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Cardioprotection of recombinant human MG53 protein in a porcine model of ischemia and reperfusion injury

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Abstract

Ischemic heart disease is a leading cause of death in human population and protection of myocardial infarction (MI) associated with ischemia-reperfusion (I/R) remains a challenge. MG53 is an essential component of the cell membrane repair machinery that protects injury to the myocardium. We investigated the therapeutic value for using the recombinant human MG53 (rhMG53) protein for treatment of MI. Using Langendorff perfusion of isolated mouse heart, we found that I/R caused injury to cardiomyocytes and release of endogenous MG53 into the extracellular solution. rhMG53 protein applied to the perfusion solution concentrated at injury

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Author Contributions

J. L., H.Z., Y. Z., Z. X., L. L., T. T., K-H. P., J. H., C. Z., D. L., R. L., J. A., and H.G. performed the experiments; J. L., H. Z., Z. L., N. W., D. Z., P. L., and J. M. designed the experiments and analyzed the data; and J. L., H. Z., N. W., and J. M. wrote the manuscript.

Disclosures

J. M. and N. W. are founders for TRIM-edicine that is developing MG53 for therapeutic application in regenerative medicine. The intellectual properties for MG53 were patented by the Rutgers University - Robert Wood Johnson Medical School.

sites on cardiomyocytes to facilitate cardioprotection. With rodent models of I/R-induced MI, we established the *in vivo* dosing range for rhMG53 in cardioprotection. Using a porcine model of angioplasty-induced MI, the cardioprotective effect of rhMG53 was evaluated. Intravenous administration of rhMG53, either prior to or post ischemia, reduced infarct size and troponin I release in the porcine model when examined at 24 hours post reperfusion. Echocardiogram and histological analyses revealed that the protective effects for rhMG53 observed following acute MI led to long-term improvement in cardiac structure and function in the porcine model when examined at 4 weeks post operation. Our study supports the concept that rhMG53 could have potential therapeutic value for treatment of MI in human patients with ischemic heart diseases.

Keywords

Myocardial infarction; cell membrane repair; TRIM72; myocardial ischemia; angioplasty

1. Introduction

Ischemic heart disease caused by coronary arteriosclerosis remains as the single largest cause of mortality in western countries and is increasingly common throughout the rest of the world. As a result of arteriosclerosis or cardiac surgery, blockade of blood flow leads to acute myocardial infarction (MI) that is associated with two types of myocardial damage, including ischemic injury induced by the initial loss of blood flow and reperfusion injury by the restoration of oxygenated blood flow[1-4]. Ischemia-reperfusion of the heart is thought to generate oxidative stress that opens the mitochondrial permeability transition pore leading to apoptosis; and lipid peroxidation leading to breakdown of the sarcolemmal membrane and cell necrosis[5-7]. While there are interventions available to reestablish coronary perfusion and to treat arrhythmias associated with MI, there are no effective treatments available to directly prevent or alleviate I/R-induced cardiomyocyte injury[8-13]. Elucidation of cardiac membrane repair mechanisms would provide insights into the etiology of myocardial remodeling and guide development of new strategies to reduce cardiomyocyte loss and minimize myocardial fibrosis.

Plasma membrane repair is of particular importance in the heart because cardiomyocytes are terminally differentiated cells with limited self-renewal capability [14]. Cardiomyocytes suffer transient membrane injuries under physiological conditions and can be exacerbated by various pathophysiological stresses[15]. In a recent series of studies, we discovered MG53, a tripartite motif (TRIM)-family protein, is an essential component of the cell membrane repair machinery [16-18]. MG53 functions in vesicle trafficking and allows for nucleation of intracellular vesicles at sites of membrane disruption, and is uniquely positioned to protect against MI associated with ischemic heart diseases. Increased vulnerability to ischemiareperfusion induced injury to the heart was observed in mouse with genetic ablation of MG53 [19, 20]. AAV-mediated delivery of MG53 gene into animal models of muscular dystrophy and cardiomyopathy could rescue certain aspects of the defective muscle and heart function[21]. While overexpression of MG53 could improve membrane repair defects in certain disease conditions, the gene therapy-based approaches necessary to pursue this effort have disadvantages. In particular, since myocardial ischemia is an acute disease that

requires immediate treatment, molecular manipulations that target expression of the intracellular MG53 protein may not be practical for treatment of MI.

We recently showed that disruption of the cell membrane leads to exposure of a signal to the external leaflet of the plasma membrane that can be detected by MG53, allowing recombinant MG53 protein to repair membrane damage when provided in the extracellular space[22]. Using several *in vivo* animal model studies, we found that intravenous delivery of the recombinant MG53 protein can repair membrane damage to skeletal muscle and lung epithelial cells and ameliorate the pathology associated with muscular dystrophy[22] and acute lung injury[23]. Here we show that recombinant human MG53 (rhMG53) protein has therapeutic value for treatment of MI involving I/R injury to the heart. We provide both *ex vivo* and *in vivo* data to suggest that application of rhMG53 either prior to ischemia or post ischemia can protect injury to the myocardium in the porcine model of cardiac injury.

2. Methods

2.1 Langendorff perfusion of mouse hearts

Wild type mouse (C57BL6/J) hearts were subjected to global ischemia/reperfusion (I/R) during Langendorff perfusion. Hearts were perfused with Krebs buffer at a flow rate of 2 ml/min and allowed to equilibrate for 30 min before the Krebs buffer was supplemented with rhMG53 (40 μg/ml) or equimolar concentration of bovine serum albumin (BSA) as a control. Perfusion flow was ceased 5 minutes after the addition of protein and the heart was maintained in an ischemic state for 30 min. To induce I/R injury, the heart was reperfused for 60 min before it was removed from the apparatus and stained using triphenyltetrazolium chloride (TTC) to indicate infarct area using standard techniques[24]. In separate studies, rhMG53 was applied to the perfusate after the mouse heart had undergone 30 min of ischemia, in order to test the protective effect of rhMG53 against reperfusion-induced injury to the cardiomyocytes.

For immunohistochemistry studies, MBP-MG53 was used in perfusate in order to differentiate endogenous and exogenous MG53 during immunostaining. At the end of 60 min reperfusion, the perfusion solution was changed from Kreb's solution containing MBP-MG53 to a solution containing FITC conjugated Annexin V (Annexin V-FITC) (BioLegend, Inc. San Diego, CA) and perfused for 1 more min. Then the hearts were fixed with perfusion of 4% paraformaldehyde for ten minutes to remove unbinding Annexin V from the heart tissue. The hearts were longitudinally cut into half and embedded using optimal cutting temperature compound (OCT) for frozen sectioning. The slides were stained with antibody against MBP for confocal microscopy imaging of colocalization of Annexin V and MBP-MG53.

2.2 Purification of recombinant human MG53 protein

Purification of the recombinant human MG53 (rhMG53) protein has been described previously[22]. The present study employed two different forms of MG53 protein, MBP-MG53 and untagged rhMG53. Untagged rhMG53 was produced by cleavage of MBP from MBP-MG53 using thrombin digestion and separation of these two using gel filtration high

pressure liquid chromatography. Untagged rhMG53 was lyophilized and stored at 4 °C as dry powder in a desiccator. The membrane protective activity of rhMG53 from each preparation was determined by our established micro-glass bead injury assay as described elsewhere [18, 22].

2.3 Cardiomyocytes live cell imaging

Ventricular myocytes were enzymatically isolated from the hearts of adult male mice (12-14 weeks) following the protocol of Wang et al[19]. The freshly isolated cardiomyocytes were plated onto coated Delta T dishes (Bioptechs inc. Butler, PA) with HEPES buffer containing (in mmol/L): 137 NaCl, 5.4 KCl, 20 HEPES, 1.8 $CaCl_2$, 15 D-Glucose, 1.3 $MgSO_4$, 1.2 NaH_2PO_4, (pH 7.4). rhMG53 and BSA were conjugated with FITC (Lightning-Link® FITC, Innova Biosciences Ltd. Cambridge, UK) and added into dishes containing cardiomyocytes to a final concentration of 25 μg/ml. A Zeiss LSM780 confocal microscope was used for live cell imaging of the translocation of FITC-labelled rhMG53 or FITC-labelled BSA. The FITC signal was recorded at a rate of 3.13 sec/frame.

2.4 Porcine model of angioplasty induced myocardial infarction

Chinese experimental miniature swine (CEMS), weighing 15±2.5kg, were provided by Beijing Experimental Animal Reproduction and Regulation Center (Grade II, Certificate No. Jing-030). All animal experiments in this study were performed in accordance with China Academy of Chinese Medical Sciences Guide for Laboratory Animals that conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Experimental pigs underwent balloon inflation of the left anterior descending (LAD) coronary artery according to established methods[25, 26] with minor modifications. Briefly, pigs were anesthetized with intravenous injection of pentobarbital (30 mg/kg) through a marginal ear vein. Animals were intubated and ventilated with a digital ventilator (SC-3, Shanghai Medical Equipment Factory), and continuously monitored for their reflexes, electrocardiography (ECG) and respiratory status. The right common carotid artery was surgically exposed, and an 8F sheath was placed in the carotid artery. Each animal was given a single dose of heparin (150 U/kg) via the arterial sheath. A coronary artery catheter was advanced to engage with the left main coronary artery under direct fluoroscopic imaging. A coronary angiogram was performed to define the anatomy of LAD. Angioplasty was accomplished by inflating a 2.5~3.5-mm balloon (1:1.2–1.3 balloon-to-artery ratio) to 8-10 atm in the LAD artery distal to the second diagonal branch. The balloon was deflated and withdrawn to allow reperfusion in distal LAD. Reperfusion was confirmed by ST-segment alterations on the ECG. The detailed operation procedures were illustrated in the **Supplemental Movie S2**. Administration of rhMG53 at different times of experimental interventions was achieved through the jugular vein. Whenever possible, experimental procedure and data analysis were conducted in a double-blinded manner.

2.5 Echocardiographic imaging in CEMS

During the ischemia/reperfusion surgery as well as different time points after surgery, echocardiograph was performed to evaluate cardiac morphology and function (Philips Medical System, Holland). Left ventricular ejection fraction (LVEF), fractional shortening

(FS), systolic inter-ventricular septal thickness (IVSs) and left ventricular posterior wall thickness (LVPWs) were evaluated to determine the global function of the left ventricle and the regional functional and structural changes of the myocardium. LVEF was determined from the apical two- and four-chamber views by using a modified Simpson's algorithm[27]. Regional wall thickness was measured at end-systole (the end of T wave of the ECG) from two-dimensional echocardiograms. Representative recordings of the echocardiography from the different experimental groups were illustrated in **Supplemental Movies S3-S8**.

2.6 Pathological examination of myocardial tissues

Either 24 hours or 4 weeks after surgery, the heart was excised and rinsed in ice-cold phosphate buffered saline (PBS) solution and sliced into 4 cross sections with equal thickness for staining with nitro blue tetrazolium (NB-T) at 25 °C for 15 min. The infarct area (NB-T non-stained area) was determined by a multimedia color pathological image analytical system (MPIAS-500, Beijing Konghai Company, China). Following this staining, control tissue from a non-ischemic area remote from the infarcted area was obtained from a superior portion of the lateral left ventricle. The infarct sample was obtained from the middle of the region characterized by the pale appearance of the myocardium[28] and another sample was taken from an area directly adjacent to the infarct zone. Part of these tissue samples were embedded in paraffin, then sectioned (4 μm thickness) and subjected to TUNEL (terminal dUTP nick end-labeling) staining with the TUNEL Apoptosis Detection Kit according to the manufacturer's instructions (EMD Millipore, MA, USA). The remaining samples were immediately processed for western blot analysis of expression of MG53.

2.7 Western Blotting

Twenty four hours after surgery, proteins from different area of porcine myocardium were collected and diluted into 0.5 mg protein/mL for the measurement of pro-survival pathway proteins. Protein content was determined with BSA as a standard according to Bradford assay. Protein samples (20 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to PVDF membranes (Millipore, Billerica, MA) through electroblotting. Blots were probed with antibodies against phosphorylated Akt (Ser473), total Akt, phosphorylated GSK-3β (Ser9), total GSK-3β, phosphorylated ERK1/2 (pThr202/Tyr204) and total ERK1/2 (Cell Signaling, Boston, MA). The blots were then developed by enhanced chemiluminescence using SuperSignal west femto maximum sensitivity substrate (Pierce, Rockford, IL). Bio-Rad Image Lab™Version 3.0 software was used to calculate the numerical value of every blot.

2.8 Statistical analysis

The results of the experiments were analyzed by several statistical methods (e.g., paired or unpaired *t*-tests, ANOVA, etc.) using commercial Prism software (GraphPad Prism). Data were presented as means ± SEM. For comparisons between two groups, significance was determined by Student *t*-test parametric analysis. For comparison of multiple groups, multifactorial analysis of variance (ANOVA) was used to determine statistical significance. A threshold of P value of <0.05 was considered statistically significant in all conditions.

3. Results

3. 1 rhMG53 protein protects myocardial infarction in isolated mouse heart preparations

While MG53 was discovered as an intracellular protein, our previous studies determined that injury to sarcolemmal membrane could lead to release of native MG53 to the serum, suggesting that detection of MG53 in the blood could be used as a potential biomarker for tissue injury[22]. As shown in **Fig. 1A**, when isolated mouse hearts were subjected to ischemia/reperfusion (I/R) injury, significant amount of endogenous MG53 protein could be detected in the perfusate. The amount of MG53 detected in the perfusate appeared to correlate with the release of creatine kinase (CK) (**Fig. 1A, 1C**), supporting the concept that I/R injury disrupts the integrity of the sarcolemmal membrane of cardiomyocytes. *In vivo* studies with I/R injury to mouse hearts also showed that the circulating MG53 level is positively correlated with the time of ischemia and reperfusion (see Supplementary **Fig. S1**).

rhMG53 protein was purified using *E. coli* fermentation and stored as a lyophilized powder (see Supplemental **Fig. S2**). The rhMG53 protein remained stable in the lyophilized powder and could be resuspended into saline solution. Mouse hearts were prepared using a modified Langendorff perfusion method and subjected to 30 minutes of global ischemia by cessation of perfusate flow, which was then restored to allow for 60 minutes of reperfusion. When rhMG53 was added to the perfusion solution, we observed significant protection of I/R-induced injury to the heart. Triphenyltetrazolium chloride (TTC) staining showed reduced infarct area in heart treated with rhMG53 (**Fig. 1B**). The protective effects of rhMG53 were observed in conditions when rhMG53 was applied prior to induction of ischemia (Pre), as well as when rhMG53 was applied post-ischemia (2 min after reperfusion, Post) (**Fig. 1B**). Significant reduction of CK was observed throughout the reperfusion phase when rhMG53 was applied either Pre or Post ischemic event (**Fig. 1C**), suggesting that rhMG53 treatment could reduce the loss of sarcolemmal membrane integrity following I/R-induced damage to the cardiac muscle.

Our previous studies showed that disruption of plasma membranes led to exposure of phosphatidylserine to the extracellular space that enables rhMG53 to concentrate at injury sites and to facilitate membrane repair[22]. To test if I/R-induced injury to cardiomyocytes caused recruitment of rhMG53 protein, we performed immunohistochemical (IHC) staining on tissue sections from the perfused mouse heart that were subjected to I/R injury. These hearts were perfused with fluorescently labeled Annexin V (Annexin V-FITC) to bind exposed phosphatidylserine (PS) at membrane disruptions in injured cardiomyocytes. To allow for differentiation of endogenous MG53 from exogenous rhMG53 we used a fusion protein of rhMG53 with a maltose binding protein (MBP) tag (MBP-MG53), which is known to have efficacy similar to unlabeled rhMG53 (see Supplemental **Fig. S2**)[22]. As shown in **Fig. 2A**, IHC staining with an anti-MBP antibody revealed overlapping patterns of MBP-MG53 and Annexin V in the injured myocardium. Confocal microscopic imaging of isolated adult mouse cardiomyocytes provided direct visualization of the interaction between rhMG53 and the injury sites on cardiomyocytes. As shown in **Fig. 2B** and **Supplemental Movie S1**, when FITC-labeled rhMG53 was added to the media of freshly isolated cardiomyocytes, it rapidly targeted the longitudinal ends and other injury sites on the

cardiomyocytes. As control, FITC labeled BSA could not decorate any membrane of the cardiomyocytes.

Taken together, these findings suggest that the exogenously applied rhMG53 can recognize the injury sites on the membrane of cardiomyocytes, which is consistent with our previous observations in skeletal muscle[22] and lung epithelial cells[23].

3.2 rhMG53 protects acute MI in the porcine model of angioplasty-induced heart injury

Initial in vivo testing of rhMG53 was performed with a rat model of MI induced by ligation of the left anterior descending (LAD) coronary artery (see Supplemental **Fig. S3**), and then a mouse model of MI induced by ligation of the coronary artery (see Supplemental **Fig. S4**). Different doses of rhMG53 protein (0.1-10 mg/kg) were applied by intravenous (IV) injection to the rats or intraperitoneal (IP) injection to the mice. The changes in acute myocardial infarct size and creatine kinase (CK) release were used as indexes of heart injury. Statistical analysis revealed dose-dependent effects of rhMG53 on the reduction of infarct size and CK release associated with I/R-induced injury to the rodent hearts. Essentially, with delivery of rhMG53 at concentration above 1 mg/kg, significant cardioprotective effect was observed with the rodent models (**Fig. S3** and **S4**). These studies provided guidance for our studies using the porcine model of angioplasty-induced MI.

The angioplasty induced MI in the porcine model has been widely used for cardiovascular research [24, 29, 30], and could provide direct assessment for the efficacy for rhMG53 in cardioprotection because of the anatomical and physiological similarity between human and porcine hearts. For establishing the Chinese experimental miniature swine (CEMS) as an MI model, we first performed a series of pilot studies by varying the position of the angioplasty balloon and the duration of the ischemia event that could lead to measurable and reproducible myocardial infarction in these animals. We ensure that placing of the balloon was to the same position (distal to second diagonal branch of LAD) by using angiography (see **Supplemental Movie S2**). Occlusion was confirmed by angiography and alterations of the ST segment in ECG. A total 9 CEMS pigs were used for the pilot experiments. These animals were divided into three groups that received ischemia events of 60 min, 45 min and 30 min, respectively. In groups with 60 min and 45 minute ischemia, we observed variable degrees of exaggerated MI and 4 out of 6 animals died within 24 hrs post-surgery. However, with 30 min ischemia, we consistently observe reproducible degree of MI in the control group, and all animals survived the experimental operations. Thus all our experiments were conducted with CEMS pigs that receive 30 min ischemia following angioplasty balloon occlusion of the LAD.

For testing the efficacy for rhMG53 in protection of I/R-induced MI in the CEMS model, four experimental groups were used: 1) I/R animals treated with saline as control (**Saline**); 2) I/R animals treated with MG53 (1.0 mg/kg) prior to ischemia (**Pre**); 3) I/R animals treated with MG53 (1.0 mg/kg) 2 min post reperfusion (**Post**); and 4) I/R animals treated with MG53 (1.0 mg/kg) 30 min after reperfusion (**Post-30**). With this CEMS model, the following four parameters were measured. First, the body surface electrocardiogram (BS-ECG) was recorded continuously throughout the experimental procedure. The summation for the elevation of ST-segment from a total of 30 surface electrodes was used to monitor

the development of ischemia in the pig heart, and for initial assessment of the cardio protective function for rhMG53. Second, we used Doppler echocardiography to monitor the changes in cardiac function at different time points following I/R injury for functional assessment of cardiac output. Third, venous blood was drawn from the pig to allow detection of serum troponin I levels as a biomarker of the extent of cardiac injury. Finally, twenty-four hours after reperfusion, the pig hearts were removed for staining to determine the size of the MI. Further biochemical studies were performed on the cardiac tissue to dissect the molecular events that underlie the cardio protective function of rhMG53.

As shown in **Fig. 3A**, staining hearts with NB-T at twenty-four hours after I/R injury revealed significant reduction of the infarct area following treatment with rhMG53. Remarkably, all three animal groups receiving treatment with rhMG53 showed significant reduction in infarct size (**Fig. 3B**). In particular, while the infarct zone appeared to be uniform and contiguous in the control group (**Fig. 3A**, *top*), the infarct zone in rhMG53 treated groups appeared to consist of isolated spots (**Fig. 3A**, *lower panels*). These spotty-signature of MI patterns were never observed in the control pigs that received saline treatment, suggesting that the rhMG53 treatment could minimize cell death within the at-risk cardiac tissue.

Cardiac specific troponin I is widely used for diagnosis of myocardial injury associated with acute MI[31]. We used ELISA to measure serum cardiac troponin I levels at different time points following I/R injury to the pig heart. As shown in **Fig. 3C**, while I/R-induced injury to the heart caused significant elevation of serum troponin I in the serum (especially at 6 and 24 hrs after reperfusion), pigs receiving treatment with rhMG53 all showed reduced troponin I levels. Significant differences in troponin I levels were measured between the saline treated group and rhMG53 treated group at both 6hrs and 24 hrs after reperfusion. These results suggest that the cardioprotective effect of rhMG53 in reduction of myocardial infarction (**Fig. 3B**) is likely associated with improved sarcolemmal membrane integrity following treatment with rhMG53. Consistent with these results, TUNEL assay also showed that treatments of rhMG53 could significantly reduce TUNEL positive nuclei (stained with brown color) in response to I/R injury (**Fig. 3D, 3E**), indicating apoptosis was inhibited by rhMG53 treatments.

Based on the NB-T staining pattern, we isolated tissue samples from three regions designated as the infarct area (negative for NB-T staining), border zone, and remote area (healthy heart tissue positive for NB-T staining). Western blotting showed that the level of MG53 protein was significantly higher in the infarct area compared with that in the border zone and remote area in animals treated with rhMG53 (**Fig. 4A**). Since there was no difference in the distribution of endogenous MG53 protein among the infarct area, border zone and the remote area in the control animal group (**Fig. 4B**), one can conclude that the elevated MG53 at the infarct area originates from accumulation of the exogenous rhMG53 in the circulating blood. Such concentration of MG53 in the infarct area further supports the observation that exogenously applied rhMG53 can recognize membrane injury sites in the heart (**Fig. 2**).

To understand the mechanisms underlying the cardio-protective effect of rhMG53, lysates from the different regions of heart were analyzed by western blot of pro-survival signaling proteins. Consistent with previous studies with overexpression of MG53 in hearts[20, 21], some of pro-survival pathway proteins, such as Akt and GSK-3β, were activated in rhMG53 treated groups (**Fig. 5**). More importantly, the activation was observed mostly in the infarct area, further confirming the targeting effect of rhMG53 in injured myocardium. Interestingly, rhMG53 treatment had minimal effect on ERK activation. This result is different from the previous study with overexpression of MG53 in isolated cardiomyocytes[20,21], suggesting possible difference between endogenous overexpression of MG53 and exogenous application of rhMG53.

3.3 Survival studies demonstrate cardioprotection of rhMG53 in the porcine model

The studies described in **Fig. 3** outlines the effect of rhMG53 in protection of acute MI associated with I/R-induced injury to the porcine heart. To test if the acute protective effect for rhMG53 could be translated into long-term maintenance of cardiac function, we performed an additional set of survival studies where pigs were subjected to I/R injury and then allowed to recover for 4 weeks. Here we analyzed three experimental groups, one treated with saline, another treated with a single dose of rhMG53 (1.0 mg/kg) prior to ischemia, and a final group treated with a single dose of rhMG53 (1.0 mg/kg) 2 min after reperfusion. We used the Doppler echocardiography to assess the changes in cardiac function in the different groups of pigs at 4 weeks post I/R injury (see **Supplemental Movies S3-S8**). As shown in **Fig. 6A**, there was significant improvement in the cardiac ejection fraction in the groups of pigs that received treatment with rhMG53 when compared with the control group. The improved cardiac output was further confirmed by the enhanced fractional shortening in the rhMG53-treated pigs (**Fig. 6B**). Such improvement was also evident at 24 hrs post I/R injury. The echocardiography measurement also provided assessment on changes in the morphology of the heart in relationship to the altered cardiac contractility. As shown in **Fig. 6C** and **6D**, the inter-ventricular septal thickness and left ventricular wall thickness in systole were significantly reduced in hearts derived from the control pigs whereas it did not occur in animal groups that received the rhMG53 treatment, suggesting the extensive loss of cardiomyocytes associated with I/R injury can be minimized by the application of rhMG53.

rhMG53-mediated improvement in heart morphology was further revealed in explanted hearts from the three groups of pigs. As shown in **Fig. 6E**, heart derived from the control group showed extensive fibrosis at the infarct area that was often accompanied with reduced ventricular wall thickness (*top panel*) due to remodeling of the myocardium following death of the cardiomyocytes near the infract area. The hearts derived from either pre-MG53 or post-MG53 treated animal groups were significantly healthier (**Fig. 6E,** *middle and bottom panels*). These observations were consistently seen in all animals tested (see also Supplemental **Fig. S5**). Further pathological analysis with Masson's Trichrome staining revealed that the excessive fibrotic remodeling in hearts derived from saline treated pigs was significantly reduced in those that received rhMG53 treatments (**Fig. 6F**). Together, these results indicate that the protective effects for rhMG53 observed following acute myocardial

injury (24 hrs after I/R injury) can result in sustained survival of cardiomyocytes that produces long-term improvement in cardiac structure and function in this porcine model.

4. Discussion

MG53 is an essential component of the membrane repair machinery in striated muscles[16, 19, 20, 32]. Recent studies from our group showed that rhMG53 protein can also protect against injury to skeletal muscle and non-muscle cell types when provided to the extracellular solution[22, 23]. Here we demonstrate a cardioprotective function for rhMG53 using multiple experimental approaches. The cardioprotective effects of rhMG53 was observed in the *ex vivo* mouse heart, and then in both the *in vivo* mouse, rat and pig models. Since the anatomy of the human heart is similar to the pig heart, our demonstration of rhMG53 in promoting survival function of the pig heart following I/R injury indicates that rhMG53 protein can potentially be an effective therapeutic agent for treatment of MI in human patients with ischemic heart disease.

When rhMG53 was applied to the heart prior to ischemia, it can minimize ischemia-induced injury to the cardiomyocytes. This suggests that rhMG53 can potentially be used as a prophylactic agent to protect tissue damage during surgical operations or other therapeutic treatments that may cause injury to the heart. Since rhMG53 was also effective in protection of MI following reperfusion (as evidenced by the strong protective effect with Post and Post-30 applications), one can envision that rhMG53 protein can have potential therapeutic value in treatment of patients suffering an acute MI.

We found that I/R-induced injury to the cardiomyocytes could cause significant release of MG53 into the circulation, thus detection of the native MG53 in the blood could potentially be used as an early diagnostic biomarker for patients with acute MI. As the native MG53 is constantly present in the blood circulation, the potential toxicological and immunological concerns for targeting MG53-mediated membrane repair in cardio protection can be minimized or controlled. Another advantage for the application of rhMG53 as a therapeutic agent is that there are established protocols for purification and scale-up production of the rhMG53 protein. The purified rhMG53 protein is stable at room temperature as a lyophilized powder, allowing for re-suspension in saline solution and delivery via different injection routes (e.g. intravenous, intramuscular or subcutaneous methods). There is certainly room for enhancing efficacy for MG53-mediated cardioprotection. For example, Kohr et al. demonstrated that oxidative stress induced oxidation of MG53 at cysteine 144 residue attenuated its stability [33]. Thus, one could generate mutant rhMG53 (e.g. $MG53_{C144S}$) or modify MG53 with s-nitrosylation[33], with the hope to enhance stability and/or protective efficacy of the protein in blood circulation.

Concentration of rhMG53 at the acute MI sites is likely an early step in the cardioprotective function for MG53. While we know that binding of rhMG53 to the lipid signals at the injury site (e.g. phosphatidylserine and cholesterol) may facility the recruitment of rhMG53 to the injury sites[16, 18, 19] and this mechanism may be shared with the intracellular action of the native MG53 protein, the mechanistic action for the exogenous rhMG53 protein is likely to be different from the intracellular MG53 protein. For example, changes in redox-state is a

key signal that trigger disulfide-bond mediated cross-linking for intracellular MG53 for nucleation of intracellular vesicles toward the membrane injury sites[16]. The exogenous rhMG53 exists in an extracellular oxidized environment and may require cofactors that differ from the intracellular MG53 to mediate the tissue repair function. Whether oligomerization of rhMG53 is involved in cardioprotection will require further studies. Since we knew that dimerization involves disulfide bond formation at cysteine 242 of MG53 protein, one approach to test this possibility can be generation of a mutant MG53 protein by replacing cysteine 242 with an alanine (C242A). With the C242A mutant protein, *in vitro* and *in vivo* studies will be required to ascertain the effect of redox-mediated MG53 oligomerization in cardioprotection. It is also possible that rhMG53 can enter the cardiomyocytes through the injury sites and directly trigger activation of the PI3K survival pathway, as demonstrated in our previous study[34]. Indeed, we observed activation of Akt upon rhMG53 treatment in infarct area, indicating a potential role of rhMG53 on Akt signaling. Thus, it is possible that, in addition to membrane repair, MG53 can directly interact with Akt pathway to facilitate survival of injured cardiomyocytes. Alternatively, rhMG53 could bind to a receptor on the sarcolemmal membrane and act indirectly through activation of second messengers. If this is the case, more future studies are required to identify such membrane receptors for rhMG53. In addition to membrane repair function, MG53 is also an ubiquitin E3 ligase[35-37]. Elucidating the involvement of E3 ligase function of MG53 in membrane repair, cell survival and metabolic control will require thorough studies in the future.

We showed that endogenous MG53 is continuously present in the serum, and the serum level for MG53 exhibit strong correlation with tissue injuries. It will be important to understand how MG53 is released to the blood stream, and how MG53 is metabolically cleared from the circulation. It is well known that exercise is beneficial for body movement and metabolic function. With exercise, the increased muscle and heart contraction activity can lead to increased release or secretion of MG53 to the circulation, which may be beneficial for prevention of chronic organ injuries associated with metabolic stresses. We have previously shown that MG53 facilitates membrane budding and vesicular trafficking[32], leading to an intriguing possibility that MG53 can be released to the circulation via a defined secretory pathway. Thus, in addition to development of the rhMG53 protein-based therapy, targeting the cellular pathway that controls circulation of the endogenous MG53 in the blood stream or the potential paracrine function for MG53 may represent an additional avenue for protection against MI and other disease states involving tissue degeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MG53	Mitsugumin53 (also known as TRIM72)
rhMG53	recombinant human MG53
MI	Myocardial infarction
I/R	Ischemia-reperfusion
TTC	triphenyltetrazolium chloride
CEMS	Chinese experimental miniature swine
LVEF	Left ventricular ejection fraction
FS	Fractional shortening
IVSs	Systolic inter-ventricular septal thickness
LVPWs	Left ventricular posterior wall thickness
NB-T	Nitro blue tetrazolium
BS-ECG	Body surface electrocardiogram
TUNEL	Terminal dUTP nick end-labeling

References

1. Kloner RA. Does reperfusion injury exist in humans? J Am Coll Cardiol. 1993; 21:537–45. [PubMed: 8426022]
2. Opie LH. Reperfusion injury and its pharmacologic modification. Circulation. 1989; 80:1049–62. [PubMed: 2571429]
3. Garcia-Dorado D, Ruiz-Meana M, Piper HM. Lethal reperfusion injury in acute myocardial infarction: facts and unresolved issues. Cardiovasc Res. 2009; 83:165–8. [PubMed: 19502282]
4. Hearse DJ, Bolli R. Reperfusion-induced injury manifestations, mechanisms, and clinical relevance. Trends Cardiovasc Med. 1991; 1:233–40. [PubMed: 21239301]
5. Walters AM, Porter GA Jr. Brookes PS. Mitochondria as a drug target in ischemic heart disease and cardiomyopathy. Circulation research. 2012; 111:1222–36. [PubMed: 23065345]
6. Kramer JH, Misik V, Weglicki WB. Lipid peroxidation-derived free radical production and postischemic myocardial reperfusion injury. Annals of the New York Academy of Sciences. 1994; 723:180–96. [PubMed: 8030864]
7. Hoffman JW Jr. Gilbert TB, Poston RS, Silldorff EP. Myocardial reperfusion injury: etiology, mechanisms, and therapies. J Extra Corpor Technol. 2004; 36:391–411. [PubMed: 15679285]
8. Hausenloy DJ, Yellon DM. The therapeutic potential of ischemic conditioning: an update. Nat Rev Cardiol. 2011; 8:619–29. [PubMed: 21691310]
9. Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. J Clin Invest. 2013; 123:92–100. [PubMed: 23281415]
10. Bell R, Beeuwkes R, Botker HE, Davidson S, Downey J, Garcia-Dorado D, et al. Trials, tribulations and speculation! Report from the 7th Biennial Hatter Cardiovascular Institute Workshop. Basic Res Cardiol. 2012; 107:300. [PubMed: 23008030]

11. Hausenloy DJ, Baxter G, Bell R, Botker HE, Davidson SM, Downey J, et al. Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations. Basic Res Cardiol. 2010; 105:677–86. [PubMed: 20865418]
12. Schwartz Longacre L, Kloner RA, Arai AE, Baines CP, Bolli R, Braunwald E, et al. New horizons in cardioprotection: recommendations from the 2010 National Heart, Lung, and Blood Institute Workshop. Circulation. 2011; 124:1172–9. [PubMed: 21900096]
13. Hausenloy DJ, Erik Botker H, Condorelli G, Ferdinandy P, Garcia-Dorado D, Heusch G, et al. Translating cardioprotection for patient benefit: position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. Cardiovasc Res. 2013; 98:7–27. [PubMed: 23334258]
14. Tam SK, Gu W, Mahdavi V, Nadal-Ginard B. Cardiac myocyte terminal differentiation. Potential for cardiac regeneration. Annals of the New York Academy of Sciences. 1995; 752:72–9. [PubMed: 7755297]
15. Clarke MS, Caldwell RW, Chiao H, Miyake K, McNeil PL. Contraction-induced cell wounding and release of fibroblast growth factor in heart. Circulation research. 1995; 76:927–34. [PubMed: 7538917]
16. Cai C, Masumiya H, Weisleder N, Matsuda N, Nishi M, Hwang M, et al. MG53 nucleates assembly of cell membrane repair machinery. Nature cell biology. 2009; 11:56–64. [PubMed: 19043407]
17. Lin P, Zhu H, Cai C, Wang X, Cao C, Xiao R, et al. Nonmuscle myosin IIA facilitates vesicle trafficking for MG53-mediated cell membrane repair. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2012; 26:1875–83. [PubMed: 22253476]
18. Zhu H, Lin P, De G, Choi KH, Takeshima H, Weisleder N, et al. Polymerase transcriptase release factor (PTRF) anchors MG53 protein to cell injury site for initiation of membrane repair. The Journal of biological chemistry. 2011; 286:12820–4. [PubMed: 21343302]
19. Wang X, Xie W, Zhang Y, Lin P, Han L, Han P, et al. Cardioprotection of ischemia/reperfusion injury by cholesterol-dependent MG53-mediated membrane repair. Circulation research. 2010; 107:76–83. [PubMed: 20466981]
20. Cao CM, Zhang Y, Weisleder N, Ferrante C, Wang X, Lv F, et al. MG53 constitutes a primary determinant of cardiac ischemic preconditioning. Circulation. 2010; 121:2565–74. [PubMed: 20516375]
21. He B, Tang RH, Weisleder N, Xiao B, Yuan Z, Cai C, et al. Enhancing muscle membrane repair by gene delivery of MG53 ameliorates muscular dystrophy and heart failure in delta-Sarcoglycan-deficient hamsters. Molecular therapy : the journal of the American Society of Gene Therapy. 2012; 20:727–35. [PubMed: 22314291]
22. Weisleder N, Takizawa N, Lin P, Wang X, Cao C, Zhang Y, et al. Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. Science translational medicine. 2012:4, 139ra85.
23. Jia Y, Chen K, Lin P, Lieber G, Nishi M, Yan R, et al. Treatment of acute lung injury by targeting MG53-mediated cell membrane repair. Nature communications. 2014; 5:4387.
24. Xiao CY, Hara A, Yuhki K, Fujino T, Ma H, Okada Y, et al. Roles of prostaglandin I(2) and thromboxane A(2) in cardiac ischemia-reperfusion injury: a study using mice lacking their respective receptors. Circulation. 2001; 104:2210–5. [PubMed: 11684633]
25. Karlsson LO, Bergh N, Li L, Bissessar E, Bobrova I, Gross GJ, et al. Dose-dependent cardioprotection of enkephalin analogue Eribis peptide 94 and cardiac expression of opioid receptors in a porcine model of ischaemia and reperfusion. European journal of pharmacology. 2012; 674:378–83. [PubMed: 22119384]
26. Andreka G, Vertesaljai M, Szantho G, Font G, Piroth Z, Fontos G, et al. Remote ischaemic postconditioning protects the heart during acute myocardial infarction in pigs. Heart. 2007; 93:749–52. [PubMed: 17449499]
27. Parisi AF, Moynihan PF, Feldman CL, Folland ED. Approaches to determination of left ventricular volume and ejection fraction by real-time two-dimensional echocardiography. Clinical cardiology. 1979; 2:257–63. [PubMed: 262574]

28. Huang S, Patterson E, Yu X, Garrett MW, De Aos I, Kem DC. Proteasome inhibition 1 h following ischemia protects GRK2 and prevents malignant ventricular tachyarrhythmias and SCD in a model of myocardial infarction. Am J Physiol Heart Circ Physiol. 2008; 294:H1298–303. [PubMed: 18192226]

29. Suzuki Y, Yeung AC, Ikeno F. The pre-clinical animal model in the translational research of interventional cardiology. JACC Cardiovasc Interv. 2009; 2:373–83. [PubMed: 19463458]

30. Schwartz RS, Edelman ER, Carter A, Chronos NA, Rogers C, Robinson KA, et al. Preclinical evaluation of drug-eluting stents for peripheral applications: recommendations from an expert consensus group. Circulation. 2004; 110:2498–505. [PubMed: 15492330]

31. Cummins B, Auckland ML, Cummins P. Cardiac-specific troponin-I radioimmunoassay in the diagnosis of acute myocardial infarction. American heart journal. 1987; 113:1333–44. [PubMed: 3591601]

32. Cai C, Masumiya H, Weisleder N, Pan Z, Nishi M, Komazaki S, et al. MG53 regulates membrane budding and exocytosis in muscle cells. The Journal of biological chemistry. 2009; 284:3314–22. [PubMed: 19029292]

33. Kohr MJ, Evangelista AM, Ferlito M, Steenbergen C, Murphy E. S-nitrosylation of TRIM72 at cysteine 144 is critical for protection against oxidation-induced protein degradation and cell death. Journal of molecular and cellular cardiology. 2014; 69:67–74. [PubMed: 24487118]

34. Zhang Y, Lv F, Jin L, Peng W, Song R, Ma J, et al. MG53 participates in ischaemic postconditioning through the RISK signalling pathway. Cardiovasc Res. 2011; 91:108–15. [PubMed: 21285295]

35. Yi JS, Park JS, Ham YM, Nguyen N, Lee NR, Hong J, et al. MG53-induced IRS-1 ubiquitination negatively regulates skeletal myogenesis and insulin signalling. Nature communications. 2013; 4:2354.

36. Song R, Peng W, Zhang Y, Lv F, Wu HK, Guo J, et al. Central role of E3 ubiquitin ligase MG53 in insulin resistance and metabolic disorders. Nature. 2013; 494:375–9. [PubMed: 23354051]

37. Nguyen N, Yi JS, Park H, Lee JS, Ko YG. Mitsugumin 53 (MG53) ligase ubiquitinates focal adhesion kinase during skeletal myogenesis. The Journal of biological chemistry. 2014; 289:3209–16. [PubMed: 24344130]

Highlights

- Treatment of ischemic heart disease is an unmet medical need.
- MG53 targets and protects injury to plasma membrane in multiple cell types.
- Recombinant human MG53 protein protects myocardium following I/R injury to the heart.
- rhMG53 might be a potential protein therapy for treatment of acute myocardial infarction.

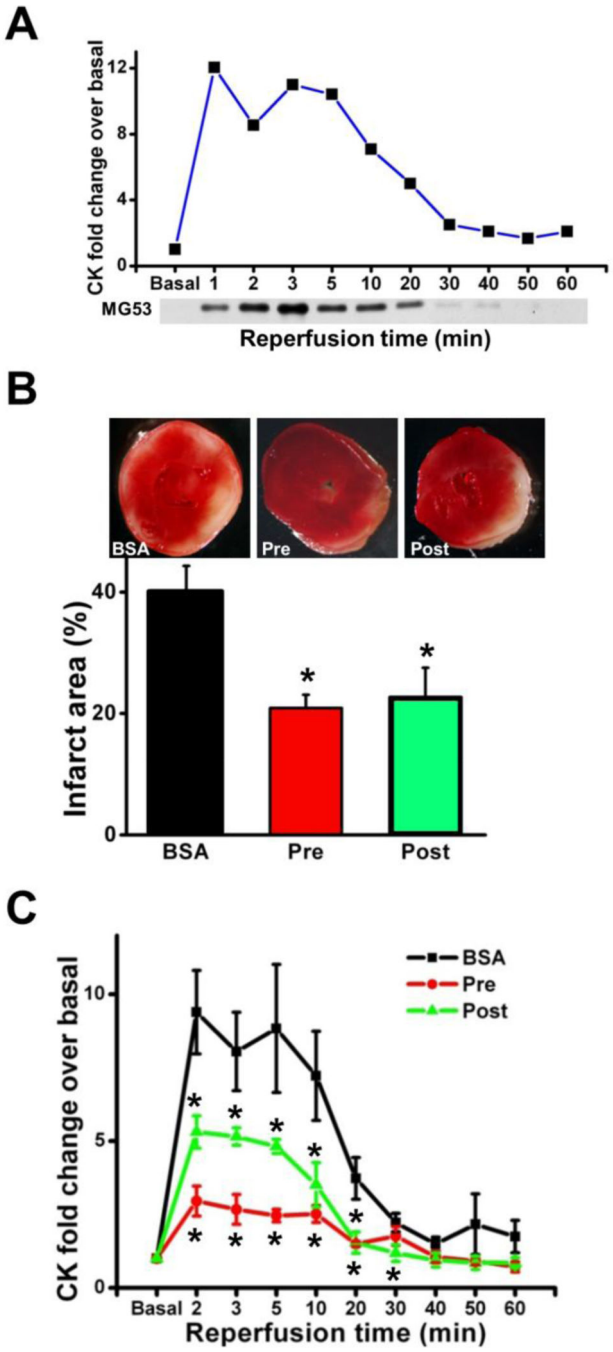

Figure 1. Cardioprotective effects of rhMG53 against I/R injury in Langendorff perfused mouse hearts

(**A**) Western blotting of perfusate collected at indicated time points (0-60 mins) following reperfusion of the mouse heart (*bottom*) shows that MG53 appears in the perfusate following I/R injury. This release occurs at a similar rate as the appearance of CK in the perfusate (*top*). (**B**) Representative images of TTC-stained heart slices from BSA (*left*) and MBP-MG53 (*center* and *right*) treated hearts. Infarct size for rhMG53 (n=4) treated hearts was significantly reduced compared to BSA treated hearts (n=4). Data presented as means ±

SEM with * indicating $p < 0.05$. (**C**) During perfusion of mouse hearts, release of CK into the perfusate was measured from effluent collected at indicated times. The significant reduction of CK with the application of MG53 provides direct support for the cardioprotective function of MG53 during ischemia-reperfusion (n=4 pairs, *p<0.05 by ANOVA).

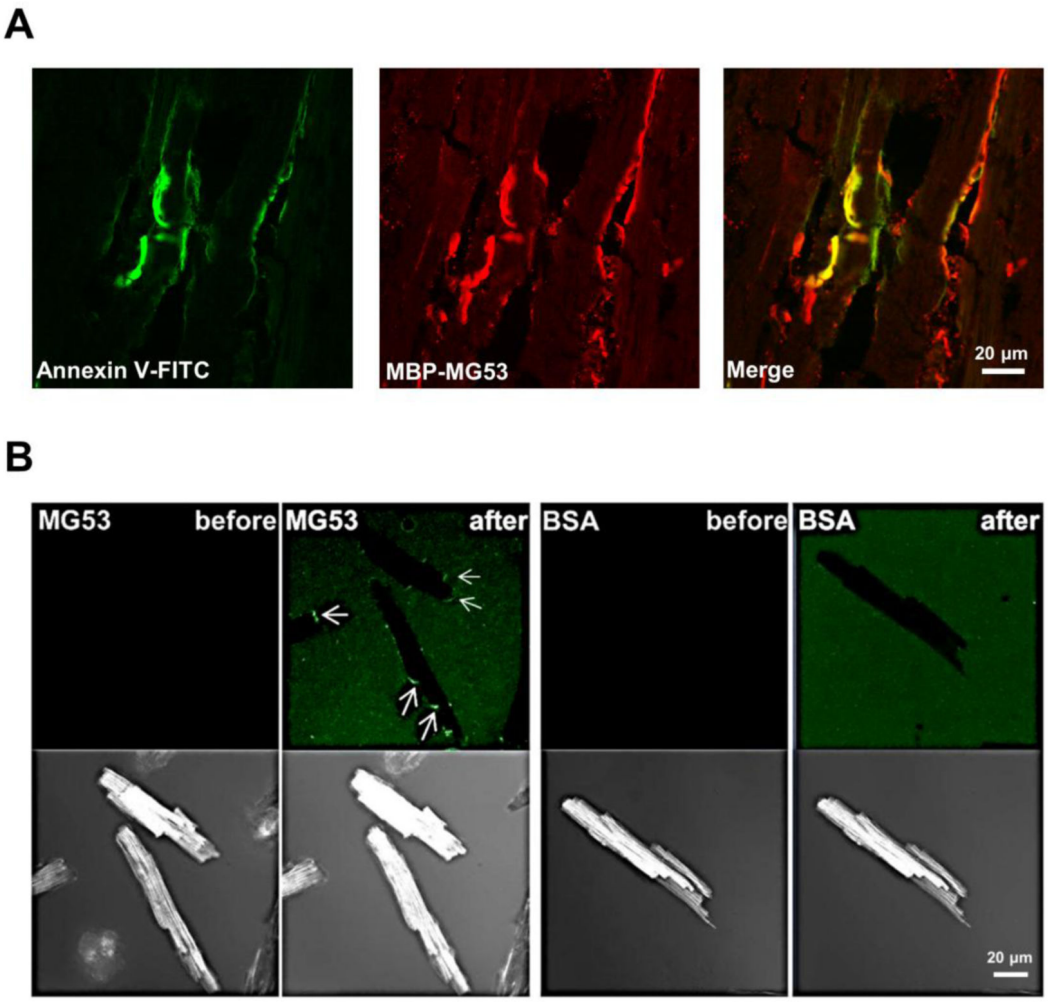

Figure 2. rhMG53 targets injury sites of cardiomyocytes when applied from extracellular space

(**A**) Cryosections from hearts perfused with Annexin V-FITC and then stained by MBP antibody to localize MBP-MG53. Examination of Annexin V-FITC labeling showed focal injury sites in the myocardium (*left*). Immunostaining for MBP-MG53 (*center*) showed that many of these injury sites also display staining for MBP-MG53 (*right*). (**B**) Representative images illustrating FITC labeled rhMG53 decorate injury sites on freshly isolated cardiomyocytes (pointed by arrows) when added to culture media. In contrast, FITC-labelled BSA did not show any labeling of the cardiomyocytes when applied to the extracellular solution. See Supplemental **Movie S1** for live cell imaging.

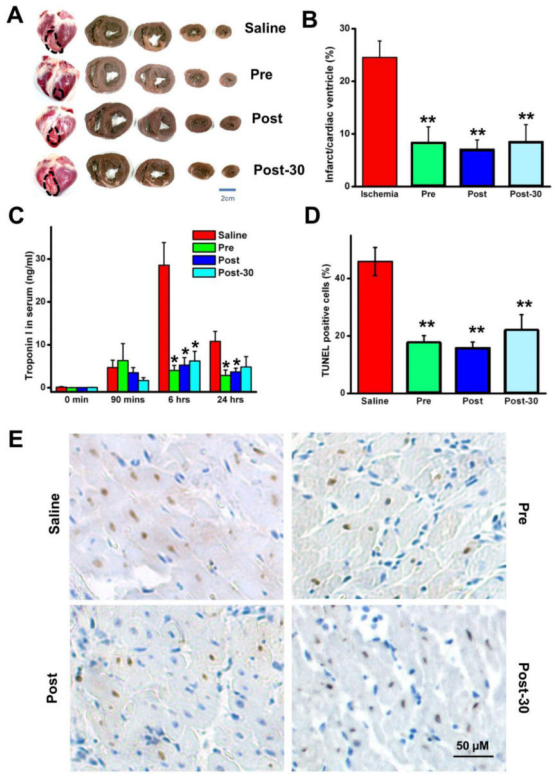

Figure 3. Cardioprotective effects of rhMG53 in the porcine model of acute MI

(**A**) Representative heart slices from pigs subjected to cardiac I/R injury. 24 hrs after I/R, the heart was explanted and local injuries to the left ventricle were shown in circled areas. NB-T staining revealed reduced infarct area following treatments with rhMG53. Saline – control group, Pre – pigs receiving rhMG53 prior to ischemia; Post – pigs receiving rhMG53 2 min after reperfusion; and Post-30 – pigs receiving rhMG53 30 min post reperfusion. (**B**) Statistical data of infarct size in pig hearts subjected to I/R. Application of rhMG53 at dose of 1 mg/kg resulted in significant reduction in infarct size, compared to hearts treated with saline as control (**p<0.01 by ANOVA). (**C**) Changes of troponin I concentration in the pig serum collected at different time points following I/R injury. Significant reduction of troponin I levels was observed at 6 hrs and 24 hrs in pigs receiving rhMG53 treatments (n=6-7, *p<0.01 by ANOVA). (**D**) Summary of TUNEL staining shows rhMG53 treatments significantly reduce apoptotic cardiomyocytes in infarct area (24 hrs after I/R injury). *p<0.01 by ANOVA. (**E**) Representative TUNEL images show rhMG53 treatments reduce TUNEL positive cells (stained in brown color).

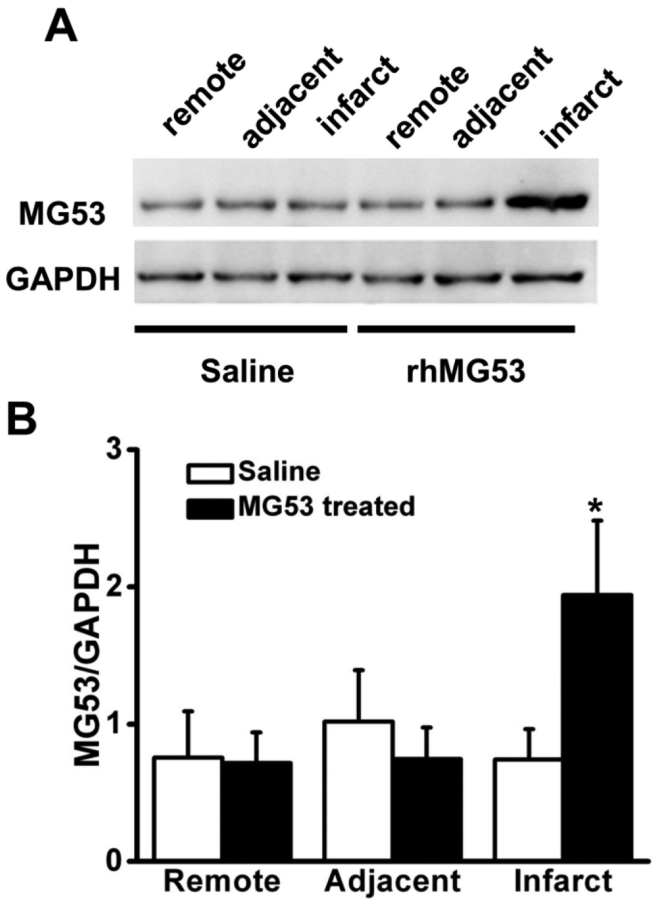

Figure 4. Concentration of rhMG53 at the infarct zone of the porcine heart

(A) Twenty-four hours after I/R injury, immunoblot of MG53 of myocardial tissues derived from the different areas of the pig heart receiving saline (as control) or rhMG53 (prior to ischemia). While MG53 levels in remote and adjacent areas are similar between control and rhMG53 treated groups, the MG53 level in the infarct area was highly elevated. (**B**) Quantitative analyses showed significant concentration of rhMG53 at the infarct zone of the heart (n=3, * $p < 0.01$).

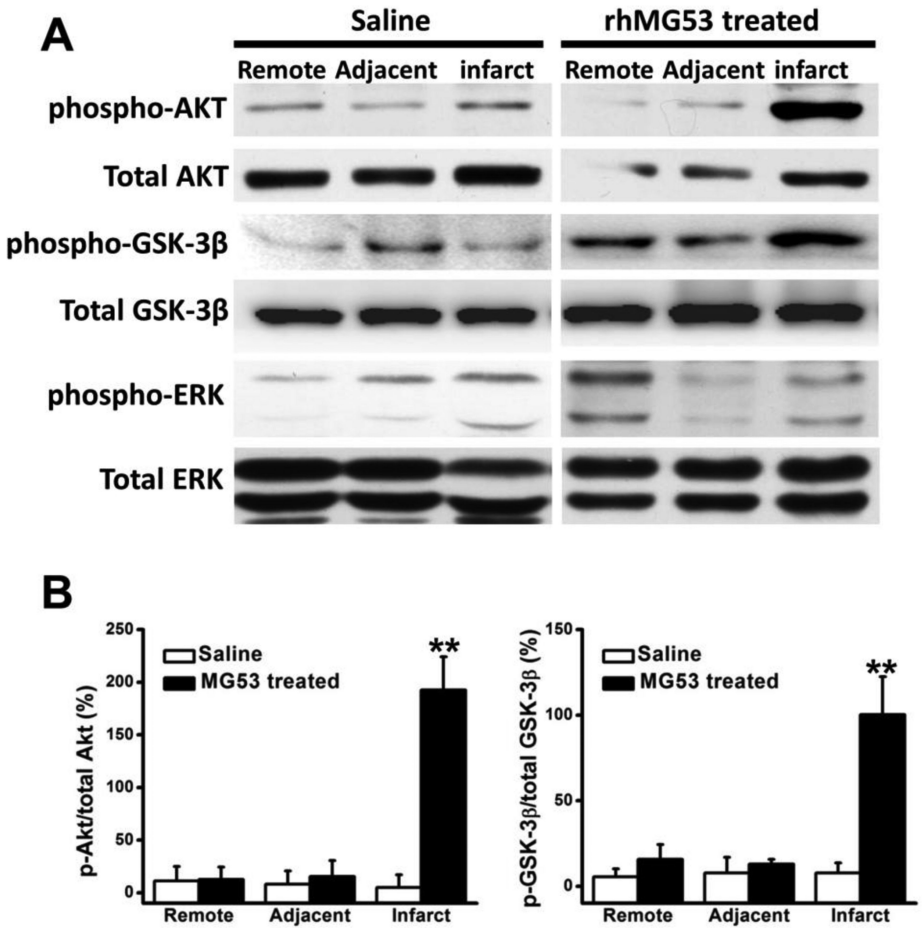

Figure 5. rhMG53 treatment activates AKT and GSK-3β survival signaling proteins in infarct zone of the hearts

(A) Twenty-four hours after I/R injury, phosphorylation of AKT and GSK-3β in different myocardial area derived from saline and rhMG53 treated groups were analyzed by Western blot. While the protein levels for AKT and GSK-3β in remote and adjacent areas were similar among control and rhMG53 treated groups, phosphorylation of AKT and GSK-3β was significantly elevated in the infarct zone of rhMG53 treated groups as compared to that in saline treated group. Phosphorylation of ERK1/2 showed no difference among different groups. (**B**) Quantitative analyses showed significant elevation of phosphorylation of AKT and GSK-3β at the infarct zone of hearts treated with rhMG53 (n=3, * $p<0.01$).

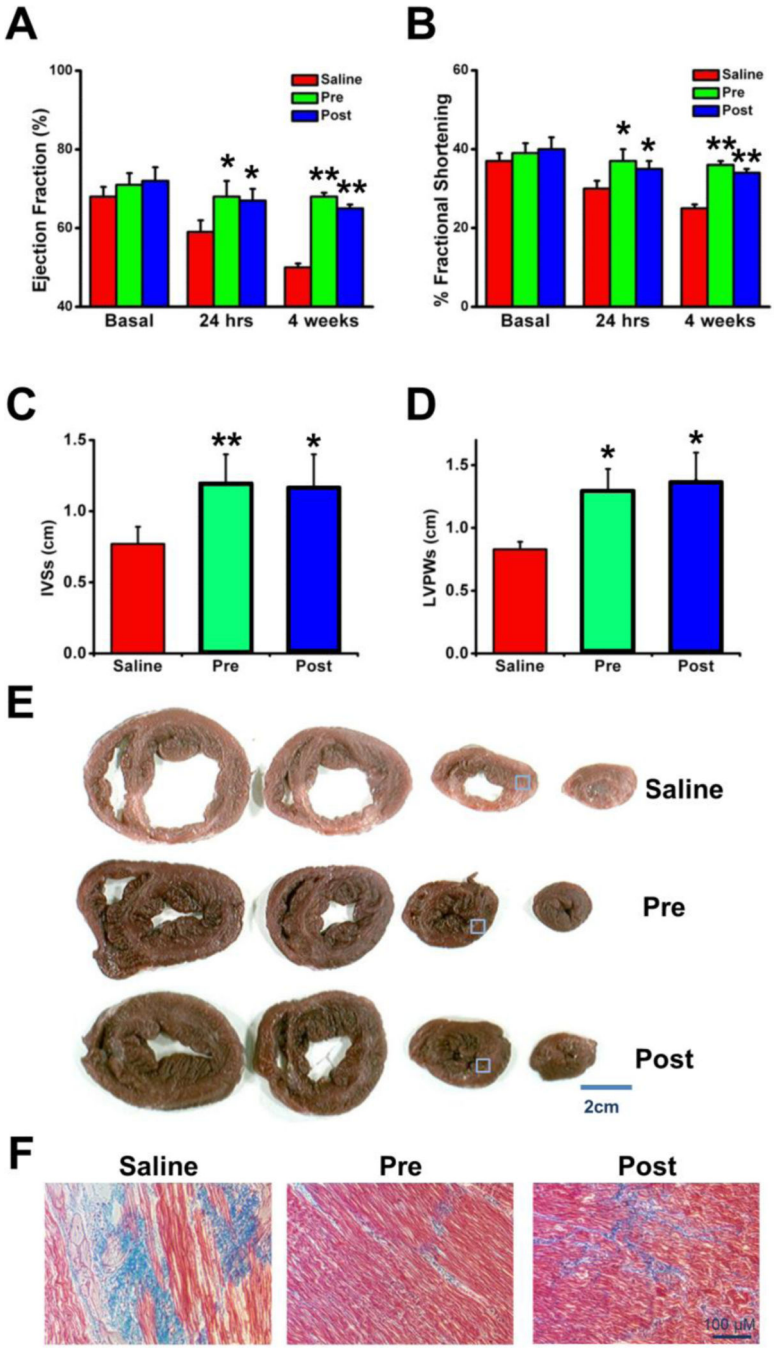

Figure 6. Sustained cardioprotective effects of rhMG53 in the porcine model of MI

Echocardiogram measurements of pigs following chronic MI at 4 weeks after undergoing coronary balloon angioplasty. The comparison of several echocardiographic parameters included the ejection fraction (EF) (**A**), the fractional shortening (FS) (**B**), the end diastolic interventricular septum (IVSs) (**C**), and the left ventricular posterior wall thicknesses (LVPWs) (**D**). Data represent mean ± SEM, n=4 per group. *P<0.05 and **P<0.01 by ANOVA. (**E**) Representative images of heart slices obtained from pigs at 4 weeks after coronary balloon angioplasty. Fibrotic myocardial tissue does not stain with NB-T and

appeared as pale color. Application of rhMG53 at dose of 1 mg/kg prior to ischemia (Pre) or prior to reperfusion (Post) improved heart morphology when compared with those receiving saline as control. Similar observation was seen in other experimental pigs (see Supplemental **Fig. S5**). (**F**) Masson's Trichrome staining reveals rhMG53 treatments reduce fibrosis in pig hearts. Paraffin embed heart sections derived from the infarct area were obtained from pigs at 4 weeks post I/R injury.