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# Amelioration of ischemia-reperfusion induced muscle injury by the recombinant human MG53 protein

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## Abstract

**Introduction—**Ischemia-reperfusion injury (I-R) in skeletal muscle requires timely treatment.

**Methods—**Rodent models of I-R injury were used to test the efficacy of recombinant human MG53 (rhMG53) protein for protecting skeletal muscle.

**Results—**In a mouse I-R injury model, we found that *mg53−/−* mice are more susceptible to I-R injury. rhMG53 applied intravenously to the wild type mice protected I-R injured muscle, as demonstrated by reduced CK release and Evans blue staining. Histochemical studies confirmed beneficial effects of rhMG53. Interestingly, rhMG53 did not protect against I-R injury in rat skeletal muscle. This was likely due to the fact that the plasma level of endogenous MG53 protein is high in rats.

**Discussion—**Our data suggest that rhMG53 may be a potential therapy for protection against muscle trauma. A mouse model appears to be a better choice than a rat model for evaluating potential treatments for protecting skeletal muscle.

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**Competing interests:**

JM founded TRIM-edicine, Inc, a university spin-off biotechnology company that is developing rhMG53 as therapeutic reagent for regenerative medicine. TT is an employee for TRIM-edicine, Inc.

**Authors' contributions:**

HZ, JH, JR, KP, TT, YZ, LL and CZ performed the experiments; HZ, JL, ZL, JM and TW conceived and designed the study; HZ, JR, KP, JM and TW analysed the research; HZ, JM and TW wrote the manuscript. All authors read and approved the final manuscript.

**Keywords**

muscle injury; TRIM72; tourniquet; creatine kinase; edema

## Introduction

Extremity trauma is a major form of injury in civilian life and in combat[1]. It often involves skeletal muscle ischemia-reperfusion injury (I-R) due to vascular damage [2] or development of extremity compartment syndrome [3], potentially leading to permanent loss of limb function, amputation, or even death[4]. I-R is a complex injury that results in vascular, neural, and muscular damage[5]. In myofibers, free radicals generated during I-R promote membrane damage[6], inciting a cascade of events that can lead to necrosis and apoptosis[4]. As such, increasing the antioxidant capacity of skeletal muscle prior to I-R injury is effective at mitigating tissue damage[7]. However, with trauma it may not be feasible to combat initial free radical production, raising the need to identify effective therapies directed at enhancing membrane repair[8].

Rats and mice have been used for many traumatic injury-related studies. Different responses to injuries between these 2 species have been observed in various models, including spinal cord injury[9,10], lung injury[11], liver injury,[12] and wound healing (observations from our laboratories). In general, rats are more resistant to injury and often recover quicker from injuries than do mice[12,10]. It is possible that rats might possess more efficient injury-repair machinery, but the exact mechanism(s) are largely unknown. Recently, Cai *et al.*[13] identified Mitsugumin 53 (MG53), a muscle-specific TRIM family protein, as an essential molecule in regulating cell membrane repair [13,14]. We further demonstrated MG53 knock-out mice develop progressive muscle weakness and defective muscle repair after exercise or injury[15]. To test the potential therapeutic application for MG53 in treatment of acute injury, we administrated recombinant human MG53 (rhMG53) to injured C2C12 myotubes or isolated myofibers *in vitro* and observed enhanced cell membrane repair[16]. Moreover, rhMG53 delivery to *mdx* and wild type mice improved the capacity to repair membrane damage caused by eccentric contractions or cardiotoxin[17,16]. Based on these observations, we hypothesized that delivery of rhMG53 would ameliorate skeletal muscle damage secondary to I-R injury. Using our standard rat tourniquet model we tested whether rhMG53 administration attenuated I-R in rats. Contrary to our expectations, histopathological measurements revealed similar muscle injury with or without the administration of rhMG53 in the rat model[18]. Interestingly, as part of the same study we found rhMG53 did improve C2C12 myotube viability upon $H_2O_2$ exposure *in vitro*[18], which was consistent with other reports that indicated a therapeutic effect of MG53 in cardiac I-R[19,20,16] and other forms of muscle injury in mice[21,16].

In this study, we provide evidence that administration of rhMG53 provides significant protection against I-R injury to skeletal muscle in the mouse model. Our data suggest that endogenous MG53 plays an important role in protecting skeletal muscle from traumatic insults, and that rhMG53 may be a potential therapeutic reagent for protection against skeletal muscle I-R.

## Methods

### Mouse Studies

Animal protocols involving mice were approved by the Ohio State University Animal Care and Use Committee. Mice (C57BL/6J, weight 25 grams, purchased from Jackson Lab) were anesthetized with isoflurane (2% isoflurane, oxygen flow rate at 1 liter per minute) throughout the ischemic period. Body temperature was maintained using a water perfused heating pad (HP3119, Hallowell, EMC)(Gaymar Heat Therapy Pump, #TP650) during the entire procedure. Ischemia was induced in the left hind limb using a tension-controlled tourniquet around the upper thigh. The tourniquet was composed of 2-0 silk, 3.0 metric suture (Ethicon, LLC); suture tension was controlled by a calibrated spring (400 g). Following 45 minutes of ischemia, the tourniquet was released for 24-hour reperfusion. In the first mouse experiment, I-R was induced in *mg53−/−* mice or littermate wild type controls. Plasma samples were collected at indicated time points for creatine kinase (CK) measurement. In a second series of experiments, the protective effect of rhMG53 administration was evaluated in adult male C57BL/6J mice. rhMG53 was prepared by dissolving lyophilized rhMG53 (provided by TRIM-edicine, Inc.) in sterile saline (2 mg/ml). It was administered via tail vein injection (6 mg/kg body weight) 5 min prior to tourniquet application and 5 min prior to release of ischemia. Sham animals received only sterile saline. Twenty-four hours after reperfusion, mice were euthanized, and muscles were harvested. Gastrocnemius (Gas) and tibialis anterior (TA) muscles were weighed before and after drying at ~50°C for 7 days, and the wet:dry ratio served as an index of edema. TA muscle sections were stained with H&E for pathological examination. Muscle fiber cell membrane integrity was determined histologically in TA by microscopic visualization of Evans blue dye (Sigma, E-2129) [EBD; 1% w/v; applied intraperitoneally (i.p.) 16 hours before injury] inclusion within damaged cells. EBD positive muscle fibers were counted, and the percentage was calculated by an individual blinded to the treatments.

### Rat Studies

Animal protocols involving rats were approved by the US Army Institute of Surgical Research Animal Care and Use Committee. This study adhered to National Institutes of Health guidelines for the care and use of laboratory animals (DHHS Publication, NIH, 86 to 23). Adult male Sprague-Dawley rats were administered either lyophilized rhMG53 dissolved in sterile saline or saline alone via tail vein injection (6 mg/kg body weight) 5 min prior to tourniquet application and 5 min prior to release. Both groups underwent 3 hour of pneumatic tourniquet induced I-R as previously described in detail [8]. Two days after injury, rats were euthanized and muscles were harvested. Muscle fiber cell membrane integrity was determined histologically in TA muscles following the same procedure described above. GAS muscle were weighed before and after drying at ~50°C for 7 days, and the wet:dry ratio served as an index of edema.

### Plasma levels of endogenous MG53 in mice, rats, and humans

For quantification of MG53 in plasma, plasma samples were collected from wild type C57BL/6J mice, Sprague Dawley rats (Harlan Laboratories, Inc. IN), and healthy, consented, human donors (samples purchased from Bioreclamation, LLC.) and analyzed by

Western blot. The densitometry of protein bands was quantified by Image J software (NIH). Ponceau S (Sigma, P7170) staining was used for loading control.

### Western blot analysis

To analyze expression levels of MG53, dysferlin, and caveolin-3 in skeletal muscles derived from mouse and rat, TA muscles were dissected from adult male C57BL/6J mice and adult male Sprague-Dawley rats. Proteins were extracted from the muscles by RIPA buffer supplied with protease inhibitor cocktail (Roche, 11697498001) and phosphatase inhibitor cocktail (Pierce, 78428). The proteins were separated by SDS-PAGE and transferred to PVDF membrane. The membranes were probed with antibodies against MG53 (1:3000, generated by our laboratory), dysferlin (1:1000, Novocastra Laboratories), and caveolin-3 (1:2000, BD Transduction Laboratories). We used 2 monoclonal antibodies derived from mouse and rabbit, both of which recognize MG53 in mouse, rat, and human tissues. Characterization of these antibodies has been reported in our previous publications[13,15,16,25]. Horseradish peroxidase conjugated secondary antibodies were incubated with the membrane. Peroxidase activity was determined with ECL kits (Pierce, 32106). The ECL signals were captured by HyBlot CL autoradiography film (Denville, Inc).

### Statistics

Statistical differences between groups were determined using independent *t*-tests for continuous data and Mann–Whitney U tests for interval data. *P*-values <0.05 were used as criteria for statistical significance.

## Results

### *mg53−/−* mice are more susceptible to I-R injury in skeletal muscle

To test the role of MG53 in protection against I-R injury in skeletal muscle, we performed comparative studies with I-R injury in muscles from *mg53−/−* mice and wild type littermate controls. As shown in Fig. 1, circulating creatine kinase (CK) in *mg53−/−* mice following I-R injury was significantly higher at multiple time points as compared with wild type littermates, indicating that MG53 plays a critical role in protecting against I-R injury to muscle.

### rhMG53 protects I-R induced muscle injury in mice

To examine if exogenous rhMG53 protein can protect against I-R injury in skeletal muscle, we delivered rhMG53 by tail vein injection to wild type mice. As shown in Fig. 2A, rhMG53 treatment reduced muscle edema significantly, as indicated by the measurement of wet:dry ratio, in I-R injured mouse skeletal muscle. The wet/dry ratio was reduced from 4.78±0.17 in the saline treatment group to 4.19±0.22 in the rhMG52 treated group ($P<0.01$). Furthermore, release of CK was also suppressed significantly by rhMG53 treatment, indicating improved membrane repair in rhMG53 treated animals (Fig. 2B).

### rhMG53 improves pathology of skeletal muscle associated with I-R injury

Similar to observations in Fig. 2A, H&E staining of the TA in the rhMG53 treated group also showed less swelling than saline controls (Fig. 3A). More necrotic fibers were evident by light-pink staining fibers in saline control muscle than in MG53 treated muscle (Fig. 3A). Evans blue dye (EBD) is commonly used to measure muscle membrane integrity [22]. This membrane-impermeable fluorescent dye stains only muscle fibers with membrane damage. As shown in Fig. 3B, rhMG53 treatment significantly reduced EBD positive muscle fibers (26.0±6.9% in rhMG53 treatment group vs 58.4±11.6% in saline control group, $P<0.01$), indicating the membrane injury induced by I-R injury can be protected and/or repaired by rhMG53 delivery.

For comparative purposes, we repeated the I-R muscle injury studies using our established rat model[18]. We noted consistently that rat muscle was more resistant to I-R induced injury, as significantly longer ischemic times were required to cause detectable muscle injury. We found that the 45 min ischemia time we used in the mouse model did not cause measurable muscle injury in the rat model (not shown). We found it necessary to use 3 hours of tourniquet ischemia in order to achieve muscle injury in the rat model comparable to that in the mouse model. Under this condition, the percentage of EBD positive muscle fibers was 55.0±5.8% (Fig. 4B) in the rat with 3 hours of ischemia, and 58.4±11.6% in the mouse with 45 minutes of ischemia (Fig. 3B). Interestingly, administration of rhMG53 did not produce significant changes in the degree of muscle injury in the rat model; the percentage of EBD positive muscle fibers was not statistically different between saline and rhMG53 treated rats (55.0±5.8% vs 47.7 ± 4.9%, respectively, $P = 0.46$) (Fig. 4B). Edema, as indicated by muscle wet:dry ratio was also similar between groups (6.97±0.34 and 7.57±0.23 for saline and rhMG53 treated, respectively, $P = 0.26$) (Fig. 4A). These data were consistent with our previous report that showed only a marginal effect of rhMG53 in the I-R induced injury to the rat muscle [18].

### Plasma derived from rat contains higher level of endogenous MG53

Such drastic differences between the responses of I-R injury and treatment of rhMG53 in the mouse and rat models raise an intriguing question that intrinsic muscle membrane repair mechanisms may be different between the 2 species. We performed a series of biochemical studies and found that several proteins that participate in repair of membrane injury, e.g. dysferlin, caveolin-3, and MG53, show similar expression levels in mouse and rat muscle (Fig. 5A). Thus, it is unlikely that difference in the intracellular membrane repair mechanism can account for the different response of I-R induced muscle injury in the 2 species. Interestingly, we found that endogenous circulating MG53 in rats is significantly higher than in mice (Fig. 5B). This finding might explain why the rats are less sensitive to skeletal muscle I-R injury. Furthermore, it might also explain why administration of rhMG53 had minimal effect on I-R injury in rat muscle.

We also conducted comparative measurements with determination of MG53 in the human plasma. As shown in Fig. 5C, the level of MG53 in human plasma is similar to that in mouse. Thus, mouse skeletal I-R injury model might be a better preclinical model for studying MG53-mediated membrane repair.

## Discussion

Extremity trauma is a large portion of both military [1] and civilian injuries [23], the majority of which include muscle trauma [24], often involving vascular injury and/or acute compartment syndrome culminating in I-R injury [25-27]. Therapeutic agents that could reduce the magnitude of I-R and/or extend ischemic time would be beneficial. Here we showed the therapeutic benefit of rhMG53 for treatment of skeletal muscle I-R in mice. This represents an exciting and important finding.

*In vitro* studies and studies using MG53 knockout mice have provided compelling evidence that MG53 is an essential component required for repairing membrane damage in cardiac [13,19,20] and skeletal muscle[28,15,29]. Furthermore, delivery of exogenous rhMG53 has been shown to ameliorate the impact of eccentric contraction in dystrophic mice and cardiotoxin injury in skeletal muscle in normal mice [16] and acute lung injury[30]. We extend these observations by demonstrating in skeletal muscle that endogenous circulating MG53 contributes to protection against I-R injury, and treatment with rhMG53 ameliorates I-R induced skeletal muscle injury.

MG53 is a muscle-specific TRIM family protein (TRIM72) involved in vesicle trafficking and fusion with the plasma membrane during normal cellular physiology[28] and during the emergency response of plasma membrane repair[13,20,19]. Through binding with phosphatidylserine, MG53 associates with intracellular vesicles underneath the sarcolemma of striated muscle. In muscle cells, MG53 interacts with dysferlin and caveolin-3 to form a dynamic vesicular complex [13]. In the event of membrane disruption, MG53 is oxidized at a specific cystine residue in response to entry of the extracellular milieu into the cell, and disulfide-bond formation between MG53 molecules provides the nucleation mechanism for vesicle translocation to the membrane injury site.

We previously reported that rhMG53 did not show a significant protective effect against I-R skeletal muscle injury in rats [18]. All published studies showing a benefit of rhMG53 administration have involved mice, suggesting that species differences may explain these disparate findings. No difference between rats and mice was found in endogenous MG53 levels in skeletal muscle. While most research has focused on MG53 levels resident in the tissue of study, endogenous MG53 is also present in circulating blood[16]. We found a more than 10-fold higher level of endogenous MG53 in rat plasma compared to mice. Based on this, we postulate that in rats baseline levels of endogenous MG53 are adequate to provide a protective effect without the need for exogenous rhMG53. This suggests that therapeutic levels of rhMG53 in mice (and possibly humans) may be much lower than those used in this and other studies and certainly warrants future study. Future studies with molecular or genetic manipulation to deplete MG53 in rats will provide a further test for the role of circulating MG53 in protection against muscle injuries. It was also important to determine what the native MG53 levels were in human blood, as high concentrations (as seen in rats) would suggest it would be of limited therapeutic value. To this end, Western blot analysis revealed that the level of plasma MG53 protein is much lower in humans than in rats, validating the pursuit of rhMG53 as a potential treatment in humans.

There are limitations to this study. We administered rhMG53 5 min prior to ischemia and again 5 minutes prior to reperfusion. While this was effective in demonstrating the beneficial effect of rhMG53, pretreatments are impractical in the context of trauma. Clearly, future experiments supporting the efficacy of post-injury treatment with rhMG53 will be required. Pretreatment is not a limitation for reconstructive surgery involving muscle flaps, functional muscle transfers, or even composite tissue allograft transplant surgery. Here pretreatment is indeed an option. In these cases rhMG53 may be of great benefit in extending surgical time. In addition, a recent study by Cea et al reported that altered expression of connexin hemichannels can enhance the permeability of sarcolemma to small molecules, such as Evans blue[31]. More studies will be needed to explore if the expression levels for connexins in skeletal muscle are different between rats and mice, and if the treatment with rhMG53 can alter the expression of connexins. This may account for some of the differences observed with Evans blue uptake in mouse vs rat muscle following I-R injuries.

Patient safety is a serious concern when considering any drug for clinical use. As already discussed, MG53 is present in circulating blood at low levels in both mouse and human. Toxicity tests in mice have reported no observable toxic effects with long-term administration of MG53 as measured by a wide range of indicators [16]. 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. 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).

Overall, this study provides additional support for the use of MG53 for protection and repair of tissue injury. Additionally, the beneficial therapeutic effect of rhMG53 in ameliorating I-R in skeletal muscle provides support for its potential as a treatment for I-R related to muscle trauma, including extremity compartment syndrome. Additional studies will be required to determine the effectiveness of rhMG53 as a post-injury treatment.

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## Abbreviations

| | |
|---|---|
| **EBD** | Evans blue dye |
| **H&E** | Hematoxylin and eosin |
| **i.m.** | intramuscular injection |
| **i.v.** | intravenous injection |

| | |
|---|---|
| **I-R** | Ischemia-reperfusion |
| **MG53** | Mitsugumin 53 |
| **rhMG53** | recombinant human Mitsugumin 53 |
| **TA** | Tibialis anterior muscle |
| **TRIM** | Tripartite motif |

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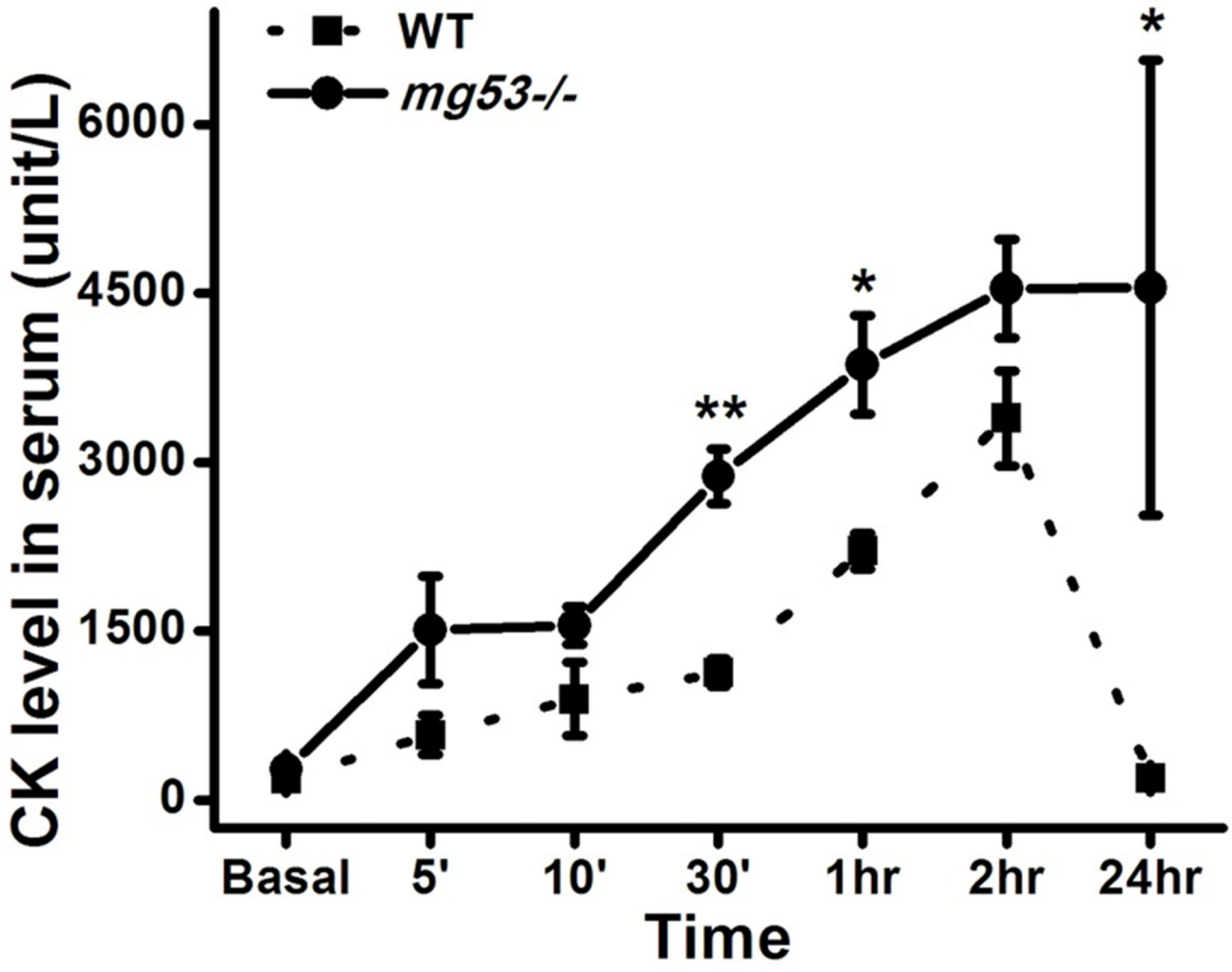


**Figure 1. *mg53*$^{-/-}$ muscle is susceptible to ischemia-reperfusion (I/R) injury**

Creatine kinase levels in serum were measured at indicated time points. *mg53*−/− mice exhibited a different pattern of serum CK changes as compared to wild type control (n=6 pairs). The serum CK elevation was quicker and remained at high levels for a longer time in *mg53*$^{-/-}$ mice. Data are presented as mean ± SEM. *: $P$<0.05.

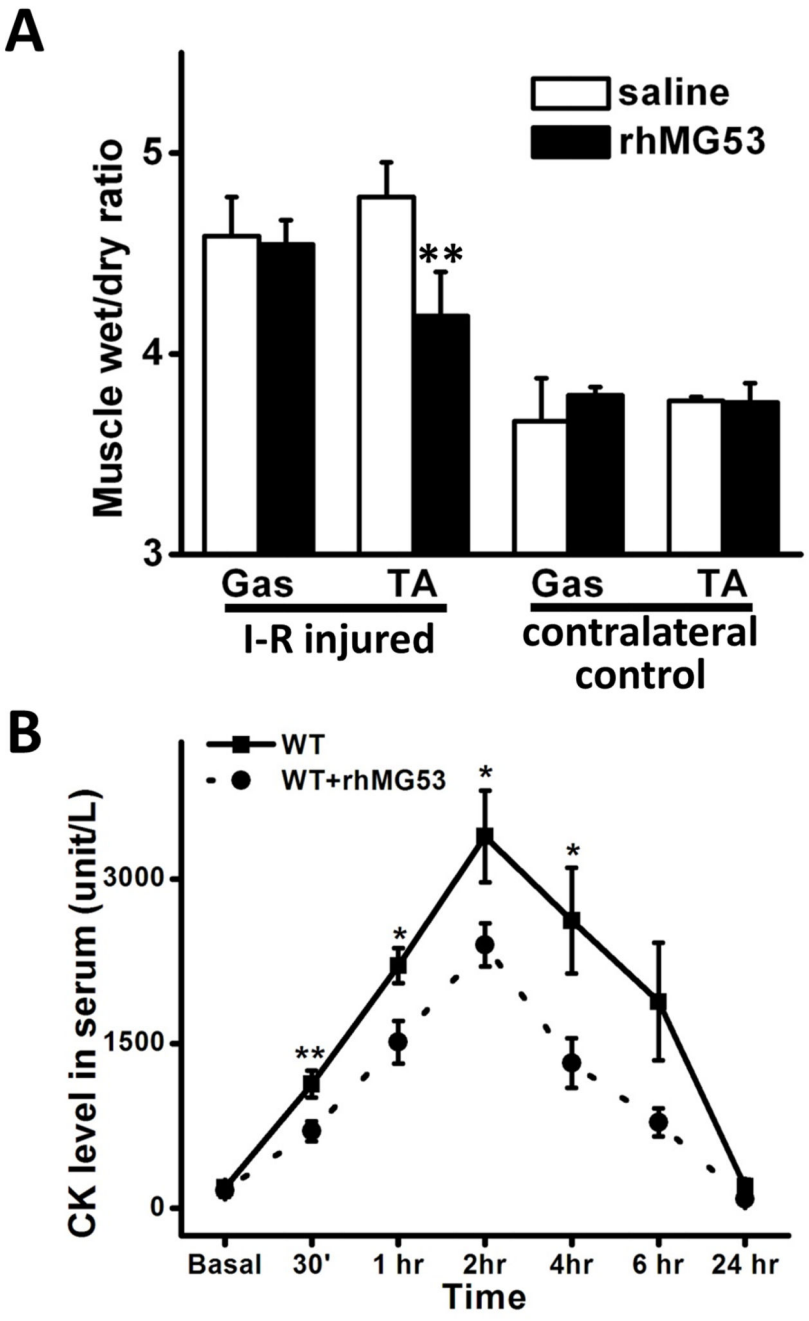


**Figure 2. rhMG53 prevents I-R induced skeletal muscle injury in mice**

(A) rhMG53 treatment (n=5) significantly reduced the wet:dry ratio of I-R injured muscle compared to saline treatment (n=4). **: $P<0.01$. (B) Serum CK concentration was measured at indicated time points (n=8 pairs). rhMG53 treated mice had lower CK in the blood than saline treated mice. Data are presented as mean ± SEM. *: $P<0.05$; **: $P<0.01$.

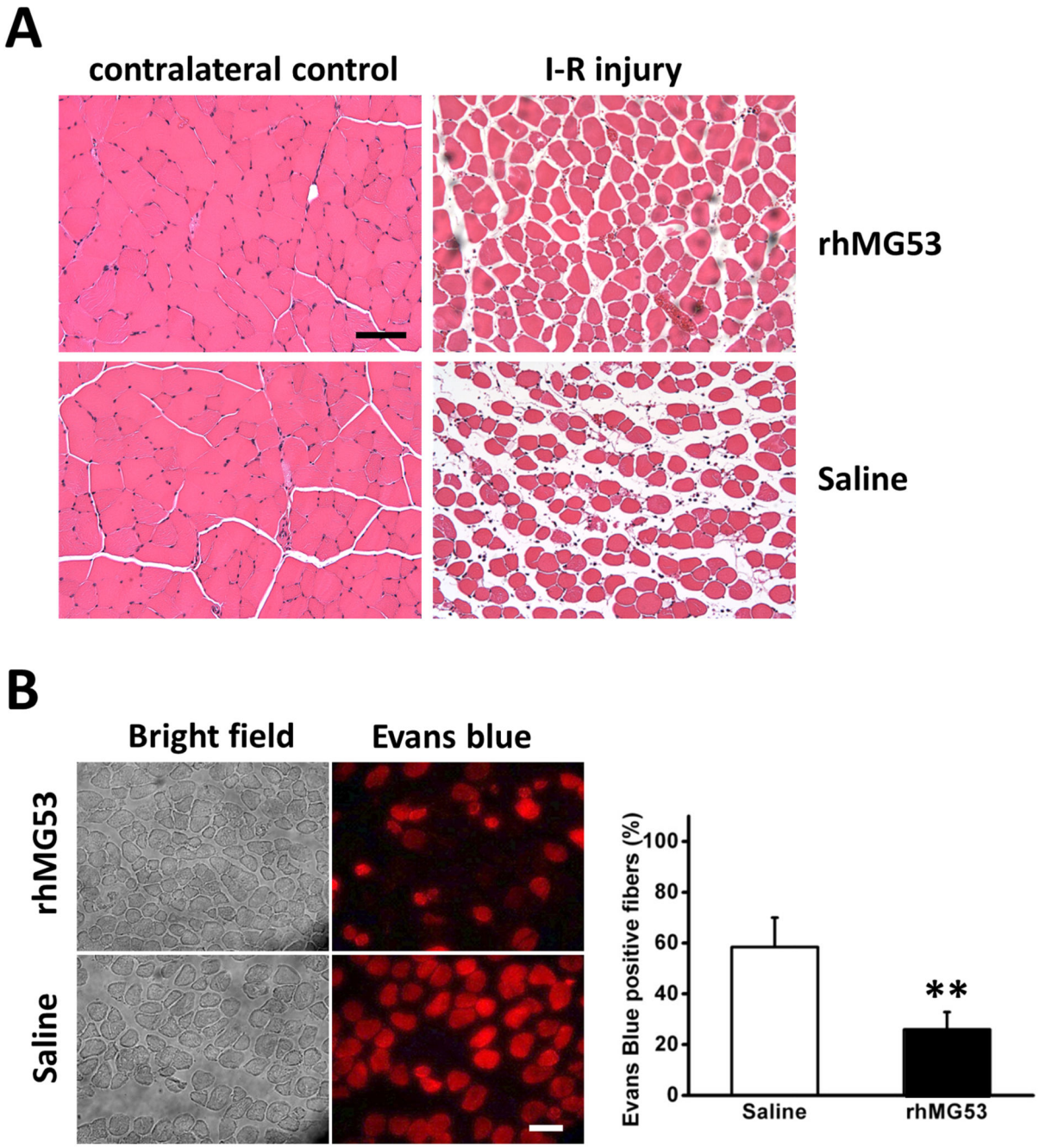


**Figure 3. rhMG53 improves muscle structure associated with I-R injury**

(**A**) H&E staining of control (*left*) and injured (*right*) muscles. More necrotic fibers were seen by light pink staining in saline control muscle than that in MG53 treated muscle. n=3 pairs. (**B**). Fluorescent images of Evans blue positive fibers (*right*) showed less injury in rhMG53 treated muscle than control. Percentage of total damaged fibers is summarized in the lower panel. The data are mean ± SEM **: $P<0.01$. n=4 pairs. Scale bars, 50 μm.

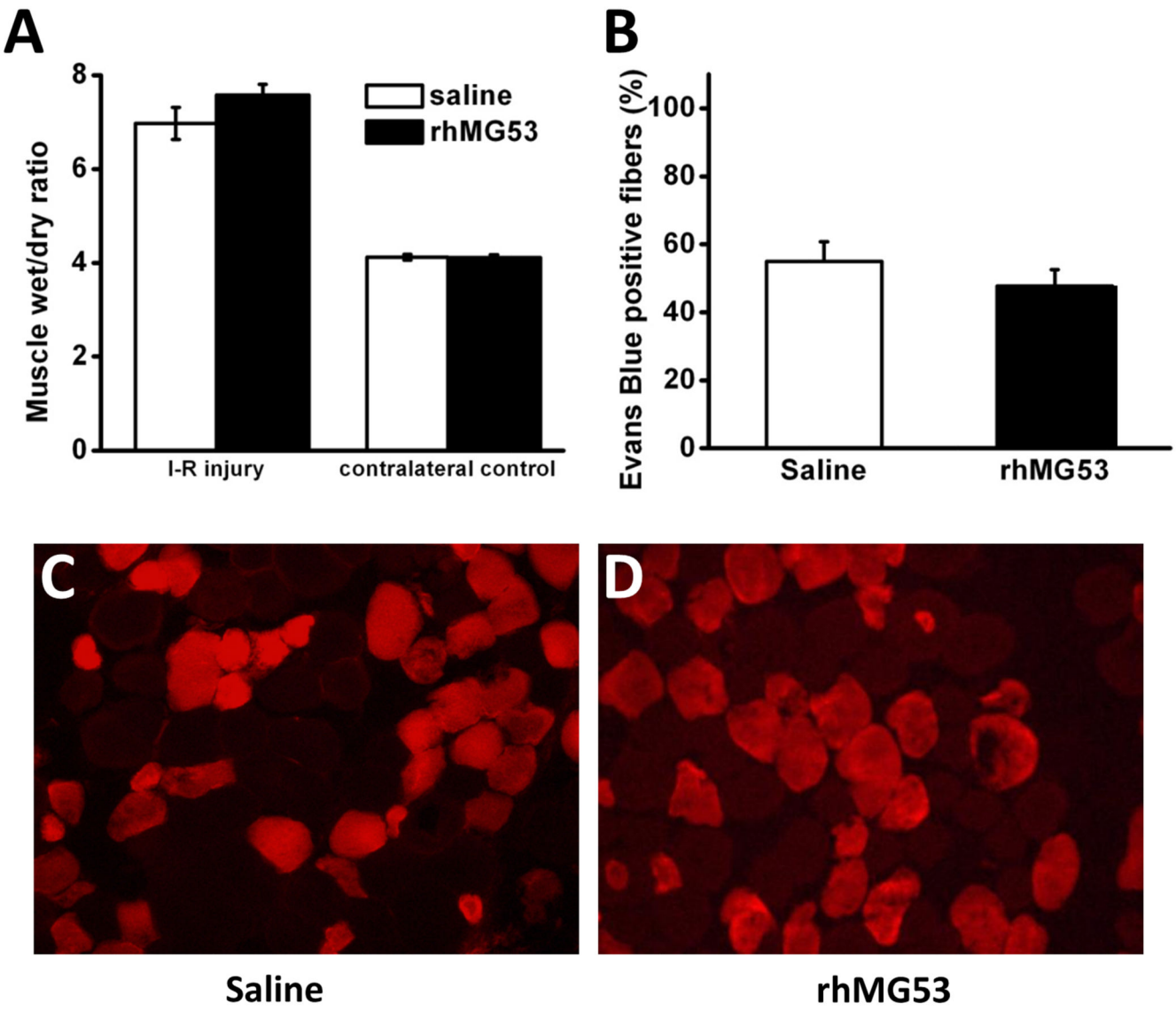


**Figure 4. rhMG53 exhibits minimal beneficial effects in I-R injury to rat skeletal muscle**

(A) Wet/dry weight in injured GAS muscle nearly doubles compared to uninjured muscle, which is unaffected by rhMG53 treatment. (B) Quantification of the number of muscle fibers that stain positive for EBD confirms the lack of effect of rhMG53 treatment in rats. (C-D) Representative fluorescent images of muscle cross-sections from (C) Control (PBS) and (D) rhMG53 treated rats clearly show similar numbers of EBD positively stained fibers (red) between treatments.

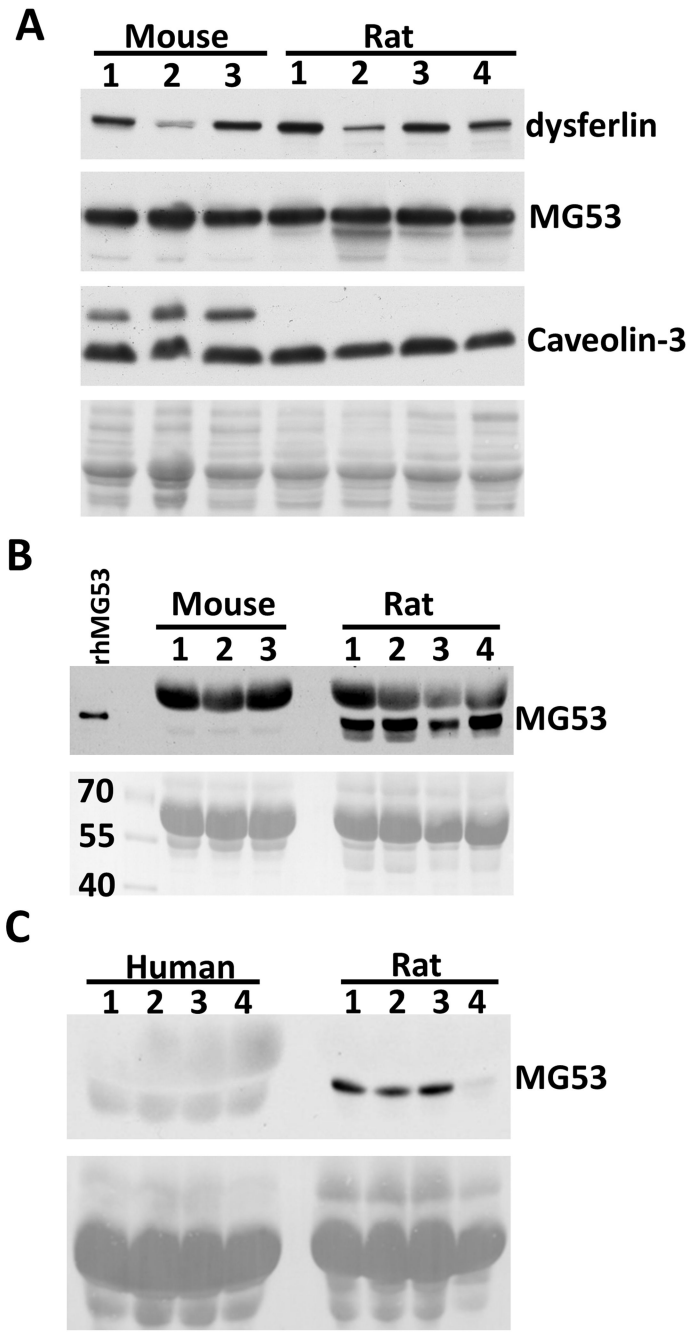


**Figure 5. Plasma samples derived from rat contain high level of endogenous MG53**

(**A**) Skeletal muscle samples from mouse and rat showed similar level of endogenous dysferlin, MG53, and caveolin-3. Ponceau S staining was used to show equal loading. Plasma samples from mice and rats (**B**) or rats and humans (**C**) were subjected to Western blot analysis. Representative Western blot figure shows that plasma derived from rat contains higher endogenous MG53 than plasma from mouse and human, indicating that mouse might be a better animal model to study MG53-mediated membrane repair. rhMG53

was used as positive control. The results were confirmed by 2 different MG53 antibodies generated from different animal species.