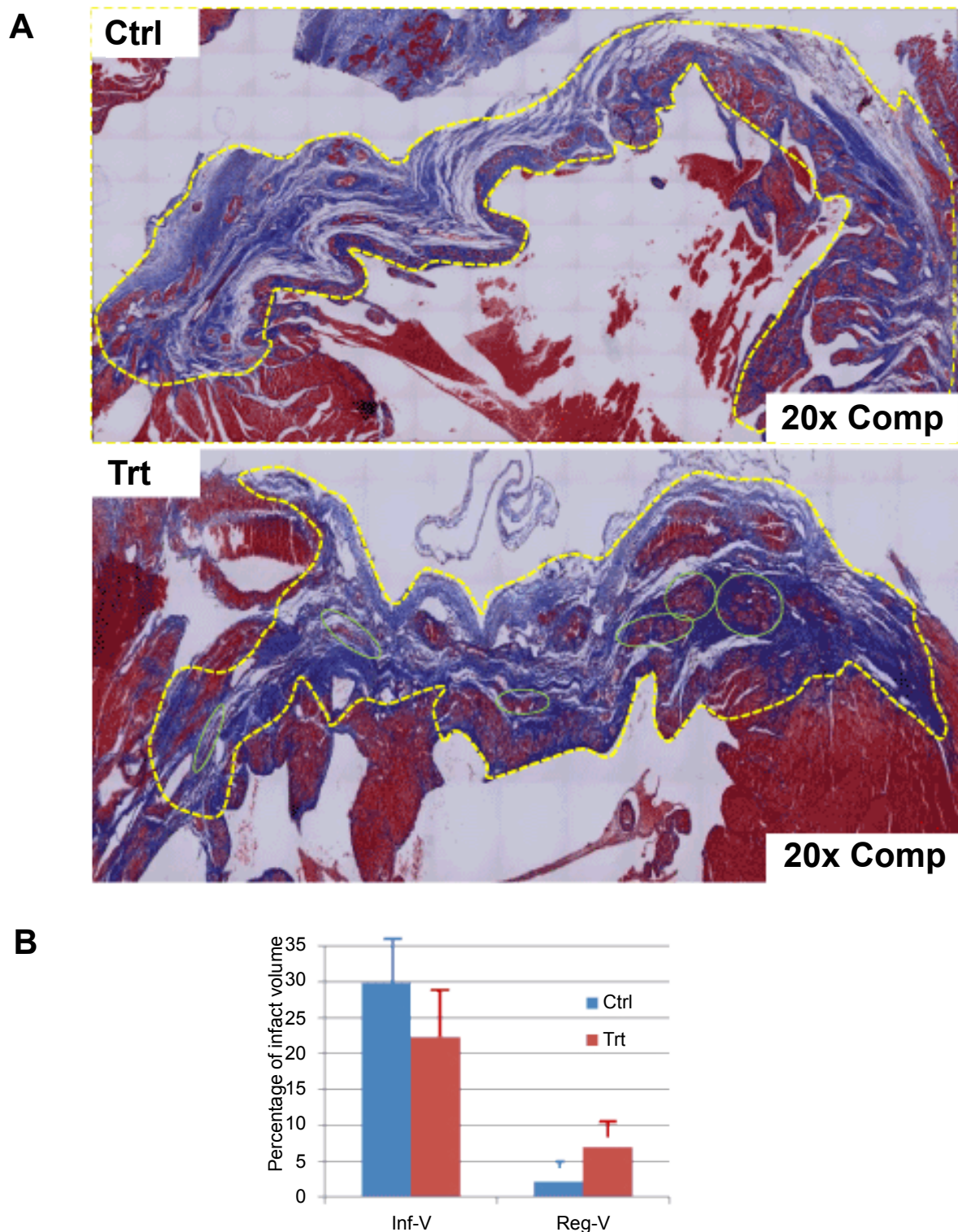
**Figure 2: Echocardiography assessment of cardiac function.** Representative M-mode and end diastolic/end systolic short axis cross sectional images of the left ventricle at the level of the papillary muscle of aFRLM-treated (I) and vehicle-treated (II) rat MI hearts. LVEDD: left ventricular end diastolic diameter; LVESD: left ventricular end systolic diameter; BI: normal rat echocardiography before MI induction; AI: images taken three days post infarction, but prior to any treatment; AT: images taken 4 weeks post either aFRLM or vehicle treatment respectively. Before the LAD ligation surgery, echocardiograms of animals in both groups showed a normal motion of structure throughout several cycles in M-mode measurements of a short axis view (I:BI & II:BI) (yellow rectangular enclosed) and normal LVEDD and LVESD (upper panels in I and II). Three days post ligation of LAD (I:AI & II:AI), anterior akinesis (yellow rectangular enclosed) and marked left ventricle dilation (red arrow heads) were observed in both groups of animals (middle panels in I & II). Four weeks of aFRLM treatment progressively improved the motion of left ventricle anterior walls (yellow rectangular) and reduced diastolic/systolic diameter (red arrow heads) (lower panel in I). By contrast, the vehicle-treated control hearts (II) remained akinesis (yellow rectangular) and progressively increased in diastolic/systolic diameter (red arrow heads) (lower panel in II). III, LVEF and LVFS measurements demonstrated the improved heart function with time by aFRLM treatment. Vehicle: vehicle-treated group; aFRLM: aFRLM-treated group; sham: open chest surgery without LAD ligation group; baseline: before ligation; B: 3 days post ligation; 2W: 2 weeks after aFRLM or vehicle treatment; 4W: 4 weeks after aFRLM or vehicle treatment. The graphs showed the restoration of the cardiac function in aFRLM-treated, but not vehicle-treated hearts.

consecutive cardiac cycles were sampled [36]. All measurements were averaged on 10 consecutive cardiac cycles per experiment and were analyzed by an experienced investigator.

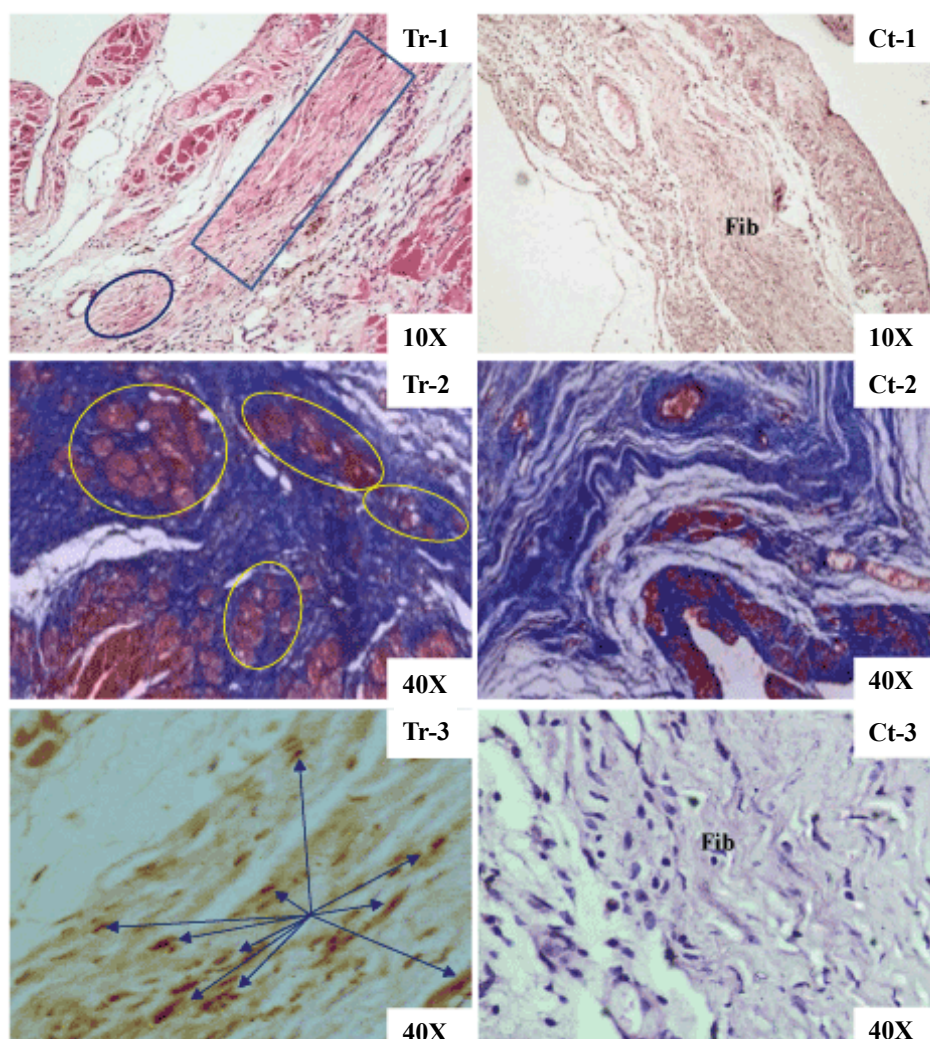
To maximize the reliability of the measurement of infarct volume of the cardiac specimen, the Masson's trichrome blue-stained areas of the cardiac specimen were digitized and quantified morphometrically. The infarct size of a particular section was calculated based on the blue

stained area and the thickness of the section and the total volume of the infarct was yielded by adding up the volumes of all sections within the infarct [5,11,24]. For identification of the regenerating myocardium, we used Ki-67 positive staining to label the newly regenerated cells and MHC positive staining to label cardiac myocytes. Therefore, counting the cells with the morphology of cardiac myocytes and the colocalization of both anti-Ki-67 and anti-MHC positive stains in the





**Figure 3: Morphometrical assessment of therapeutic effect of aFRLM on MI.** A: The representative figures with the whole cross-field of infarction stained by Masson's trichrome method were composed by 130 (Trt) and 160 (Ctrl) consecutive microscopic photos (20X). The vehicle-treated MI heart (Ctrl) showed a blue-stained large and thinned infarct area (yellow dashed line surrounded). The fibrous scar of the infarct was stained blue. By contrast, aFRLM-treated MI heart showed a smaller and less thinned infarct area (yellow dashed line surrounded). More interestingly, many red-stained myocyte-like cell clusters (green circles) replaced the blue-stained fibrous scar and reduced the infarct volume in aFRLM-treated MI heart. B: Inf-V, the infarct volumes in both aFRLM- (Trt) and vehicle- (Ctrl) treated hearts. Reg-V, the regenerating myocyte volumes in hearts of both groups. Note, some newly formed cardiac myocyte clusters (A: green circles) replaced about one fourth ( $6.9 \pm 2.3\%$ ) of the original necrosed cardiac tissues reducing the average infarct volume to  $22.3 \pm 7.0\%$  of the left ventricle volume in aFRLM-treated group. By contrast, the average infarct volume occupied about  $29.8 \pm 7.3\%$  with less than  $2.1 \pm 1.1\%$  regenerating myocyte-like cells observed in vehicle-treated hearts ( $P < 0.01$ ).



**Figure 4: Morphological assessment of the therapeutic effect of aFRLM on MI.** Representative micrographs of aFRLM-treated (4 weeks) MI heart (Tr-1) showed that many red-stained myocyte-like cell clusters (blue rectangular surrounded) in the central area of the infarct were found that replaced the infarcted cardiac tissues. By comparison, the large area of fibrous scar was found throughout the whole infarct region (Fib) in vehicle-treated MI heart (Ct-1) with few myocyte-like cell clusters found. Masson's trichrome staining demonstrated that although the infarcted cardiac tissues were replaced blue-stained fibrous scar tissues, many red-stained myocyte-like cell clusters were found in the central infarct region (tr-2). However, in the vehicle-treated MI heart (Ct-2), the blue-stained fibrous scar replacement of the infarcted cardiac tissues was found throughout the whole infarcted region with few red-stained myocyte-like cells clusters observed. Some of the myocyte-like cells in the central infarct (blue arrows) were positively stained by both Ki-67 (brown nuclei) and MHC (yellow cytoplasm) specific antibodies in aFRLM-treated MI hearts (Tr-3). Some of the regenerating myocytes joined together in tandem forming myocardium-like tissue. By contrast, fibrous scar replacement was found throughout the infarct region (Fib) with few both Ki-67 and MHC positively stained myocyte-like cells found (Ct-3).

infarct region are the criteria for regenerating myocytes. As a result, the reliability of the results derived from the current tightly controlled experiment is considerably high.

Since aFRLM showed its ability to induce cardiogenic differentiation of MSCs *in vitro* and the sizes of the newly regenerated cardiac myocytes in aFRLM treated MI hearts were approximately one-third smaller than those of the normal myocytes, the cellular origin of the regenerating myocytes are likely derived from circulating MSCs [38-40]. However, this assumption remains to be verified by further studies. To test this hypothesis, we examined the effect of aFRLM in inducing cardiogenic differentiation of bone-marrow-derived MSCs *in vitro*. We found that approximately 20% (7 days) or 30% (14 days) of the aFRLM treated MSCs became elongated and refractive forming rod-like phenotype in comparison with the flat, low refractive, irregular, and asymmetry

morphology in vehicle-treated MSCs. Furthermore, more than 30% of these myocyte-like cells were positively stained by MHC specific antibodies after 14 days treatment of aFRLM indicating the potential ability of aFRLM in promoting cardiogenic differentiation of MSCs. By contrast, few MHC positively stained cells in vehicle treated wells were found at the same time point.

This study represents the first report that aFRLM can be used for repair of infarcted heart through enhancing myocyte regeneration in subclinical MI animal model. The possible mechanism underlying how oral administration of aFRLM in the MI model could significantly improve cardiac function and induce myocardial regeneration remains unknown. Occlusion of a major coronary artery, such as LAD, would cause a significant loss of functional cardiac myocytes through necrosis, intrinsic and extrinsic apoptosis pathways and autophagy. Repair of



the infarcted heart tissues with newly regenerated cardiac myocytes remains a forbidden area. Therefore, the molecular regulation of these cellular events is extremely complicated. For instance, the nuclear factor NF- $\kappa$ B super family of transcription factors has been implicated in the regulation of cell survival and inflammation in many cell types, including cardiac myocytes. Recent studies have shown that NF- $\kappa$ B is cardioprotective during acute hypoxia and reperfusion injury. However, prolonged activation of NF- $\kappa$ B appears to be detrimental and promotes heart failure by eliciting signals that trigger chronic inflammation through enhanced elaboration of cytokines including tumor necrosis factor, interleukin-1, and interleukin-6, leading to cell death. Further studies indicated that the timing and duration of activation, and cellular context may explain mechanistically the differential outcomes of NF- $\kappa$ B signaling in the heart that may be essential for future development of novel therapeutic interventions.

In our preliminary studies, we found that aFRLM treatment induced transient up-regulation of NF- $\kappa$ B (12 hours and 3 days reached the peak level), then the NF- $\kappa$ B level decreased on day 7 and further decreased on day 14. Therefore, we hypothesize that the possible underlying mechanism of substantial treatment of MI and induction of growth of new myocytes by aFRLM treatment is probably attributed to the multi-properties of aFRLM in anti-inflammation, anti-apoptosis, anti-oxidative stress induced injuries, increased cell survival and the prevention of ischemic reperfusion damage. As demonstrated in our preliminary studies, activation of NF- $\kappa$ B in the first three days post infarction may enhance the homing of resident Cardiac Stem Cells (CSCs) or circulatory mesenchymal stem cells to the injured myocardium, which consequently leads to an accumulation of CSCs/ MSCs in the infarct region [41]. After three days post infarction, the NF- $\kappa$ B level decreased in the aFRLM treated hearts. The aFRLM treatment-induced sequential up- and down-regulation of NF- $\kappa$ B expression in the infarcted hearts may at least partly involve the concerted efforts for repair of the damaged heart. Namely, the transient up-regulated expression of NF- $\kappa$ B (12 hours-3days post aFRLM treatment) may help the migration and accumulation of CSCs/ MSCs to the infarct zone, the ensuing down-regulation of NF- $\kappa$ B may contribute to the regulation of inflammation response (i.e. anti-inflammation, anti-apoptosis, and anti-oxidative stress) that would create a cardiogenesis inductive environment for cardiogenic differentiation and maturation of the accumulated CSCs/ MSCs [41-43].

The nature of aFRLM derived from the fruit of an edible plant and the convenience and safety of using aFRLM through oral administration demonstrated in this study make immediate development of a new remedy for MI possible. In order to accelerate the development of aFRLM into a substantial clinical therapy, it requires further studies including investigations of pharmacodynamic and pharmacokinetic properties, and metabolism of aFRLM *in vivo*. It is also of interest and of wider clinical significance to investigate whether aFRLM is beneficial for old MI and ischemic cardiomyopathy.

The demonstrated therapeutic effect of aFRLM in an animal MI model and the enhanced cardiogenic differentiation efficiency of MSCs *in vitro* by aFRLM suggest that aFRLM can be potentially developed into a potent therapeutic drug for the effective treatment of MI. Further identification of its active molecule and the signaling pathways involved lays the foundations for the immediate development of new treatment strategies for coronary heart diseases.

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