Cardioprotection of Ischemia/Reperfusion Injury by Cholesterol-Dependent MG53-Mediated Membrane Repair

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Rationale: Unrepaired cardiomyocyte membrane injury causes irreplaceable cell loss, leading to myocardial fibrosis and eventually heart failure. However, the cellular and molecular mechanisms of cardiac membrane repair are largely unknown. MG53, a newly identified striated muscle-specific protein, is involved in skeletal muscle membrane repair. But the role of MG53 in the heart has not been determined.

Objective: We sought to investigate whether MG53 mediates membrane repair in cardiomyocytes and, if so, the cellular and molecular mechanism underlying MG53-mediated membrane repair in cardiomyocytes. Moreover, we determined possible cardioprotective effect of MG53-mediated membrane repair.

Methods and Results: We demonstrated that MG53 is crucial to the emergency membrane repair response in cardiomyocytes and protects the heart from stress-induced loss of cardiomyocytes. Disruption of the sarcolemmal membrane by mechanical, electric, chemical, or metabolic insults caused rapid and robust translocation of MG53 toward the injury sites. Ablation of MG53 prevented sarcolemmal resealing after infrared laser–induced membrane damage in intact heart, and exacerbated mitochondrial dysfunction and loss of cardiomyocytes during ischemia/reperfusion injury. Unexpectedly, the MG53-mediated cardiac membrane repair was mediated by a cholesterol-dependent mechanism: depletion of membrane cholesterol abolished, and its recovery restored injury-induced membrane translocation of MG53. The redox status of MG53 did not affect initiation of MG53 translocation, whereas MG53 oxidation conferred stability to the membrane repair patch.

Conclusions: Thus, cholesterol-dependent MG53-mediated membrane repair is a vital, heretofore unappreciated cardioprotective mechanism against a multitude of insults and may bear important therapeutic implications. (Circ Res. 2010;107:00-00.)

Key Words: membrane repair • MG53 • cholesterol • ischemia/reperfusion injury • heart

In eukaryotic cells, the plasma membrane partitions a ~10,000-fold Ca2+ gradient and prevents loss of vital intracellular constituents, thus representing the last line of defense for cell integrity, homeostasis, and function. Physical, chemical or metabolic disruption of the plasma membrane leads to a repair-or-die emergency of the cell. Although the natural tendency to reseal the lipid biomembrane acts constitutively, recent studies indicate that plasma membrane disruption requires active emergency response mechanisms to mend the broken membrane.1 In the heart, plasma membrane repair is of particular importance because cardiomyocytes are terminally differentiated cells, displaying only very limited self-renewal capability.2 Cardiomyocytes undergo transient membrane injuries that occur as accidents under physiological conditions and can be exacerbated by various pathophysiological stresses.3 Progressive necrotic and apoptotic cell death causes onset of myocardial fibrosis and undermines cardiac contractile and electrophysiological performance, ultimately leading to heart failure.4,5

Ironically, little was known about cardiac membrane repair until recently when several molecular components in striated muscles were identified.6–10 In particular, 2 muscle-specific proteins, dysferlin7 and MG53,9 have been implicated in the repair of sarcolemmal membrane in skeletal muscles. Similar to the skeletal muscle, dysferlin-deficient mice display defective cardiomyocyte membrane repair ability that is linked to increased susceptibility to cardiomyopathy,8 whereas the molecular function of dysferlin in membrane repair has not been fully elucidated. For example, it is unknown whether the formation of
a membrane repair patch is associated with translocation of dysferlin toward the injury site in cardiac muscle. Our previous studies have shown that MG53 is an essential component of membrane repair machinery in skeletal muscle, as MG53 ablation results in defective membrane repair and progressive skeletal myopathy. In addition, we have found that MG53 can interact with dysferlin and is required for dysferlin movement to the acute membrane injury sites. However, the role of MG53 in membrane repair and progressive skeletal myopathy. In addition, we have found that MG53 can interact with dysferlin and is required for dysferlin movement to the acute membrane injury sites. However, the role of MG53 in the heart has not been determined and cardiac membrane repair remains largely elusive.

Here, we investigate the cellular and molecular mechanism of MG53-mediated membrane repair in the heart and demonstrate its cardioprotective role against various insults. We show that genetic ablation of mg53 prevents membrane resealing and increases susceptibility to ischemia/reperfusion (I/R)-induced myocardial damage. Rapid and robust MG53 translocation toward the injury sites occurs in cardiomyocytes subjected to either local or global plasma membrane disruption, whereas physiological process of excitation-contraction coupling or membrane deformation is unable to trigger such MG53 translocation. Furthermore, we show that unfurled membrane cholesterol is indispensable in initiating MG53-mediated membrane repair, uncovering a novel signaling role of membrane cholesterol. As an important cardioprotective response to stress, the cholesterol-dependent MG53-mediated membrane repair may provide a valuable therapeutic target for the treatment of heart disease.

**Methods**

Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and protocols were approved by the Institutional Animal Care and Use Committee of Peking University accredited by AAALAC International. The mg53−/− mice were generated as described and wild-type (WT) littermate mice were used as controls. Single ventricular myocytes were enzymatically isolated from the hearts as described. Adenovirus containing GFP or mg53(C242A) mutant gene was used to infect cardiomyocytes. Plasma membrane injury was elicited mechanically, electrically, or chemically in cardiomyocytes. The whole-heart membrane repair assay using two-photon excitation microscopy was as described by Han et al. with minor modifications, and the I/R protocol used was as described previously.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**MG53 Mediates Membrane Repair in Intact Heart**

MG53 protein expression in mouse heart was detectable during embryonic development and was progressively increased during the first month after birth (Online Figure I, A). At the age of 12 to 14 weeks, abundant expression of MG53 was found in muscular walls of all 4 chambers (right and left atria and ventricles) (Online Figure I, B). To test whether MG53 participates in membrane repair in cardiac muscle, we used a membrane repair assay modified from Han et al (Figure 1) and a mouse model with genetic ablation of mg53. Langendorff-perfused beating hearts from mg53−/− and WT littermate mice were subjected to infrared laser irradiation applied to a band of 500 μm² (910 nm, 650 mW, 57 seconds). The process of membrane resealing was visualized by local accumulation of FM1-43, a membrane-impermeable dye that becomes fluorescent when it partitions into a membrane. In WT heart, time-lapse two-photon imaging revealed that local FM1-43 fluorescence leveled off after an initial rise following laser irradiation, indicative of a halt in entry of FM1-43 after membrane resealing. In contrast, mg53−/− mouse hearts exhibited a continuous, high-rate elevation of FM1-43 fluorescence over much broader bands at the injury site (Figure 1), indicating an impairment of membrane resealing. These results establish an important role of MG53 in membrane repair in intact heart.

**Deficiency of MG53 Exacerbates I/R-Induced Loss of Cardiomyocytes**

We next sought to determine the possible cardioprotective function of MG53-mediated membrane repair in the heart. In...
In this regard, mg53−/− mice at young age (12 to 14 weeks old) did not exhibit detectable changes in cardiac morphometrics and contractile functions when compared to WT littermate controls (Online Figure II). Histological failure to reveal any significant myocardial pathology, either (Online Figure III). The absence of overt cardiac phenotypes in young mg53−/− mice raised under normal laboratory conditions is consistent with the finding in dysferlin-deficiency mice and supports the hypothesis that membrane repair serves as an emergency response against detrimental stimuli to the heart.

To assess potential MG53-mediated cardioprotection under stress conditions, we subjected the Langendorff-perfused mouse hearts to an I/R protocol (30 minutes/30 minutes) and assessed cardiac damage in the presence and absence of MG53-mediated membrane repair. Because loss of mitochondrial membrane potential (∆Ψm) is an early event of cell death,13,14 we used ∆Ψm as a sensitive readout of cardiomyocyte damage and performed simultaneous confocal measurement of tetramethyl rhodamine methyl ester (TMRM) fluorescence (index for ∆Ψm) and Evans blue dye (EBD) uptake (index for loss of membrane integrity) (Figure 2A). Before the I/R treatment, there was a trend of increased number of cardiomyocytes showing a loss of ∆Ψm in mg53−/− compared to WT mouse hearts (10.15±0.95% in mg53−/− versus 5.54±2.00% in WT, P>0.05, n=6 hearts with 220~250 image frames for each group), but the difference was not statistically significant (Figure 2B). Confocal microscopy revealed that, after the I/R injury, local multicellular areas showing complete loss of ∆Ψm were interspersed with areas of intact ∆Ψm, giving rise to a sharply delimited mosaic pattern (Figure 2A). Importantly, whereas the I/R injury caused a significant increase in the number of ∆Ψm-lost cardiomyocytes in both mg53−/− and WT mouse hearts, the number of ∆Ψm-lost cardiomyocytes in mg53−/− hearts was greater than that in WT controls by 140% (Figure 2B), indicating a cardioprotective role of MG53. Detailed analysis further revealed that a higher percentage of cardiomyocytes that had lost ∆Ψm was EBD-positive in the absence of MG53 (69.62±5.94% in mg53−/− versus 44.23±8.18% in WT, P<0.05, n=6 hearts with 220~250 image frames for each group) (Figure 2B), suggesting that MG53 retards the transition from reversible to irreversible cardiomyocyte damage.

Furthermore, we used conventional assessments to evaluate the cardioprotective effect of MG53 in response to I/R injury. As shown in Online Figure IV, MG53 ablation increased infarct size (42.32±3.06% in mg53−/− versus 31.73±2.11% in WT, assessed in Langendorff-perfused heart subjected to global I/R injury, P<0.05, n=6 hearts for each group), TUNEL-positive nuclei (15.64±1.60% in mg53−/− versus 9.14±1.17% in WT, P<0.01, n=6 hearts for each group) and also lactate dehydrogenase release by 2-fold. Importantly, plasma membrane translocation of endogenous MG53 did occur when the intact heart was subjected to I/R insult (Figure 2C), suggesting that MG53-mediated repair mechanism is activated by this stress.

**Figure 2. MG53 deficiency exacerates I/R-induced loss of mitochondrial membrane potential (ΔΨm) and cardiomyocyte death.** A, Simultaneous confocal imaging of ΔΨm (TMRM, shown in green) and cardiomyocyte death (EBD uptake, shown in red) in Langendorff-perfused heart after I/R injury. Note that all ΔΨm-intact cells are EBD-negative, and all EBD-positive cells show loss of ΔΨm but not vice versa. Scale bars: 50 μm. B, Statistics. From left to right, percentage of ΔΨm-lost cells before and after I/R, EBD-positive cells after I/R, and EBD-positive cells among ΔΨm-lost cells. Data are expressed as means±SEM; n=6 hearts with 220~250 image frames for each group. **P<0.01 before vs after I/R; #P<0.05 before vs after I/R; *P<0.01 KO vs WT after I/R; **P<0.01 before vs after I/R. C, MG53 immunostaining in heart slices. Note the translocation of endogenous MG53 to the sarcolemma in WT heart after I/R injury and the absence of MG53 immunofluorescence in KO heart. Scale bars: 10 μm.

**MG53 Translocation During Acute Membrane Injury**

As shown in Figure 2C, MG53 translocated and formed extensive patches on the sarcolemma in clusters of cardiomyocytes in WT heart after the I/R injury, supporting that MG53 plays a crucial role in mending broken membrane. To investigate the underlying mechanism, we used adenoviral expression of a MG53 fusion protein with an amino-terminal GFP (GFP-MG53) in cultured rat cardiomyocytes (Online Figure V). Under the present experimental conditions, GFP-MG53 was expressed at a level similar to that of endogenous MG53 (Online Figure V, A), and high-resolution confocal microscopy allowed for real-time visualization of intracellular GFP-MG53 trafficking. Figure 3A shows that focal electroporation of the sarcolemmal membrane caused rapid enrichment of GFP-MG53 at the wound site. Local GFP-MG53 fluorescence rose biexponentially with time constants of 12.0 and 166 s, respectively (Figure 3B and Online Video I). Mechanical injury by microelectrode penetration of the plasma membrane also triggered GFP-MG53 translocation to the injury site, and repetitive injuries at different locations...
resulted in multiple patches of enriched GFP-MG53 (Online Figure VI), indicating that MG53 translocation ensues wherever and whenever the plasma membrane is disrupted.

Global membrane permeabilization by a chemical detergent, saponin (50 μg/mL), induced robust GFP-MG53 translocation over the entire sarcolemmal membrane (Figure 3C and Online Video II). The gain of GFP-MG53 at the membrane mirrored a near-complete loss of GFP-MG53 in the cytoplasm, both displaying a time constant of 4.4 seconds (Figure 3D). This result indicates that most of the GFP-MG53 molecules are available and responsive when damage occurs at the sarcolemmal membrane of the cardiomyocytes. In GFP-expressing cells, however, complete loss of GFP following chemical permeabilization occurred without any appreciable staining of the sarcolemmal membrane (Figure 3C and Online Video III). Similar results were obtained with another membrane disruptive reagent, Triton X-100 (0.1% vol/vol) (Online Figure VII). Notably, immunofluorescence of native MG53 was concentrated at the damaged plasma membrane in a manner similar to that of GFP-MG53 (Figure 3E). Furthermore, in GFP-MG53-expressing cells, the intrinsic fluorescence from GFP-MG53 overlapped with the anti-MG53 immunofluorescence with a colocalization coefficient of 0.91 ± 0.02 (n=9 cells). Similarly, immunofluorescence of endogenous MG53 was also found to concentrate at membrane injury sites wounded by electroporation (Online Figure VIII, A) and microelectrode penetration (Online Figure VIII, B). These results corroborate that GFP-MG53 and endogenous MG53 behave similarly during the process of membrane translocation.

Furthermore, consistent with Figure 2C, hypoxia-reoxygenation treatment on cardiomyocytes induced membrane translocation of GFP-MG53 (Online Figure IX), substantiating that MG53-mediated membrane repair protects heart from I/R induced injury.

Figure 3. MG53 translocates to injured sarcolemma in cardiomyocytes. A, Patchy GFP-MG53 translocation induced by focal electroporation via a pair of microelectrodes (inset). B, Time course of GFP-MG53 accumulation at the electroporation site. Arrowhead indicates time of electroporation. Normalized fluorescence F/F₀, where F₀ refers to the level before electroporation, is expressed as mean ± SEM; n=9 cells. C, Subcellular distribution of GFP and GFP-MG53 before and after treatment with 50 μg/mL saponin. D, Sarcolemmal and cytosolic GFP-MG53 during saponin permeabilization. Arrowhead denotes time of saponin administration. Data are expressed as means ± SEM; n=5 cells. E, MG53 immunostaining in Ad-GFP– or Ad-GFP-MG53–infected cells after saponin treatment. Note that GFP fluorescence (shown in green) is completely lost in Ad-GFP infected cells. In cells expressing GFP-MG53, distributions of GFP fluorescence from GFP-MG53 and of TRITC immunofluorescence (shown in red) from native and GFP-fused MG53 are virtually identical. Scale bars: 10 μm.

MG53 Translocation Requires Membrane Disruption

What is the exact signal that is sensed by MG53 and promotes its plasma membrane translocation? To this end, the above experiments in Figure 3 were performed in a Ca²⁺-free external solution (with the benefit of preventing cell contracture after membrane damage), suggesting that Ca²⁺ entry is not a prerequisite for MG53 translocation in cardiomyocytes, as is the case in skeletal muscle.⁹ To further test whether intracellular Ca²⁺ release participates in regulating MG53 translocation, we used caffeine (20 mmol/L) to deplete the intracellular sarcoplasmic reticulum Ca²⁺ store before cell wounding. Figure 4A shows that GFP-MG53 translocation induced by saponin treatment remained intact even in the absence of intracellular Ca²⁺ release. Conversely, we also found no accumulation of GFP-MG53 at the sarcolemmal membrane despite physiological Ca²⁺ transients and cell contraction elicited by electric pacing at 1 or 5 Hz (Figure 4B). Taken together, we conclude that neither Ca²⁺ entry nor intracellular Ca²⁺ transients are necessary or sufficient for GFP-MG53 translocation. This finding reinforces the idea that MG53 translocation is an early event before Ca²⁺-dependent vesicle trafficking and membrane fusion steps in the membrane repair cascade.¹⁵–¹⁸
Further studies showed that membrane stretching or altered membrane curvature does not result in MG53 translocation to the cell membrane. Here we used a patch pipette to create the “I” shaped membrane deformation by negative pressure, as is the practice during the formation of a gigaseal for electrophysiological recording. No GFP-MG53 localization was evident in spite of the sharp membrane deformation and local stretch stress (Figure 4C). Only after the membrane was ruptured by suction did GFP-MG53 translocate to membrane at the patch pipette tip (Figure 4C). Thus, these data show that plasma membrane disruption is a prerequisite for membrane translocation of MG53.

Oxidation Confers Stability to the MG53 Repair Patch in Cardiomyocytes

Our previous study in skeletal muscle has shown that entry of extracellular oxidants is necessary for MG53 translocation, and the cysteine residue of 242 amino acid of MG53 serves a redox-sensor for MG53 oligomerization or crosslinking. In this study, we extended this finding by showing that redox regulation exerts differential effects at different phases of MG53 translocation in cardiomyocytes. Specifically, application of the membrane permeable oxidant H2O2 with different concentrations (0.1, 0.5, 1 mmol/L) failed to recruit GFP-MG53 to the plasma membrane (Online Figure X), even at a high dose (5 mmol/L) that caused cell damage and contracture (Figure 5A). Inclusion of the reducing reagent dithiothreitol (DTT) did not affect the initial GFP-MG53 translocation, but nascent GFP-MG53 matrix was quickly dissociated from the membrane in the presence of DTT (Figure 5B), suggesting that oxidation is required to stabilize the MG53 repair patch. To further investigate this phenomenon, we expressed GFP-MG53(C242A) mutant (the cysteine of 242 amino acid was mutated to alanine),
which lacks the redox-sensing ability,\(^9\) to a MG53-null background of cardiomyocytes from \(\textit{mg}53\)–/– mouse heart (to avoid possible interference of endogenous MG53). In response to saponin-induced membrane disruption, GFP-MG53(C242A) robustly translocated to the membrane but was unable to form a stable repair patch (Figure 5C), as was the case with DTT treatment. Hence our data suggest that initial movement of MG53 on membrane disruption is redox-independent, whereas oxidative regulation of MG53 at cysteine of 242 amino acid plays a critical role in stabilizing the MG53 repair patch.

**Membrane Cholesterol Is Necessary to Signal MG53 Translocation**

Given that MG53 discriminates between intact and injured membrane, we hypothesized that MG53 interacts with the hydrophobic core of the lipid bilayer membrane, which is exposed only in the event of membrane disruption. In this regard, cholesterol came into the picture because it is embedded in the hydrophobic core and accounts for \(\approx 40\text{%}\) of the lipid molecules of the plasma membrane in animal cells.\(^9\) Besides, the majority (\(\approx 90\text{%}\)) of it is enriched in plasma versus organelle membranes.\(^9,20\) We therefore tested the hypothesis that membrane cholesterol, when unfurled, signals MG53 to translocate to the membrane.

Staining cholesterol with filipin uncovered a dense colocalization of GFP-MG53 and cholesterol to the membrane site injured by electrode penetration, but an absence of GFP-MG53 in intact cholesterol-rich plasma membrane (Figure 6A). Strikingly, when membrane cholesterol was depleted with methyl-\(\beta\)-cyclodextrin (M-\(\beta\)-CD), the GFP-MG53 translocation after focal electroporation was completely abolished (Figure 6B and 6C). The effects of Triton X-100 as a membrane-disrupting reagent were similar to that of electroporation (Figure 6B). Restoration of cholesterol by incubation the cells with M-\(\beta\)-CD complexed with cholesterol largely revived the GFP-MG53 translocation to damaged membranes (Figure 6B and 6C).

Because cholesterol is particularly enriched in the membrane microdomains called lipid rafts and caveolae,\(^21,22\) we investigated whether such substructures are required for cholesterol regulation of MG53 translocation. By filipin disruption of the lipid rafts and caveolae without depleting membrane cholesterol, we found that GFP-MG53 translocation to membrane damage sites remained unaffected (Figure 6B and 6C), suggesting that MG53 recognizes unfurled membrane cholesterol regardless of the integrity of lipid rafts and caveolae. Thus, membrane cholesterol is obligatory for MG53 translocation, and exposure of membrane cholesterol likely acts as an initial step of the MG53-mediated membrane repair.

**Discussion**

**Cardioprotection by MG53-Mediated Membrane Repair**

As a muscular pump undergoing constant contractile activity, the heart is an organ under intense mechanical stress with high metabolic demands. As a result, cardiomyocytes are under constant threat of mechanical injury, oxidative stress, and metabolic insult. Because cardiomyocytes cannot be replaced in large numbers, lost cardiomyocytes are replaced by fibroblasts, resulting in myocardial fibrosis in response to a number of etiologies, including myocardial infarction caused by coronary heart disease. Thus, the heart must develop intrinsic defensive mechanism to survive such injuries by preventing cardiomyocytes death. In this regard, the present study has identified MG53 as a potent cardiac protector: MG53 ablation exacerbated the I/R-induced myocardial damage, as manifested by markedly worsened mitochondrial dysfunction and loss of cardiomyocytes.
Several lines of evidence have demonstrated that cardioprotection by MG53 is mediated, at least in part, by its membrane repair function. First, we have shown that local or global membrane disruption evokes MG53 to translocate exclusively to the injured membrane in cardiomyocytes. Second, a membrane repair assay using FM1-43 fluorescence revealed that MG53 deficiency impaired membrane resealing after two-photon irradiation damage in intact heart. That MG53 is inert to physiological Ca\textsuperscript{2+} transients and cardiac contraction is also consistent with the idea that MG53 is normally a bystander but plays a key role in the emergency response to rescue the cell from disastrous membrane disruption.

One other muscle specific protein thought to contribute to this membrane repair response in striated muscle is dysferlin.\textsuperscript{6–8} Dysferlin has been shown to be essential for membrane repair in skeletal muscle;\textsuperscript{7} however it requires the function of MG53 to translocate to sites of membrane injury.\textsuperscript{11} Thus, MG53 can nucleate the assembly of repair machinery at sites of membrane damage, whereas dysferlin likely operates at a downstream stage of the membrane resealing process. Our findings that MG53 is important in protecting the heart from I/R injury is in contrast to previous report that dysferlin is not sufficient to trigger the MG53 translocation process. As highly hydrophobic, compactly ring-rigid structure, cholesterol is an indispensable molecular player for the initiation of membrane repair.\textsuperscript{11} Here we have identified yet another fundamental role of membrane cholesterol in signaling membrane repair. Depletion of membrane cholesterol abolished, and its recovery restored MG53 translocation on membrane electroporation or chemical permeabilization. Remarkably, cholesterol regulation of MG53 translocation remained intact even after filipin disruption of membrane lipid rafts or caveolae, suggesting that cholesterol-mediated hydrophobic interaction between MG53 and the disrupted membrane is not confined to specialized membrane microdomains. Our previous study in skeletal muscle showed that MG53 mediates membrane repair through direct interaction with membrane phosphatidylserine,\textsuperscript{9} here we further found that cholesterol, as another membrane lipid, is obligatory for MG53-mediated membrane repair. Because MG53 can interact with phosphatidylserine directly but not cholesterol in the lipid profiling assay,\textsuperscript{9} cholesterol-dependent secondary membrane structure or other yet-to-be identified partner may contribute to this role of cholesterol.

In determining whether the cholesterol-dependent MG53 translocation is sensitive to redox regulation as promoted by studies in skeletal muscle,\textsuperscript{9} we demonstrated 2 steps for formation of the MG53 repair patch in cardiomyocytes: trigger of MG53 translocation and stabilization of the translocated MG53. The initial MG53 translocation to membrane injury sites is redox-independent, but MG53 oxidation at cysteine of 242 amino acid appears to be necessary for stabilizing the nascent MG53 repair patch. In skeletal muscle, the initial MG53 translocation phase appears to be very brief or the MG53 repair patch is very unstable, such that MG53 translocation was seen as only a small blip in the time course plot under reducing conditions (see figure 4f and 4g in the article by Cai et al\textsuperscript{9}). Also, the Ca\textsuperscript{2+} independence of MG53 translocation in cardiomyocytes is consistent to that in skeletal muscle which showed that extra-cellular Ca\textsuperscript{2+} entry is not essential for MG53 translocation to membrane injury sites (see figure 6e and 6f in the article by Cai et al\textsuperscript{9}).

Intuitively, this cholesterol dependence of MG53 translocation serving to initiate cardiac membrane repair would be robust and fool-proof, because membrane cholesterol is exposed if and only if the membrane is disrupted. Given the abundance of membrane cholesterol, it confers one additional advantage of efficiency to membrane repair. Both robustness and effectiveness are important features in a situation where speed and fidelity of the response is a matter of survival of the cell.

In summary, we have shown that MG53 membrane translocation constitutes the initial step of repairing disrupted cardiomyocyte plasma membrane in the heart. Mechanistically, membrane cholesterol exposed in the disrupted membrane signals MG53 translocation in a redox-independent manner, whereas MG53 oxidation confers stability to the repair patch. As a result, cholesterol-dependent MG53-mediated membrane repair pro-

Role of Membrane Cholesterol in MG53-Mediated Membrane Repair

In searching for the exact signal that triggers MG53 translocation, we have demonstrated, for the first time, that membrane cholesterol is an indispensable molecular player for the initiation of MG53 translocation in cardiac membrane repair. As highly hydrophobic, compactly ring-rigid structure, cholesterol is an important component of the plasma membrane. It is embedded in the hydrophobic core of the lipid bilayer, and thus acts to rigidify the membrane, reduce the passive permeability, and increase the mechanical durability.\textsuperscript{21} Recent investigation of lipid rafts has unraveled an important role of cholesterol in assembling these membrane microdomains critical for the regulation of signal transduction and membrane trafficking.\textsuperscript{21,22}
ects heart from loss of mitochondrial function and subsequent irreplaceable loss of cardiomyocytes under stress conditions such as myocardial I/R injury. These findings uncover an important heretofore unappreciated cardioprotective mechanism that involves cardiac membrane repair, and might help to develop therapeutic strategies to ameliorate cardiomyopathy and to retard, or potentially reverse, the progression of heart failure.

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Disclosures
None.

References

Novelty and Significance

What Is Known?
- Plasma membrane repair is an active emergency response to mend broken membrane.
- MG53 is an essential component of membrane repair machinery in skeletal muscle.
- Deficiency of dysferlin-mediated membrane repair leads to cardiomyopathy.

What New Information Does This Article Contribute?
- MG53 plays crucial roles in membrane repair response of heart.
- MG53-mediated membrane repair protects the heart from ischemia/reperfusion injury.
- Cholesterol is necessary for signaling MG53-mediated membrane repair response.

Cardiomyocytes undergo membrane injury happens under physiological conditions and this injury could be exacerbated by pathophysiological stress. Unrepaired cardiomyocyte membrane injury causes irreparable cell loss, leading to myocardial fibrosis and eventually heart failure. However, the cellular and molecular mechanisms of cardiac membrane repair are largely unclear. In this study, we demonstrate that MG53, a newly identified striated muscle-specific protein, constitutes an essential component of the membrane repair machinery in heart. MG53-mediated membrane repair serves as a powerful cardio-protection mechanism in preventing ischemia-reperfusion injury. Furthermore, we find that the redox status of MG53 does not affect initiation of MG53 translocation, whereas MG53 oxidation confers stability to the membrane repair patch. Unexpectedly, we reveal a crucial role of membrane cholesterol in signaling MG53 to mend the broken membrane. We uncover a new role of plasma cholesterol and, for the first time, link cholesterol, MG53, and membrane repair with endogenous mechanisms defending against the life-threatening cardiac accident. As an important cardioprotective response to stress, the cholesterol-dependent MG53-mediated membrane repair may provide a valuable therapeutic target for the treatment of heart disease.
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SUPPLEMENTAL MATERIAL

Detailed Methods

Adult Cardiomyocyte Isolation, Culture, and Adenovirus Infection

Animals were treated in compliance with the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and protocols were approved by the Institutional Animal Care and Use Committee of Peking University accredited by AAALAC international. The *mg53*-/- mice were generated as described ¹ and wild type (WT) littermate mice were used as controls. Single ventricular myocytes were enzymatically isolated from the hearts of adult male Sprague–Dawley rats (200–250 g) or of adult male mice (12-14 weeks), as described previously.² Freshly isolated cardiomyocytes were plated on culture dishes coated with laminin (Sigma) for 1 hour and then the attached cells were cultured in M199 medium (Sigma) added with (in mmol/L) 5 creatine, 2 L-carnitine, 5 taurine, 25 Hepes (all from Sigma), and insulin-transferrin-selenium-X (Gibco). Cardiomyocytes were then infected with adenovirus carrying the target gene at an m.o.i. of 20 and the efficiency of infection was nearly 100% as assessed by expression of fluorescent proteins after 60 - 72 hours culture.

Recombinant Adenovirus Production

The *mg53* or *mg53(C242A)* mutant gene was cloned and inserted into pEGFP-C1 vector (Clontech), and then the *gfp-mg53* or *gfp-mg53(C242A)* fusion gene was cloned and constructed into pShuttle-CMV vector (Strategene). The adenovirus was produced using the AdEasy system (Strategene). Briefly, the recombinant plasmid pShuttle-CMV-*gfp-mg53* or pShuttle-CMV-*gfp-mg53(C242A)* was transformed into BJ5183-AD competent cells by electroporation to produce recombinant Ad plasmid. The adenovirus was produced in HEK293A cells by transfection of the recombinant Ad plasmid. The adenovirus containing the *gfp* gene alone served as control.

Manipulation and Staining of Membrane Cholesterol

Cholesterol depletion was carried out by incubation of cardiomyocytes with 5 mmol/L methyl-β-cyclodextrin (M-β-CD) (Sigma) for 1 hour at 37°C, as previously described.³,⁴ For cholesterol recovery, membrane cholesterol-depleted cardiomyocytes were incubated with 2.5 mmol/L M-β-CD complexed with 0.25 mmol/L cholesterol (Sigma) for 1 hour at 37°C. Disruption of cholesterol-sphingolipid rafts and caveolae without loss of membrane cholesterol was achieved by incubation with 2 μg/ml filipin (Sigma).⁵,⁶ To visualize cholesterol, cells were stained with filipin as described.⁷ Briefly, cardiomyocytes were fixed with 4% paraformaldehyde for 30 min and subsequently incubated with 50 μg/ml filipin for 1 hour at room temperature (20-22°C). The filipin fluorescence was then viewed under Zeiss LSM510 microscope equipped with a CCD camera and UV filter set (Zeiss).

Membrane Injury

In cardiomyocytes, plasma membrane injury was elicited mechanically, electrically, or chemically. For chemical membrane injury, cardiomyocytes were treated with either 50 μg/ml saponin (Sigma), which aggregates membrane cholesterol and makes pores to the membrane, or 0.1% (v/v) Triton X-100 (Sigma), a non-ionic detergent which dissolves the lipid bilayer. To electroporate the plasma membrane locally, a pair of patch pipettes (tip diameter ~2 μm) were placed within 2 μm of the cell surface and a 25-ms square-wave DC current of 50 μA was applied to effect focal electroporation. Mechanical
membrane injury was produced either by rapid penetration of the patch pipette 3-5 µm into the cell or, under the patch-clamp conditions, by rupture of the patched membrane with strong suction. See below for membrane damage induced by infrared laser irradiation in intact heart.

Confocal Imaging

A Zeiss LSM510 confocal microscope (Zeiss) was used for single-cell and whole-heart imaging (not including quantitative whole-heart membrane repair experiments). To alleviate cell contracture, single-cell membrane injury experiments were performed in Ca²⁺-free Tyrode’s solution containing (in mmol/L) 134 NaCl, 4 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 10 HEPES, 11 D-glucose (pH 7.4), unless specified otherwise. GFP, GFP-MG53 and GFP-MG53(C242A) were excited at 488 nm and the fluorescence emission was collected at >505 nm. For simultaneous measurement of intracellular Ca²⁺ concentration ([Ca²⁺]), cardiomyocytes were loaded with 5 µmol/L rhod-2 AM (Invitrogen) for 10 min, rinsed twice and then bathed in Tyrode’s solution with 1 mmol/L CaCl₂. Field stimulation (1 or 5 Hz)-elicited [Ca²⁺] transients were measured as the self-ratio of rhod-2 fluorescence ΔF/F₀, where F₀ refers to diastolic fluorescence, and fractional shortening was derived from edge-tracking analysis of confocal line scan image of the cell. In this experiment, GFP-MG53 and rhod-2 were excited at 488 and 543 nm, and their fluorescence emission was collected at 505-530 and >560 nm, respectively. All experiments were done at a room temperature of 20–22°C.

Adult mouse heart (12-14 weeks old) was excised, and the ascending aorta was cannulated with a customized needle. The heart was perfused in the Langendorff configuration under constant perfusion pressure (1000 mm H₂O) with oxygenated (100% O₂) Tyrode’s solution (pH 7.4, at 37°C). The Langendorff-perfused heart was placed on the stage of the confocal microscope and the images were taken ~30 µm deep into the epicardium of left ventricle. Motion artifacts due to spontaneous beating of the heart (~480/min) were minimized by 10 µmol/L blebbistatin (Sigma). After a 20-min stabilization period, the heart was perfused with 500 nmol/L tetramethyl rhodamine methyl ester (TMRM) (an indicator of mitochondrial membrane potential ΔΨm; Invitrogen) and 0.1% evans blue dye (EBD) (an indicator of plasma membrane integrity). The ischemia-reperfusion (I/R) protocol used was as described previously.8 Ischemia (30 min) was achieved by clamping the perfusion line and reperfusion (30 min) by releasing the clamp. Confocal images were taken from multiple, randomly selected regions prior to and after the I/R, at excitation wavelengths of 543 and 633 nm and emission collection wavelengths of 565-595 and >650 nm for TMRM and EBD, respectively.

All the above observations were executed under a 40x, 1.3NA oil immersion objective. For different excitation, the sources of lasers are respectively: 405 nm, Diode laser, 25mW; 488 nm, Ar-laser, 30 mW; 543 nm, HeNe laser, 1 mW; 633 nm, HeNe laser, 5 mW. Images were acquired with 512 pixels per line and 512 lines per frame. For whole-heart imaging, pixel size was fixed at 0.64 µm; for single-cell imaging, pixel sizes were adjusted between 0.16 to 0.64 µm to accommodate different experimental needs. Scanning pixel dwell time is 2.56 µs. Pinhole was adjusted to keep the thickness of optical slices to be about 2 µm. For multi-color imaging, each fluorescence was excited and collected emission individually with switching per line between fluorescence channels.

Whole-heart Membrane Repair Assay with a Two-photon Imaging System

The membrane repair assay used two-photon excitation microscopy, as described by Han et al.9 Specifically, Langendorff-perfused heart stained with FM 1-43 (10 µmol/L included in the Tyrode’s solution; Invitrogen) was placed on the stage of a two-photon
laser-scanning microscope (Fluoview FV1000MPE, Olympus) equipped with a 60x, 0.8NA water immersion objective. Membrane damage was inflicted by 2,000 scans (~57 s) at full laser power at 910 nm (MaiTai BB, Spectra Physics; ~650 mW) over a narrow band of 3×400 pixels (~2 µm×250 µm) on the surface of the heart. Then, time-lapse images of FM 1-43 fluorescence (excited at 910nm with 2% laser power; collected at 510-550nm) were captured with 512×512 pixels for 1,000 s at 20 s intervals. The pixel size is about 0.62 µm and pixel dwell time is about 2 µs.

**Hypoxia-reoxygenation Treatment of Cardiomyocytes**
Cultured rat cardiomyocytes infected with GFP-MG53 adenovirus were resuspended in glucose-free Tyrode solution and exposed to hypoxia (95% N₂+5% CO₂) for 1 hour followed by reoxygenation (95% O₂+5% CO₂) for 2 hours. The location of GFP-MG53 fusion protein was visualized using Zeiss LSM510 confocal microscope at 488nm excitation and >505nm emission.

**Myocardial Infarct Size Measurement**
After ischemia-reperfusion (30min/120min) treatment, the mice Langendorff-perfused hearts were cut into 5-6 slices and stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution. Each slice was photographed and the infarct size was measured using Image J software. Infarct size was expressed as a ratio of the infarct area to the area at risk.

**Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) Assay**
TUNEL assay was performed on myocardial slices fixed in formaldehyde solution using a CardioTACS™ in situ apoptosis detection kit (#TA5353; R&D Systems Inc.). Apoptosis was determined as a ratio of the number of TUNEL positive nuclei to total nuclear number.

**Lactate Dehydrogenase (LDH) Determination**
The perfusion effluent at the time of 10-min reperfusion was collected and used for LDH release assessment using a LDH determination kit (Shanghai Gensource Co. Ltd.) according to the manufacturer’s instructions.

**Western Blotting**
Whole-tissue or cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Membranes were blocked with 5% non-fat dry milk and incubated with primary antibody overnight at 4°C. Polyclonal anti-MG53 antibody ¹ (1:5000 dilution to about 50ng/ml) and anti-GAPDH antibody (Santa Cruz) (1:2000 dilution to 100ng/ml) were used. Blots were visualized using secondary antibodies conjugated with IRDye (LI-COR) (1:2000 dilution) and an Odyssey imaging system (LI-COR).

**Immunofluorescence Assay**
Paraffin-embedded slices of heart tissue or cardiomyocytes were rinsed with PBS, and blocked with 10% normal goat serum. The sections were incubated with a polyclonal anti-MG53 antibody ¹ (1:5000 dilution to about 500ng/ml) for 2 hours at room temperature, washed with PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Santa Cruz) (1:100 dilution) or tetra rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Santa Cruz) (1:100 dilution) for 1 hour at room
temperature. The immunofluorescence staining was visualized using Zeiss LSM510 confocal microscope at 488 (FITC) and 543 nm (TRITC) excitation and 505-530 and >560nm emission.

**Echocardiography**

Echocardiography was performed in mice anesthetized with trichloroacetaldehyde monohydrate (0.5 g/kg) using a Doppler echocardiographic system equipped with a L15/6-MHz transducer (Sonos 5500, Philips Medical Systems, Andover, Massachusetts). The heart was first imaged using the two-dimensional mode in the parasternal long-axis and short-axis views. The short-axis views, at the level of papillary muscles, were used to position the M-mode cursor perpendicular to the ventricular septum and LV posterior wall. Measurements of the LV internal end-diastolic diameter (LVIDd) were taken at the time of the apparent maximal LV diastolic dimension, whereas measurements of the LV internal end-systolic diameter (LVIDs) were taken at the time of the most anterior systolic excursion of the posterior wall. LV ejection fraction (EF) was calculated by the cubic method: \( EF(\%) = \frac{((LVIDd)^3 - (LVIDs)^3)}{(LVIDd)^3} \times 100 \), and LV fractional shortening (FS) was calculated by \( FS(\%) = \frac{(LVIDd - LVIDs)}{LVIDd} \times 100 \). The data were averaged from 5 cardiac cycles.

**Statistics**

Digital image processing used IDL software (Research Systems) and custom-devised computer algorithms. Statistical data are expressed as mean ± s.e.m. and Student’s t test was applied to determine the statistical significance of the differences. A \( p \) value less than 0.05 was considered statistically significant.
Online Figure I. Expression of MG53 in heart. A, Western blotting analysis of cardiac MG53 expression during embryonic (E18, E19) and neonatal days (D1, D3), two-week (2W) and one-month (1M) after birth. B, MG53 expression in walls of left and right atria (LA, RA) and ventricles (LV, RV) in 12-14 weeks old mice.
Online Figure II. Echocardiography analysis of cardiac morphology and function in *mg53*-null (KO) and wild type (WT) littermate mice. IVSd and IVSs: intraventricular septal thickness in diastole and systole, respectively; LVIDd and LVIDs: left ventricular internal dimension in diastole and systole; LVPWd and LVPWs: left ventricular posterior wall thickness in diastole and systole; EF: ejection fraction; and FS: fractional shortening (FS). Data are expressed as mean ± s.e.m., n =6 for WT and n=6 for KO. Mice were 12-14 weeks old.
Online Figure III. Representative hematoxylin and eosin (H&E) stainings of heart sections from wild type (WT) and mg53-null (KO) mice under normal laboratory conditions. Scale bars: 20µm. Mice were 12-14 weeks old.
Figure A: Comparison of WT and KO hearts with infarct size (% of area at risk).

Figure B: TUNEL positive nuclei (% of total) in WT and KO.

Figure C: LDH release (U/L) before and after I/R in WT and KO.
Online Figure IV. MG53 ablation exacerbates ischemia-reperfusion (I/R) induced injury of Langendorff-perfused heart. A. Infarct size assessment. Left panel: typical TTC staining of wild type (WT) and mg53-null (KO) hearts subjected to I/R injury; right panel: statistical data, n=6 hearts for each group. *, p<0.05 KO versus WT. B. TUNEL staining data of WT and KO hearts subjected to I/R injury. N=6 hearts for each group. **, p<0.01 KO versus WT. C. LDH release of WT and KO hearts subjected to I/R injury. N=9 hearts for KO and n=6 hearts for WT. *, p<0.05 KO versus WT; #, p<0.05 after I/R versus before I/R for each group.
**Online Figure V. Expression of GFP-MG53 in cultured rat cardiomyocytes.** A, Western blot for MG53 in adult rat cardiomyocytes. Data were obtained 60-72 h after adenoviral infection of Ad-GFP or Ad-GFP-MG53 at m.o.i.=20. The polyclonal antibody used recognizes both GFP-MG53 and endogenous MG53. B, Confocal imaging for intracellular distribution of GFP-MG53. Note that GFP-MG53, but not GFP, was excluded from the nuclei. Scale bars: 10μm.
Online Figure VI. GFP-MG53 translocation induced by mechanical injury of the membrane.

Labels “1” and “2” denote sites injured by two consecutive microelectrode penetrations. Sale bars: 10μm.
Online Figure VII. GFP-MG53 translocation induced by Triton X-100 injury of the membrane. Data shown were at 4.7s after Triton X-100 (0.1%, v/v) administration. The GFP fluorescence in Ad-GFP infected cells was completely lost in another 10 s. Scale bars: 10 μm.
Online Figure VIII. Endogenous MG53 translocates to membrane injury sites.

Immunostaining of endogenous MG53 in cardiomyocytes injured by electroporation (A) or microelectrode penetration (B). Arrowheads denote the injury sites of sarcolemme. Scale bars: 10μm.
Online Figure IX. Translocation of GFP-MG53 to cardiomyocyte membrane in response to hypoxia-reoxygenation. Cardiomyocytes infected by Ade-GFP-MG53 were injured by hypoxia-reoxygenation treatment (60 min hypoxia by perfusing 95%N₂ + 5%CO₂ and 120 min reoxygenation by perfusing 95%O₂ + 5%CO₂). Cardiomyocytes with obvious membrane translocation of GFP-MG53 were marked by arrows. “Before” denotes before hypoxia-reoxygenation treatment; “After” denote after hypoxia-reoxygenation. Scale bars: 20 μm.
Online Figure X. Effect of H$_2$O$_2$ on the membrane translocation of GFP-MG53.

Cardiomyocytes were treated with different concentrations of H$_2$O$_2$ for 20 min.
Supplemental References


Legends for Video files

**Video I.** GFP-MG53 translocation to plasma membrane site of injury induced by focal electroporation.

**Video II.** Membrane translocation of GFP-MG53 in cardiomyocytes in response to saponin permeabilization.

**Video III.** Loss of GFP in cardiomyocytes in response to saponin permeabilization.