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Invited Mini Review

Dual function of MG53 in membrane repair and insulin signaling

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MG53 is a member of the TRIM-family protein that acts as a key component of the cell membrane repair machinery. MG53 is also an E3-ligase that ubiquinates insulin receptor substrate-1 and controls insulin signaling in skeletal muscle cells. Since its discovery in 2009, research efforts have been devoted to translate this basic discovery into clinical applications in human degenerative and metabolic diseases. This review article highlights the dual function of MG53 in cell membrane repair and insulin signaling, the mechanism that underlies the control of MG53 function, and the therapeutic value of targeting MG53 function in regenerative medicine. [BMB Reports 2016; 49(8): 414-423]

INTRODUCTION

Skeletal muscle, whose main function is to control locomotion, comprises about 40% of the human body mass. Associated with muscle contraction-relaxation is injury to the sarcolemma membrane that necessitates an active repair mechanism to maintain integrity of the muscle fiber. Skeletal muscle injury-repair and regeneration is a multi-cellular process that involves repair of the acute injury to the sarcolemma, mobilization of satellite cells to replace the lost-muscle fibers, and control of fibrotic remodeling for maintenance of muscle function. In muscular dystrophy, compromised sarcolemma integrity or membrane repair triggers the cascade of muscle degeneration that incurs progressive, severe morbidity, and ultimately mortality. Thus, developing therapeutic approaches to improve the sarcolemma integrity while facilitating regeneration of injured muscle fibers remain a major challenge in muscle physiology research.

In addition to functioning as a contractile machine, skeletal muscle plays an important role in regulating glucose homeostasis, accounting for ~80% of insulin-stimulated glucose disposal (1). Development of insulin resistance is the main underlying cause for metabolic disorders such as obesity and type 2 diabetes. Although metabolic disorders primarily induce defects in nutrient regulation, their burden on the human body extends to other physiological systems (2-4). A less explored complication of metabolic syndrome is its impact on tissue repair. For example, patients with metabolic diseases often are more susceptible to tissue injury, and slow to recover after injuries (5, 6).

Repair of injury to the plasma membrane is an important aspect of normal cellular physiology (3, 6-8), and disruption of this process can result in pathophysiology in a number of different tissues (3, 6, 9-11). MG53 is a tripartite motif protein (TRIM72) that presides over the mechanisms of plasma membrane repair (12, 13). It facilitates the nucleation of intracellular vesicles to sites of membrane injury to form a repair patch. Genetic ablation of MG53 causes compromised cell membrane repair that can lead to progressive skeletal myopathy and a decreased regenerative capacity of cardiomyocytes (14, 15). Research efforts have been devoted to translate this basic discovery into clinical applications towards treatment of muscular dystrophy (16, 17), acute lung injury (18), myocardial infarction (15, 19-21), and acute kidney injury (22).

Besides a role in cell membrane repair, MG53 also participates in modulation of insulin signaling. MG53 contains the conserved RING motif with intrinsic E3-ligase enzymatic activity to induce ubiquitination and down-regulation of insulin receptor substrate 1 (IRS-1), which negatively impact insulin signal strength in skeletal muscle (23-26). It has been suggested that interventions that target direct interaction between MG53 and IRS-1, without the disruption of membrane repair function for MG53, may be a novel therapeutic approach for the treatment of human diseases that are associated with insulin resistance (22, 25).

In this review, we highlight the dual function of MG53 in cell membrane repair and insulin signaling, the mechanism that underlies the transcriptional control of MG53, and the

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therapeutic value of targeting MG53-mediated cell membrane repair in regenerative medicine.

IMMUNO-PROTEOMIC IDENTIFICATION OF MG53 PROTEIN FROM SKELETAL MUSCLE

Collaborative study between Drs. Hiroshi Takeshima and Jianjie Ma utilized an immuno-proteomic approach for investigation of proteins involved in myogenesis, Ca signaling, and maintenance of membrane integrity in striated muscle (27-30). The immuno-proteomic approach involved a combination of monoclonal antibody immunohistochemistry, cDNA library screening and gene knockout techniques, leading to the discovery of a group of novel proteins termed mitsugumins (MG) that play important roles in muscle physiology, regenerative medicine, and cardiovascular diseases. For example, MG29 is a synaptophysin-related membrane protein that is essential for the maturation and development of the transverse-tubule membrane structure in the skeletal muscle (31-34). MG29 may act as a molecular marker of aging that can shield skeletal muscles against aging-related decreases in Ca homeostatic capacity (28-32, 35, 36). We have identified TRIC, a sarco/endoplasmic reticulum (SR/ER)-resident membrane protein that forms a homo-trimeric molecular complex to create a cation selective channel to regulate the efficient Ca release process during excitation-contraction coupling in muscle cells (37, 38). Another important molecule isolated from our immuno-proteomic library is junctophilin (JP), which possesses the property of physically linking the transverse-tubule and SR membrane, allowing the formation of triad junctions that provide the structural framework for excitation-contraction coupling in skeletal and cardiac muscles (39). Further studies have linked alteration of JP and polymorphisms of JP in cardiovascular diseases (40).

In 2005, Dr. Takeshima cloned the MG53 gene using the immuno-proteomic library. Sequence alignment showed that MG53 belongs to the TRIM-family protein, and the primary amino acid sequence of MG53 is highly conserved across the animal species. Biochemical and RNA-hybridization studies revealed a predominant expression of MG53 in mouse skeletal and cardiac muscles. Interestingly, genetic ablation of MG53 gene did not appear to affect the behavior and reproduction of the animals under normal physiological conditions. Subsequent studies in Dr. Ma's laboratory identified a distinct phenotype with the *mg53* −/− mice. When the animals were subjected to down-hill running exercise, the absence of MG53 led to excessive injury to the skeletal and cardiac muscles due to the defective membrane repair capacity. Toward understanding the biological function of MG53, Cai *et al.* performed expression of the MG53 cDNA in heterologous cells, and observed that overexpression of MG53 increased the trafficking of intracellular vesicle to the plasma membrane, leading to the appearance of extensive filapodia-like structures (41). These observations led to the hypothesis that MG53-containing vesicles may participate in formation of a repair patch following acute injury to the plasma membrane. Next followed a series of studies, that culminated in the realization of MG53 as an essential component of the cell membrane repair machinery (12, 13).

Parallel to the immuno-proteomic approach, independent studies from Dr. Young-Gyu Ko's laboratory also identified MG53 from C2C12 myotubes using comparative two-dimensional electrophoresis of detergent-resistant lipid rafts (23, 42). Lipid rafts or caveolae enrich a myriad of receptors and signaling molecules, and function as a harbor for cell signal transduction. Insulin receptor β (IRβ), insulin-like growth factor receptor (IGFR), insulin receptor substrate-1 (IRS-1), phosphatidylinositol-3-kinase (PI-3-K) and Akt are all enriched in the lipid rafts (43, 44). Studies have shown that disappearance of caveolae and the decrease in insulin-elicited phosphorylation of IRβ in the skeletal muscle of cav-3 or cavin-1-disrupted mice, leads to insulin resistance (45-47). These data indicate that caveolae are critical membrane compartments for insulin signaling. Thus, proteomic analysis of the lipid rafts is a powerful tool for identifying novel signaling molecules (48). Cavin-1, gC1qR, raftlin and ezrin have been found from different mammalian cell lines by comparative lipid raft proteomics (23, 49-52).

Lee *et al*. isolated lipid raft proteins from C2C12 myoblasts and myotubes and compare them by two-dimensional electrophoresis for identifying novel signaling molecules involved in C2C12 myogenesis (23). Using these approaches, MG53 was identified in the lipid rafts of C2C12 myotubes. Further studies showed that MG53 is co-localized with caveolin-3 in C2C12 myotubes, and mouse skeletal and cardiac muscles. The molecular interaction of MG53 with lipid raft proteins, such as caveolin-3, cavin-1, dysferlin, IRS-1 and IRβ, has been demonstrated by endogenous immunoprecipitation in mouse skeletal and cardiac muscles, indicating that MG53 is a real lipid raft protein (14, 23, 24, 41, 53).

MG53 protein harbors the N-terminal tripartite motif (TRIM) and C-terminal PRY-SPRY (spIA and ryanodine receptors), making it a member of the TRIM family (12, 23). There are more than 70 TRIM proteins with a RING finger motif, a B-box and one or two coiled-coil domains. MG53 is named as TRIM72 (Fig. 1A) (54). The RING finger motif has a consensus sequence $C\text{-}X_2\text{-}C\text{-}X_{[9\text{-}39]}\text{-}C\text{-}X_{[1\text{-}3]}\text{-}H\text{-}X_{[2\text{-}3]}\text{-}C\text{-}X_2\text{-}C\text{-}X_{[4\text{-}48]}\text{-}C\text{-}X_2\text{-}C$, where X is any amino acid. With its unique "cross-brace" arrangement with 2 zinc ions, the RING finger motif has E3 ligase activity, transferring from E2 enzyme to their specific target proteins. Indeed, RING domain of MG53 has E3 ligase activity for IRS-1 ubiquitination with the help of E2 ligase UBE2H, because RING domain-disrupted MG53 mutants (C14A and ΔRING) do not induce IRS-1 ubiquitination and degradation (24, 25). B-boxes also contain zinc-finger motifs and may be linked to cell membrane repair and wound healing (55, 56). Coiled-coil domains show typical hyper-secondary structures formed by intertwining multiple

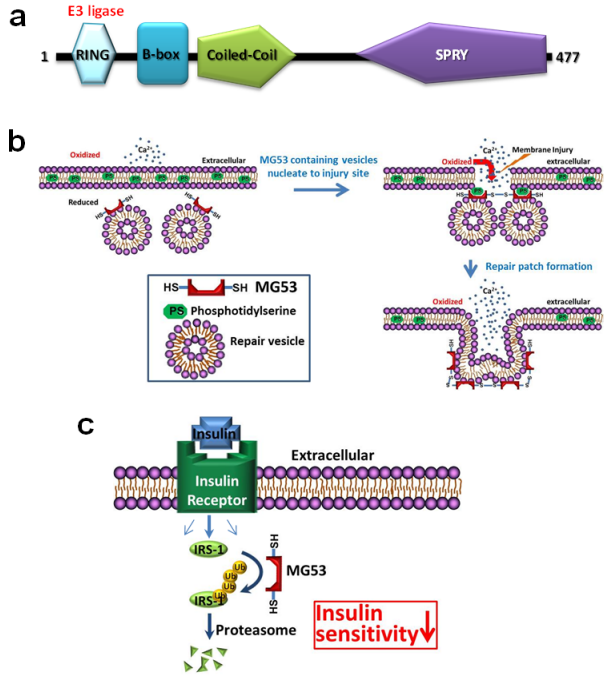

Fig. 1. MG53 function in cell membrane repair and insulin signaling. (A) MG53 contains 477 amino acids with RING (Really Interesting New Gene), B-box, and Coil-coil domain at the amino terminus, plus a SPRY domain at the carboxyl terminus. (B) MG53 participates in the nucleation of transport vesicles to sites of membrane injury. It binds to phosphatidylserine (PS) and undergoes oligomerization when the cell changes from reduced to oxidized environment at the injury site. Entry of extracellular Ca^{2+} enables fusion of vesicles for formation of a repair patch. (C) MG53 contains an E3 Ligase activity that facilitates the ubiquitination of IRS-1. The proteasome-mediated degradation of IRS-1 contributes to the regulation of insulin signaling in muscle cells.

α-helices, and are known to be required for homo- or hetero-oligomerization of TRIM family proteins and interacting with different binding partners. Indeed, MG53 mutants without the coiled-coil domain (ΔCC) loses its ability to form homo-oligomer and interact with IRS-1 (23, 57). PRY-SPRY domain of MG53 might be involved in protein-protein interaction, since its structure has a pocket for an unknown binding partner (58).

MG53 FUNCTION IN CELL MEMBRANE INJURY-REPAIR

Prior to the finding of MG53, studies from other investigators have identified several molecular components involved in membrane repair, particularly those specific to cardiac and skeletal muscles (59, 60). Bansal *et al*. showed that dysferlin plays an important role in maintenance of sarcolemmal membrane integrity (9). Several mutations in the dysferlin gene have been linked to human muscular dystrophy (9, 61, 62). It was proposed that dysferlin can function as a fusogen to allow vesicles to form a membrane repair patch (63). This was based on immunostaining observations that dysferlin concentrates at the injury sites of isolated muscle fibers and the muscular dystrophy that appears in dysferlin knockout mice (9). However, since the initial study by Bansal and colleagues, there has been no indication that dysferlin itself can facilitate the rapid translocation of vesicles associated with acute membrane damage. Indeed, *dysferlin* $^{-/-}$ muscle maintains the capacity for vesicle translocation to damage sites on the sarcolemma. This suggests that although dysferlin may participate in the final membrane resealing process, proteins other than dysferlin are likely required for nucleation of intracellular vesicles toward the acute injury sites.

While MG53 is a soluble protein in nature, it can tightly associate with intracellular vesicles and the inner leaflet of the sarcolemma of striated muscle, likely through binding with phosphatidylserine (PS) that is enriched in these membrane surfaces (12, 64). Under live cell imaging, a traffic of intracellular vesicles containing MG53 can be observed through the cell, fusing with the sarcolemma and entering the extracellular space (12, 41). Cai *et al*. first observed that MG53 containing vesicles can translocate to sites of membrane damage following mechanical or laser-induced injury to the plasma membrane (12). Muscle fibers derived from *mg53* $-/-$ mice display defective membrane repair following such injuries. A role for MG53 in muscle membrane repair was further established through the ultrastructural analysis using electron microscopy, which revealed a lack of vesicle accumulation at sites of injury to the *mg53* $-/-$ muscle fibers. Inspired by these initial observations in 2006, it took us nearly 2 years to resolve the mechanism that underlies the nucleation process for MG53-mediated cell membrane repair (12). MG53 appears as a monomer under reduced conditions, as in the cytoplasm of an intact cell. In response to changes in the redox-status, as it occurs during acute damage of the cell membrane, MG53 forms oligomer complexes under oxidized conditions. Through targeted mutagenesis and functional studies, we determined that MG53 oligomer formation involves a critical cystidine residue (C242). Mutation of C242 into alanine (C242A) disrupted the MG53 oligomer formation, caused defective trafficking of MG53-containing vesicles to injury sites, and pre-dominantly, it negatively impacted the wild type MG53 function in muscle membrane repair. These studies support a model where the entry of oxidized milieu triggers activation MG53 to facilitate vesicle translocation to the site of membrane disruption. Subsequent entry of extracellular Ca and activity of other fusogenic factors, such as dysferlin or annexin (12), can facilitate fusion of these vesicles at the membrane injury sites to create a membrane repair patch (Fig. 1B).

Over the past 7 years, we made significant progress in understanding the molecular mechanisms for MG53-mediated

membrane repair in muscle physiology and disease. MG53 contains two leucine zipper motifs in the coiled-coil domain (LZ1 - L176/L183/L190/V197 and LZ2 - L205/L212/L219/L226), which are highly conserved across the different animal species. Mutagenesis and chemical cross-linking studies show that LZ1 is critical for MG53 homodimerization, whereas LZ2 is not (57). We found that *in vivo* modification of cysteine residues with alkylating reagents can prevent the nucleation process of MG53-meidated cell membrane (57). Thus, in addition to the leucine zipper-mediated intermolecular interaction, redox-dependent cross talk between MG53 remains an obligatory step for membrane repair. On the basis of these studies, we propose a model for MG53 function during cell membrane repair. Through leucine zipper-mediated intermolecular interaction, MG53 exists as a dimer under healthy condition with intact cell membrane structure. The leucine zipper-mediated MG53 dimer formation also provides accessibility of active sulfhydryl groups (e.g., Cys242) that forms a larger oligomeric complex during assembly of the cell membrane repair machinery.

We also found that PTRF (polymerase I and transcript release factor) can function as a docking protein for MG53 during membrane repair by binding exposed cholesterol at the injury site (53). Cells lacking endogenous PTRF exhibit defective trafficking of MG53 to the injury sites. Mutations in PTRF associated with human muscular dystrophy result in abnormal subcellular distribution of PTRF and disrupt the membrane repair function of MG53. Furthermore, mutagenesis studies identified that zinc-binding to the RING and B-box motifs of MG53 contributes to muscle membrane repair and wound healing (55, 56). AAV-mediated gene delivery showed that enhanced expression of MG53 in the δ-Sarcoglycan-deficient hamsters improved the membrane repair and ameliorated muscular dystrophy and heart failure in this animal model (16).

TRANSCRIPTIONAL REGULATION MG53 IN SKELETAL AND CARDIAC MUSCLE

MG53 mRNA and protein are predominantly expressed in mouse skeletal and cardiac muscle tissues (12, 23). The MG53 mRNA and protein levels are gradually increased during the myogenesis of C2C12 cells, MyoD-overexpressing mouse embryonic fibroblasts, human primary skeletal muscles, and mouse satellite cells (23, 25). All these findings indicate that MG53 transcription is regulated by MyoD which is a master transcription factor for skeletal myogenesis.

Using promoter luciferase reporter and chromatin immunoprecipitation (ChIP) assays, MG53 promoter was found to harbor two functional E-boxes (23). The MG53 transcription during C2C12 myogenesis is activated by an IGF-activated PI3K-Akt pathway because MG53 promoter activity is dramatically decreased by PI3K inhibitor (LY294002) or Akt knockdown but not by mTOR, GSK3β or MAPK inhibitor (rapamycin, LiCl or PD98059), and increased by constitutively active Akt. Indeed, IGF-activated PI3K-Akt pathway has known to be required for the formation and activation of myogenic transcriptional complex, since active Akt induces direct phosphorylation of p300 and association of MyoD, MEF2, p300 and P/CAF acetyltransferases (68, 69).

Myocyte enhancer factor 2 (MEF2) is another myogenic transcription factor which is critical for muscle type remodeling from fast-twitch glycolytic fibers to slow-twitch oxidative fibers (70, 71). For example, lacZ transgenic mice with MEF2-binding sites in its promoter show that only oxidative fibers have strong β-galactosidase activity (72). MEF2C- or MEF2D disruption reduces the number of oxidative fibers in mouse soleus (73). Among different skeletal muscles, MG53 protein level is the highest in soleus (which is mainly composed of slow-twitch oxidative fibers), and low in gastrocnemius (which is mainly composed of fast-twitch glycolytic fibers) (23, 42). These data indicate that MG53 transcription might be dependent on MEF2 transcriptional activity. Indeed, MG53 promoter is found to contain a functional MEF2-binding site as determined by the promoter luciferase reporter assay, chromatin immunoprecipitation (ChIP) assay and electrophoretic mobility shift assay (EMSA) (42). Chung *et al*. also demonstrate that as like oxidative fiber-specific genes such as MHC-I, and IIa, troponin-I (slow) and myoglobin, MG53 is synergistically transcribed by the combination of MyoD and MEF2 or Mgn and MEF2 during C2C12 myogenesis (42).

MG53 SECRETION AND THERAPEUTIC APPLICATION IN TREATMENT OF MUSCLE INJURY

During skeletal muscle contraction and relaxation, injury to the sarcolemmal membrane occurs under physiological conditions. Compromised membrane repair capacity has been linked to a number of different disease states including muscular dystrophy (74). Although native MG53 protein is predominantly expressed in striated muscle tissues, with overexpression of MG53 beneficial effects that protect against cellular injuries are present in non-muscle cells (17). In addition to the intracellular action of MG53, membrane injury exposes lipid signals at the cell surface that can be recognized by MG53, allowing exogenous MG53 protein to repair membrane damage when provided in the extracellular space. In both muscle and non-muscle cells, the recombinant human MG53 (rhMG53) protein purified from *Escherichia coli* provides dose-dependent protection against chemical, mechanical, or ultraviolet-induced damage. Intravenous administration rhMG53 can ameliorate eccentric contraction-induced injury in dystrophic mice, and cardiotoxin-induced injury to skeletal muscle in normal mice (17).

We conducted additional *in vivo* studies with rhMG53 and found that intravenous administration of rhMG53 was effective in protection against myocardial infarction (20), acute lung

injury (18), and acute kidney injury (22) in rodent and large animal models of these diseases. These data support the concept of targeting cell membrane repair in regenerative medicine, and present MG53 as a potential biological reagent for restoration of membrane integrity in a broad range of human diseases.

While MG53 is an intracellular protein, a small portion of native MG53 is detected in the circulating blood, which can be elevated following damaging exercise training (17). Similar observation was made when we challenged mice with myocardial infarction injury through ligation of anterior coronary artery (76). Since native MG53 is present in blood circulation at levels directly correlating with injury or secretory activity of the muscle and heart (17, 20, 76), systemic administration of rhMG53 would unlikely induce an immune response and potentially be a safe biologic reagent. We conducted pharmacokinetic and toxicological evaluations of rhMG53 in murine and canine models, and found that repetitive intravenous administration of rhMG53 did not produce any adverse effects in these animals (17, 22).

Interestingly, when using ischemia/reperfusion (I/R) to injure the skeletal muscle, we found that rat muscles were much more resistant to I/R injury as compared to the mouse muscle (76). Through biochemical studies, we found that key proteins that participate in repair of membrane injury, e.g. dysferlin, caveolin-3, and MG53, do not show significant difference in mouse and rat muscle (76), suggesting that difference in the intracellular membrane repair mechanism cannot account for the different response of I/R induced muscle injury in the two species. Interestingly, MG53 protein present in the bloodstream is significantly higher in rats than that in mice. These results suggest that circulating MG53 might play a role in muscle injury-regeneration, and targeting the extracellular MG53 function can have beneficial effects on human diseases associated with defective membrane repair capacity.

MG53 AS AN E3-LIGASE TO MODULATE INSULIN SIGNALING

Lee *et al*. investigated the cellular function of MG53 on myogenesis by using various approaches such as adenoviral overexpression, RNA interference and systemic gene disruption (23, 25). C2C12 myogenesis is compromised by MG53 overexpression but enhanced by MG53 knockdown. Myogenesis of human satellite cells is also highly enhanced by MG53 knockdown. MG53 disruption enhances the myogenesis in mouse satellite cells and MyoD-overexpressing mouse embryonic fibroblasts (23, 25). Moreover, the overexpression of RING domain-disrupted MG53 mutants (C14A and ΔRING) enhances the C2C12 myogenesis, indicating that MG53-mediated protein ubiquitination is required for the negative feedback regulation of MG53 on skeletal myogenesis (25).

It has been well known that IGF-IGFR-PI3K-Akt signalling axis is essential for skeletal myogenesis (77, 78). For example, the knockdown of IRS-1 prevented, but the overexpression of constitutively active Akt enhanced the C2C12 myogenesis (23). Lee *et al*. investigated the molecular mechanism of MG53 on the negative feedback inhibition of myogenesis by analyzing IGF signalling after MG53 overexpression and knockdown in C2C12 cells, by using adenoviral overexpression and RNA interference (23). The IGF-elicited activation of IRS-1, PI3K and Akt is decreased in MG53-overexpressing C2C12 myoblasts, and increased in MG53-knockdown C2C12 myotubes. The molecular interaction between MG53 and IRS-1 is also demonstrated by two independent research groups (23, 24). These data demonstrated that IRS-1 is a real target of MG53 for regulating skeletal myogenesis. Because IGF-elicited the activation of IGFR, ERK1/2 is not changed by MG53 overexpression and knockdown (23), and IGF-stimulated Ras-Raf-ERK1/2 pathway is diverted from IGFR but not from IRβ, it was concluded that IGFR is not the target of MG53.

Because IRS-1 is a convergent signaling molecule for both IGF and insulin, MG53-mediated IRS-1 inactivation also leads to the blockage of insulin signaling. For example, MG53 overexpression prevents the insulin-elicited phosphorylation of IRS-1, Akt and ERK1/2 in myoblasts without affecting the insulin-elicited phosphorylation of IRβ (25). Because insulin-stimulated Ras-Raf-ERK1/2 and PI3K-Akt pathways are diverted from IRS-1 and MG53 inactivates IRS-1, both pathways are inactivated by MG53. The insulin-elicited phosphorylation of IRS-1, Akt and ERK1/2 is increased in the skeletal muscles such as soleus and gastrocnemius-plantaris of mg53−/− mice (24, 25). However, insulin-elicited phosphorylation of IR1β remains unchanged by MG53 disruption (25). These data indicate that MG53 negatively regulates IRS-1 but not IRβ in the skeletal muscle.

During the myogenesis of C2C12 cells and MyoD-overexpressing MEFs, MG53 protein level is greatly increased and IRβ and IGFR protein level is constant, whereas IRS-1 protein level is decreased in spite of the slight increase of IRS-1 mRNA level (25). MG53 overexpression decreases IRS-1 protein level in C2C12 myoblasts, and MG53 knockdown increases the IRS-1 protein level in C2C12 myotubes without changing IRβ and IGFR protein level (24, 25). Moreover, MG53 disruption increases IRS-1 protein level in mouse skeletal muscles such as soleus and gastrocnemius-plantaris. These data indicate that MG53 mediates the degradation of IRS-1 but not of IRβ and IGFR. The MG53-induced IRS-1 degradation is abrogated in the presence of MG132, a proteasome inhibitor, indicating that MG53 induces IRS-1 ubiquitination. Interestingly, the MG53-induced IRS-1 degradation is abolished by the overexpression of C14A and ΔRING in HEK293 cells, showing that C14A and ΔRING work as a dominant negative form of MG53 because both C14A and ΔRING interact and form oligomer with MG53.

Yi *et al*. demonstrated a molecular interaction between MG53 and UBE2H (an E2 ligase enzyme) by endogenous and exogenous immunoprecipitation; the UBE2H knockdown

abrogates MG53-induced IRS-1 ubiquitination, indicating that MG53 induces IRS-1 ubiquitination with the help of E2 ligase UBE2H (25). Based on these studies, we propose a model for MG53-mediated ubiquitination of IRS-1 and its implication in insulin signaling in skeletal muscle. MG53 interacts with IRS-1 in the lipid rafts or caveolae of the sarcolemma and induces IRS-1 ubiquitination with the help of E2 ligase enzyme, UBE2H (Fig. 1C). The ubiquitinated IRS-1 is degraded by proteasome. In the presence of MG53, insulin signaling is decreased because of the low level of IRS-1 protein, leading to the blockage of PI3K-Akt pathway. Thus, GLUT4-containing intracellular vesicles do not fuse with the plasma membrane, leading to the lack of GLUT4 in the plasma membrane and reduced glucose uptake in the skeletal muscle. Glucose and insulin tolerance test and serum level of insulin, leptin, triacylglycerol, free fatty acids and cholesterol show that the HFD-induced insulin resistance was reduced in MG53-disrupted mice.

DEVELOPMENT OF RECOMBINANT MG53 PROTEIN AS THERAPEUTIC AGENT FOR REGENERATIVE MEDICINE

The primary amino acid sequence of MG53 does not contain glycosylation or other post-translational modification motifs, raising the feasibility of using an *E. coli* expression system to produce functional rhMG53 proteins (17, 18, 20, 22, 56, 76, 79). The chemistry, manufacture and control (CMC) process for rhMG53 has been established that allows for scale-up production of rhMG53 to support our pre-clinical and future clinical studies. The purification protocol of rhMG53 protein contains three chromatography steps. The expressed rhMG53 protein is first enriched by an anion-exchange column from the soluble supernatant of *E. coli* lysate under a defined buffer solution. Fractions that contain rhMG53 protein were pulled and subjected to a hydrophobic column, which resulted in purification of rhMG53 to greater than 85% purity. After passing through the anion-exchange column for the second time, a final preparation of rhMG53 with greater than 97% purity was achieved, through elution by a slightly different buffer composition. The purified rhMG53 protein can be stored as a lyophilized powder, and the lyophilized rhMG53 protein be functionally reconstituted in saline solution, which allows for convenient application as an injectable reagent.

To quantify the effect of rhMG53 in ameliorating membrane damage, we developed an *in vitro* cell population membrane damage assay. In this assay, we measure the quantity of lactate dehydrogenase (LDH), which leaks from the cell interior into the extracellular solution following injury to the cell membrane (17, 18, 56). We validated this assay with several different forms of injury, including anoxia/reoxygenation, chemical, and mechanical damage with mirco-glass beads. We found that applying rhMG53 to the extracellular environment can reduce the degree of membrane damage in a dose-dependent manner. The boiled rhMG53 protein is inefficient due to denaturation of the protein. Using this assay we showed that rhMG53 is more potent at minimizing membrane injury at lower concentrations than other agents, such as poloxamer 188 (P188) (17).

As part of our translational effort, it is important that the safety and pharmacokinetic (PK) property of rhMG53 in circulation was established. Preliminary evaluation of rhMG53 in mice pharmacology studies demonstrated no overt signs of toxicity (17). When MG53 was administered intravenously (i.v.) once daily (6 mg/kg) to mice for two weeks, no changes in body weight, serum levels of inflammatory cytokines (IL-6 and TNFα), serum levels of ALT (alanine aminotransferase), LDH (lactate dehydrogenase), or changes in the histopathology of the liver, kidney, lung, spleen and cardiac muscle were reported (17). Recently, we conducted a pilot study with repetitive i.v. dosing of rhMG53 in a beagle dog model (22). Histological analyses did not reveal gross abnormality within major vital organs, including heart, lung, kidney, liver, brain and spleen, indicating that the dogs could tolerate repetitive exposure to rhMG53. ELISA determination of serum levels of rhMG53 showed the PK of MG53 remained unchanged from the beginning (first dose) to the end of i.v. administration (seventh dose), with a half-life of ~1.4 h at both time points. Thus, systemic administration of rhMG53 did not appear to produce neutralizing antibodies, as peripheral tolerance to MG53 had already occurred in body circulation (22). From this study, we can conclude that rhMG53 can be a safe biological agent for treatment of tissue injuries.

One advantage to the use of rhMG53 as a protein therapeutic regent in regenerative medicine is that it can be produced effectively in different host cells, including both bacteria and mammalian cells, which can simplify production of the protein for preclinical studies and clinical trials. We also showed that rhMG53 can be applied to animal models using subcutaneous injection (17), thus the protein could potentially be self-administered by patients without the need for professional hospital visits. From the pharmacokinetic measurement of MG53, we know that rhMG53 has limited half-life in circulation (~1.5 hour) in rodents and canines (17, 22). Thus, a sustainable delivery mechanism will be necessary for prevention of chronic muscle injury.

To further understand the role of circulating MG53 as a myokine for tissue protection, we recently developed a transgenic mouse model that allows for tailored control of MG53 secretion, providing ways to examine the efficacy for circulating MG53 in ameliorating the pathology of skeletal myopathy in animal models of muscular dystrophy, as well as for evaluating the safety profile for sustained MG53 in affecting other organ functions. We constructed a transgene by adding a tissue plasminogen activator (tPA) leader sequence ahead of the MG53 cDNA (tPA-MG53) to allow for secretion of MG53 into the bloodstream (79). The tPA-MG53 transgene was cloned behind a muscle-specific promoter for generation

of the transgenic mouse model. The tPA-MG53 mice are healthy with enhanced lifespan, and display improved muscle function under stress conditions with lesser degree of fibrotic remodeling compared with the wild type mice. Moreover, the tPA-MG53 mice are resistant to ischemia-reperfusion induced brain injury, suggesting a neuroprotective role for the circulating MG53 in the bloodstream (79).

CONCLUSION

Identification of the dual function of MG53 as a component of cell membrane repair and modulator of insulin signaling opens many avenues of cell biological research and translational applications. Given the key contribution of compromised membrane integrity and repair to the pathophysiology of muscular dystrophy and other human diseases, the application of rhMG53 as a "molecular bandage" (80) may have significant value for improving the regenerative capacity of the injured cells to prevent or treat the underlying cause of degenerative human diseases. While the expression of native MG53 is primarily restricted to striated muscle cells, one can express the MG53 cDNA in non-muscle cells and recapitulate the expected membrane repair function to boost their regenerative capacity under stress conditions. As an E3-ligase that controls function of IRS-1 and insulin signaling in muscle cells, MG53 can potentially modulate glucose uptake into skeletal muscle and the overall glucose metabolism and homeostasis. Molecular or pharmacological approaches that target MG53 and IRS-1 interaction without disruption of membrane repair function for MG53 may offer effective means for treating metabolic disorders that are associated with insulin resistance.

Song *et al.* (24) reported MG53 expression was markedly elevated in animal models of insulin resistance. However, their proposed role for MG53-mediated IRS-1 down-regulation in the manifestation of metabolic disorders lacks a biological base. Separate report by Yi *et al.* (25) and other investigators (81-84) presented no evidence for MG53 upregulation in diabetes, and muscle samples derived from human diabetic patients and mice with insulin resistance showed normal expression of MG53. There are three additional homologous proteins in the IRS family, IRS-2, IRS-3, and IRS-4, and all contribute to insulin signal transduction. Studies by Terauchi *et al.* (85) and Tamemoto *et al.* (86) showed that an absence of IRS-1 is not sufficient to induce type II diabetes. IRS-3 deficiency also does not alter glycemic regulatory capabilities (87). Other elegant studies also demonstrate that mice with double ablation of IRS-1 and IRS-2 do not exhibit abnormalities in glucose homeostasis (87, 88). Interestingly, only through a combination of IRS-1 and IRS-3 deficiency are diabetic phenotypes observed, indicating that IRS-1 and IRS-3 serve overlapping physiological functions in insulin signaling (87). Thus, MG53-mediated IRS-1 down-regulation cannot induce type II diabetes through insulin resistance, as the existence of IRS-3 compensates for IRS-1 absence.

We recently showed that circulating MG53 levels actually decrease in animals with metabolic syndrome, and sequestration of MG53 at the mitochondria may serve protective roles for MG53 in metabolic diseases (81). In a recent study, Liu *et al.* (89) showed that gross cardiac-specific overexpression of MG53 induces diabetic cardiomyopathy via transcriptional activation of PPAR-α. In their mhc-MG53 transgenic model, the protein level for MG53 was increased by more than 5 fold in the heart, and could lead to uncontrolled activation for MG53 in the nucleus. Importantly for our studies, in our tPA-MG53 mice, we found no evidence of cardiomyopathy, even at the age of 32 months of age (79). These results highlight the cardioprotective function for MG53 in the circulation, and also suggest possible different mechanisms of action for MG53 in intracellular vs extracellular activities. Clearly, more studies are required to dissect the role for the E3-ligase activity MG53 in regulation of metabolic function, because this is fundamental for our effort to translate the basic findings into clinical applications.

The TRIM family contains over 70 members in the human genome; however, the biological function of most of these proteins remain unexplored. It is possible that other TRIM family proteins may act in a similar fashion as MG53 in the membrane repair process, or they can interact with MG53 to regulate the overall cell membrane repair response. Searching for the interacting partners of MG53 and understanding their intracellular and extracellular actions in tissue repair and regeneration will require dedicated efforts of interdisciplinary approaches.

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