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Glucose-Sensitive Myokine/Cardiokine MG53 Regulates Systemic Insulin Response and Metabolic Homeostasis

Editorial, see p 915

Hong-Kun Wu, BS*
Yan Zhang, MD, PhD*
Chun-Mei Cao, MD, PhD*
et al

BACKGROUND: Mitsugumin 53 (MG53 or TRIM72), a striated muscle-specific E3 ligase, promotes ubiquitin-dependent degradation of the insulin receptor and insulin receptor substrate-1 and subsequently induces insulin resistance, resulting in metabolic syndrome and type 2 diabetes mellitus (T2DM). However, it is unknown how MG53 from muscle regulates systemic insulin response and energy metabolism. Increasing evidence demonstrates that muscle secretes proteins as myokines or cardiokines that regulate systemic metabolic processes. We hypothesize that MG53 may act as a myokine/cardiokine, contributing to interorgan regulation of insulin sensitivity and metabolic homeostasis.

METHODS: Using perfused rodent hearts or skeletal muscle, we investigated whether high glucose, high insulin, or their combination (conditions mimicking metabolic syndrome or T2DM) alters MG53 protein concentration in the perfusate. We also measured serum MG53 levels in rodents and humans in the presence or absence of metabolic diseases, particularly T2DM. The effects of circulating MG53 on multiorgan insulin response were evaluated by systemic delivery of recombinant MG53 protein to mice. Furthermore, the potential involvement of circulating MG53 in the pathogenesis of T2DM was assessed by neutralizing blood MG53 with monoclonal antibodies in diabetic *db/db* mice. Finally, to delineate the mechanism underlying the action of extracellular MG53 on insulin signaling, we analyzed the potential interaction of MG53 with extracellular domain of insulin receptor using coimmunoprecipitation and surface plasmon resonance assays.

RESULTS: Here, we demonstrate that MG53 is a glucose-sensitive myokine/cardiokine that governs the interorgan regulation of insulin sensitivity. First, high glucose or high insulin induces MG53 secretion from isolated rodent hearts and skeletal muscle. Second, hyperglycemia is accompanied by increased circulating MG53 in humans and rodents with diabetes mellitus. Third, systemic delivery of recombinant MG53 or cardiac-specific overexpression of MG53 causes systemic insulin resistance and metabolic syndrome in mice, whereas neutralizing circulating MG53 with monoclonal antibodies has therapeutic effects in T2DM *db/db* mice. Mechanistically, MG53 binds to the extracellular domain of the insulin receptor and acts as an allosteric blocker.

CONCLUSIONS: Thus, MG53 has dual actions as a myokine/cardiokine and an E3 ligase, synergistically inhibiting the insulin signaling pathway. Targeting circulating MG53 opens a new therapeutic avenue for T2DM and its complications.

*Drs Wu, Y. Zhang, and Cao contributed equally.

The full author list is available on page 912.

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Clinical Perspective

What Is New?

- We have provided multiple lines of evidence that mitsugumin 53 (MG53) is myokine/cardiokine secreted from striated muscle in rodents and humans in response to high glucose or insulin via a canonical secretory pathway.
- Serum MG53 levels are elevated in rodents and humans with obesity and type 2 diabetes mellitus.
- Intravenous administration of recombinant MG53 inhibits insulin response in multiple organs, whereas neutralizing blood MG53 with monoclonal antibodies ameliorates hyperglycemia and enhances insulin sensitivity in diabetic *db/db* mice.
- Circulating MG53 binds to the extracellular domain of the insulin receptor, allosterically inhibiting insulin signaling.

What Are the Clinical Implications?

- Our findings define MG53 as a metabolism-regulated myokine/cardiokine and the increase in circulating MG53 as a pathogenic factor of systemic insulin resistance and type 2 diabetes mellitus.
- Circulating MG53 constitutes an important therapeutic target and a biomarker of insulin resistance and type 2 diabetes mellitus.
- Targeting extracellular MG53 by immunotherapy as demonstrated in this study or by chemical blockade opens an exciting new avenue for the treatment of type 2 diabetes mellitus and its complications.

Nutrient overload and physical inactivity cause metabolic syndrome, which features systemic insulin resistance, hyperglycemia, dyslipidemia, hypertension, and central obesity.[1,2] Metabolic syndrome predisposes to cardiovascular disease and type 2 diabetes mellitus (T2DM), and a combination of these conditions (ie, cardiometabolic diseases) is the leading cause of death around the world.[3] Accounting for 40% of the body mass of lean individuals, skeletal muscle plays a pivotal role in maintaining whole-body metabolic homeostasis. In particular, nutrient-derived, insulin-dependent glucose uptake occurs mainly in skeletal muscle via glucose transporter 4–mediated transport. Previous studies have shown that muscle insulin resistance is the earliest defect of various metabolic diseases, including obesity and T2DM.[4] Emerging evidence marks a striated muscle-specific protein, mitsugumin 53 (MG53; also known as TRIM72), as an important pathogenic factor of metabolic syndrome and the resultant cardiometabolic diseases.[5–7] As a muscle-specific E3 ligase, MG53 impairs the insulin signaling pathway via targeting the insulin receptor (IR) and IR substrate 1 (IRS1) for ubiquitin-dependent degradation, resulting in muscle insulin resistance.[6,7] However, muscle insulin resistance per se is insufficient to cause obesity and full-blown metabolic syndrome.[8] A key question is how MG53 from muscle regulates whole-body insulin sensitivity and metabolic homeostasis.

It has been shown that striated muscle secretes proteins as myokines or cardiokines that can act in an autocrine, a paracrine, or an endocrine fashion to regulate whole-body metabolic processes, suggesting a central role of muscle in the interorgan regulation of insulin responsiveness and energy homeostasis.[9–11] For example, skeletal muscle secretes myostatin, irisin, myonectin, and interleukin-6,[12–15] whereas the heart secretes atrial natriuretic factor, brain natriuretic factor, adiponectin, follistatin-like 1, and growth differentiation factor-15.[16–20] Some of the secreted proteins circulate in the bloodstream and alter whole-body energy homeostasis.[9–11] Our hypothesis is that MG53 from striated muscle may act as a myokine/cardiokine in response to metabolic stimuli, leading to systemic insulin resistance and metabolic disorders.

Here, we have provided in vivo and in vitro evidence that high glucose or high insulin induces MG53 secretion from striated muscle. Under disease circumstances, blood MG53 level is elevated in humans and rodents with diabetes mellitus. Increased circulating MG53 suffices to induce systemic insulin resistance and metabolic syndrome, whereas neutralizing blood MG53 with a monoclonal antibody has therapeutic effects in diabetic *db/db* mice. Mechanistically, we have shown that MG53 binds to the IR extracellular domain (ECD), allosterically inhibiting the receptor. These findings demonstrate that, as a myokine/cardiokine, circulating MG53 antagonizes whole-body insulin signaling, acting in synergy with intracellular MG53 E3 ligase activity in striated muscle. Targeting extracellular MG53 opens a new avenue for the treatment of diabetes mellitus and its complications.

METHODS

The data, analytical methods, and study materials are available to other researchers for purposes of reproducing the results or replicating the procedure. All the materials are available in our laboratory in Peking University. Detailed methods are provided in the online-only Data Supplement.

MG53 Antibody Therapy for *db/db* Mice

Diabetic *db/db* mice (8–10 weeks old) were randomly allocated to either immunoglobulin G (IgG) or MG53 monoclonal antibody (anti-MG53) treatment (1.5 mg IV per animal). Body weight and blood glucose were recorded daily for 1 week, and insulin tolerance tests (2 U/kg) were performed 7 days after treatment.

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Statistical Analysis

Statistical parameters and significance are reported in the figures and the figure legends.

Data are expressed as mean±SEM. Statistical analysis was performed with GraphPad Prism version 5.01 and the SPSS 18.0 software package (SPSS Inc). Data sets were tested for normality of distribution with the Kolmogorov-Smirnov test. Data groups (2 groups) with normal distributions were compared by use of 2-sided unpaired Student *t* test, unless specifically indicated. Comparisons between multiple groups were assessed by 1-way ANOVA with Bonferroni post hoc analysis. Pearson correlation coefficient and *P* value were used to evaluate the degree of correlation between 2 groups of variables. No statistical method was used to predetermine sample size.

Study Approval

All procedures involving experimental animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Peking University, Beijing, China, and conformed to the *Guide for the Care and Use of Laboratory Animals* by *Association for Assessment and Accreditation of Laboratory Animal Care* (8th edition, 2011).

RESULTS

High Glucose– and High Insulin–Induced MG53 Release From Isolated Perfused Rodent Hearts

We first determined whether MG53 is released into the extracellular fluid and subsequently enters circulation in a regulated fashion. In the isolated rodent hearts under Langendorff perfusion with modified Krebs-Henseleit solution, we detected robust MG53 release by Western blotting analysis of the outlet perfusate accumulated over 30-minute periods in rat hearts and 60-minute periods in mouse hearts (Figure 1 and Figure IA in the online-only Data Supplement). High glucose (25 mmol/L) augmented rat myocardial MG53 release by 3-fold in the first 30 minutes after stimulation (Figure 1A). Elevated MG53 release continued during the second 30 minutes (30–60 minutes) of glucose challenge and was graded in a glucose concentration–dependent manner (Figure 1A and 1B). Meanwhile, the myocardial MG53 level was decreased after a 60-minute high-glucose treatment (Figure IB in the online-only Data Supplement). In sharp contrast, L-glucose, which cannot be utilized by organisms, failed to alter myocardial MG53 release (Figure 1C). To mimic the conditions of metabolic syndrome and T2DM, we investigated the effect of combinatorial stimulation by glucose and insulin. Insulin stimulation with a basal concentration of glucose (11.1 mmol/L) augmented the myocardial MG53 release in isolated perfused rat hearts in a time- and concentration-dependent manner (Figure 1D and 1E). The copresence of high glucose and insulin stimulated MG53 release at a rate ≈3.4-fold that at baseline (Figure 1F). In contrast to the previous reports that circulating MG53 is the result of passive leakage from damaged muscle,[21,22] the MG53 release triggered by high glucose plus insulin occurred in the absence of any change in myocardial lactate dehydrogenase or creatine kinase release (indexes of loss of cell membrane integrity) in perfused rat hearts (Figure IC and ID in the online-only Data Supplement), indicating that metabolism-regulated MG53 release is not caused by myocardial damage. Furthermore, using MG53 overexpression (*mg53 TG*) and knockout (*mg53*$^{-/-}$) mouse models[6] (Figure IE in the online-only Data Supplement), we demonstrated that MG53 release induced by combined glucose and insulin stimulation was more than doubled from the *mg53 TG* heart compared with the wild-type (wt) heart but was undetectable in the *mg53*$^{-/-}$ heart (Figure 1G and 1H), validating the identity of the released protein detected by Western blotting.

High Glucose and Insulin Trigger MG53 Release in Isolated Skeletal Muscle and In Vivo

In addition to myocardium, the combined treatment of high glucose (25 mmol/L) and insulin (10 U/L) effectively triggered MG53 release from isolated perfused skeletal muscle (soleus) from wt mice (Figure 2A and Figure IF in the online-only Data Supplement) but not *mg53*$^{-/-}$ mice (Figure IG in the online-only Data Supplement). Glucose-induced MG53 release from skeletal muscle occurred in the absence of any increase in the perfusate lactate dehydrogenase or creatine kinase concentration (Figure IH and I, part I in the online-only Data Supplement). This result indicates that metabolic stimulation (high glucose or insulin) enhances skeletal muscle and myocardium to release MG53 in a physiological context. Because skeletal muscle accounts for ≈40% of body mass of lean individuals, we speculate that high glucose–induced MG53 release would be sufficient to elevate circulating MG53 levels in vivo. Currently, no reliable ELISA kits are available for the quantification of mouse or human serum MG53. Therefore, we screened >40 custom-made monoclonal antibodies and identified a few high-affinity antibodies (anti-MG53 No. 3 and anti-MG53 No. 90) that specifically reacted with only mouse (No. 3) or both mouse and human (No. 90) MG53 (Figure IIA in the online-only Data Supplement). Western blotting with anti-MG53 No. 90 revealed that serum MG53 was undetectable in *mg53*$^{-/-}$ mice (Figure IIB in the online-only Data Supplement) but was enriched in *mg53 TG* mice (Figure IIC in the online-only Data Supplement), validating the robustness and specificity of the custom anti-MG53 for serum MG53 measurements. Intraperitoneal injection of glucose into wt mice elevated the serum MG53 level, which was maintained for at least 4 hours and returned

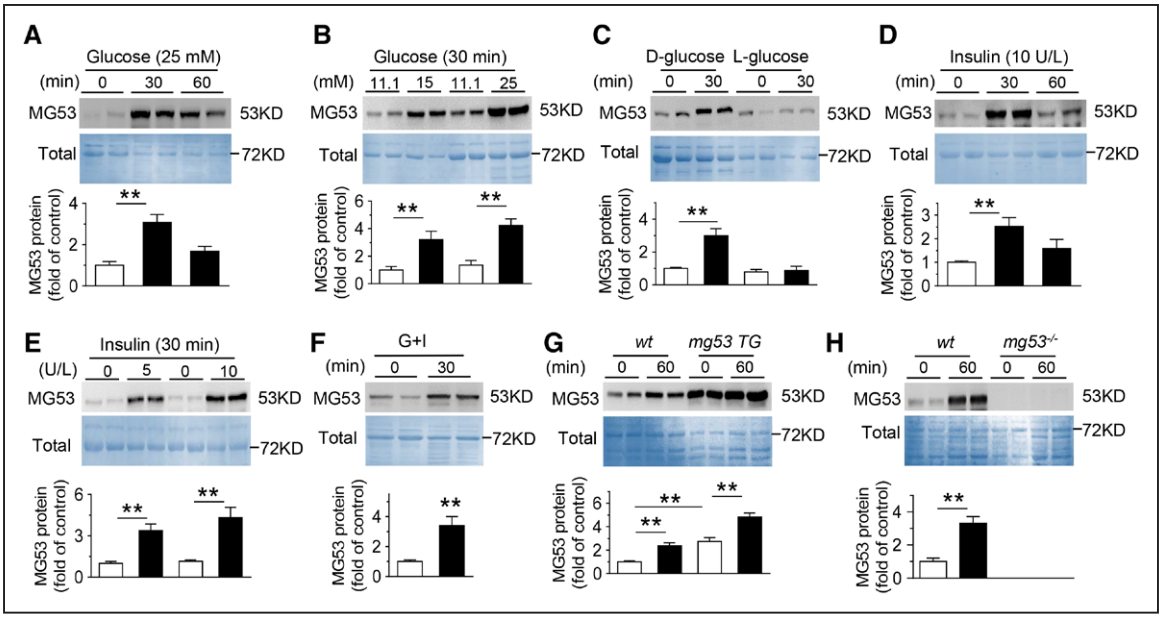

Figure 1. High glucose– and insulin–induced mitsugumin 53 (MG53) release in Langendorff-perfused rodent hearts.
A and **B**, Representative Western blots (**top**) and averaged data (**bottom**) showing that high glucose increased MG53 levels in perfusate in a time- and concentration-dependent manner in isolated perfused rat heart (n=12 hearts for **A** and 10 for **B**). **C**, Perfusate MG53 in isolated rat heart perfused with high D-glucose (25 mmol/L) or L-glucose (13.9 mmol/L L-glucose in the presence of basal D-glucose at 11.1 mmol/L; n=10 per group). Data were obtained under basal conditions (0 minutes, 11.1 mmol/L D-glucose) and at 30 minutes after treatment. **D** and **E**, As in **A** and **B** except with insulin stimulation (n=11 for **D** and 6 for **E**). **F**, Metabolic stimulation with high glucose (25 mmol/L) combined with insulin (10 U/L) (G+I) in isolated perfused rat heart (n=15). **G** and **H**, Perfusate MG53 was more abundant in *mg53 TG* mouse heart than in wild-type (wt) control heart under basal and metabolically stimulated conditions (G+I, 25 mmol/L glucose combined with 10 U/L insulin; **G**) but was undetectable in *mg53*$^{-/-}$ mouse heart (**H**; n=8 per group). In all bar graphs, data are normalized to the corresponding nonspecific bands (Total), which were obtained through Brilliant Green staining, and are presented as mean±SEM. $^{**}P<0.01$, 1-way ANOVA (**A** through **E**, **G**, **H**) or Student *t* test (**F**). The control of each bar graph is the first pair of samples of each gel.

to the baseline within 6 hours (Figure 2B and Figure IID in the online-only Data Supplement). More importantly, oral administration of glucose also significantly elevated serum MG53 levels (Figure 2C), along with increased blood glucose levels, in healthy humans in the absence of change of serum lactate dehydrogenase concentrations (Figure IIE–IIG and Table I in the online-only Data Supplement). Hyperglycemia-associated increases in serum MG53 in humans were validated in mice and humans by commercially available antibodies (Figure IIIA–IIIE in the online-only Data Supplement). Because recent studies have shown that the human heart expresses little or no MG53,[22] high glucose–induced increase in serum MG53 levels may originate mainly from skeletal muscle in human. Thus far, we have provided ex vivo and in vivo evidence indicating that MG53 is a glucose-sensitive myokine in both rodents and humans.

Glucose/Insulin-Induced MG53 Release Is Mediated by a Regulated Secretory Pathway

Using pharmacological and genetic approaches, we deciphered the mechanism underlying the glucose- or insulin-induced MG53 release. First, to distinguish regulated MG53 secretion from passive leakage, we evaluated the effect of Brefeldin A, a protein transport inhibitor.[23] Brefeldin A suppressed high glucose–induced MG53 release in a dose-dependent manner in C2C12 cells infected with adenovirus expressing MG53 (Figure 2D). Similar results were observed in HEK293 cells expressing MG53-myc (Figure IIH in the online-only Data Supplement). In isolated perfused rat hearts, Brefeldin A (30 μmol/L) also decreased glucose-induced MG53 release (Figure 2E), suggesting that glucose-triggered MG53 release is mediated by a canonical secretory pathway. In most cases of regulated secretion, a rise in the cytosolic Ca^{2+} concentration is a prerequisite for fusion of the secretory-vesicle membrane with the plasma membrane and the release of the vesicle contents by exocytosis.[24] Indeed, buffering extracellular Ca^{2+} with EDTA (0.75 mmol/L) markedly reduced the high glucose combined with insulin–triggered MG53 release (Figure 2F). Furthermore, the SNARE-binding protein synaptotagmin 7 is an important Ca^{2+} sensor involved in neuronal and hormonal exocytosis[25,26] and is expressed in mouse heart and skeletal muscle (Figure II, part I in the online-only Data Supplement). In the *syt7*$^{-/-}$ mice,[27] MG53 release stimulated by high glucose plus insulin was markedly attenuated in isolated perfused hearts and in vivo (Figure 2G and 2H and Figure IIJ in the online-only Data Supplement), indicating that the SNARE-binding protein synaptotagmin 7 is required for metabolism-regulated MG53 secretion. Taken together, we conclude that high glucose–induced MG53 release is mediated by a Ca^{2+}- and SNARE binding protein–de-

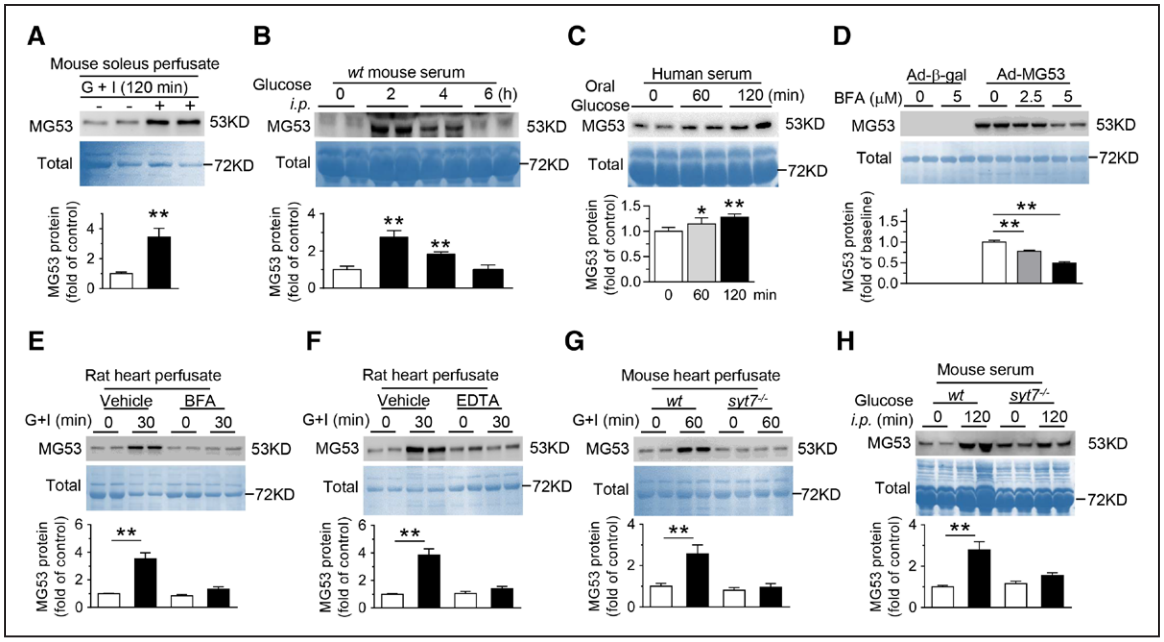

Figure 2. High glucose– and insulin–induced mitsugumin 53 (MG53) secretion in isolated skeletal muscle and in vivo.
A, Representative Western blots (**top**) and averaged data (**bottom**) showing MG53 level in the perfusate of mouse soleus muscle with or without a high concentration of glucose (25 mmol/L) and insulin (10 U/L) (G+I) treatment. (n=12). **B**, Representative Western blots and averaged data showing that hyperglycemia (glucose, 2 g/kg IP, 3 repeats at 40-minute intervals) increased serum MG53 in wild-type (wt) mice (n=8). **C**, Representative Western blots and averaged data of serum MG53 from healthy humans before and after oral glucose administration (75 g twice at a 30-minute interval; n=8). **D**, Brefeldin A (BFA) dose-dependently suppressed MG53 release in C2C12 cells. Cells were infected with adenovirus expressing β-galactosidase (Ad-β-gal) or adenovirus expressing MG53 (Ad-MG53) and incubated with high glucose (25 mmol/L) for 3 hours (n=8). **E**, BFA (30 μmol/L) blocked the increase of perfusate MG53 induced by high glucose (25 mmol/L) combined with insulin (10 U/L) (G+I) in isolated perfused rat heart (n=10). **F**, EDTA-Na (0.75 mmol/L) blocked the increase of perfusate MG53 induced by metabolic stress (G+I, 25 mmol/L glucose combined with 10 U/L insulin) in isolated perfused rat heart (n=10). **G**, Metabolic stimulation (G+I, 25 mmol/L glucose combined with 10 U/L insulin) elevated MG53 release from wt but not *syt7*$^{-/-}$ mouse heart (n=10). **H**, Hyperglycemia (glucose, 2 g/kg IP, 3 repeats at 40-minute intervals) increased serum MG53 in wt but not *syt7*$^{-/-}$ mice (n=10). In all **bottom** panels, data are normalized to the corresponding nonspecific bands (Total), which were obtained through Brilliant Green staining, and are presented as mean±SEM. **P*<0.05, ** *P*<0.01, Student *t* test (**A**) or 1-way ANOVA (**B** through **H**).

pendent secretory mechanism, as is the case for many types of cytokines.[28]

Diabetic Hyperglycemia Is Accompanied by Increased Serum MG53 Levels

To appraise the potential physiological and pathological relevance of the above findings, we measured serum MG53 together with other metabolic parameters (including blood glucose, insulin, and lipid levels) in humans and animals with T2DM. The serum MG53 level was assessed by Western blotting with the anti-MG53 No. 90 and multiple commercially available antibodies reacting with both mouse and human MG53 (Figures IIA and Figure IIIA–IIIE in the online-only Data Supplement). We found that serum MG53 level was increased in patients with T2DM and that the serum MG53 abundance correlated with blood glucose and insulin levels (Figure 3A and Figure IIIB–IIIE in the online-only Data Supplement). Similar results were observed in mice on a high-fat diet for 35 weeks (Figure 3B). In both T2DM humans and high-fat diet–treated mice, the increases in circulating MG53 were accompanied by increased body mass index, hyperglycemia, and hyperinsulinemia (Figure IIIF–IIIK and Table II in the online-only Data Supplement). Next, taking advantage of an ELISA kit that works in rats, we determined whether there is a quantitative correlation between serum MG53 and other metabolic readouts in Zucker diabetic fatty rats. On a Purina diet for 4 weeks, Zucker diabetic fatty rats developed T2DM, as manifested by severe hyperglycemia and glucose intolerance (Figure IVA and IVB in the online-only Data Supplement), as well as hyperinsulinemia, dyslipidemia, and increased body weight (Figure IVC–IVF in the online-only Data Supplement). Serum MG53 levels were significantly elevated in the diabetic rats compared with the control group (Figure 3C). T2DM-associated increase of MG53 was closely correlated with fasting blood glucose and insulin levels, as well as body weights (Figure 3D–3F). Thus, diabetic hyperglycemia and hyperinsulinemia are positively correlated with elevated serum MG53 levels.

Cardiac-Specific Overexpression of MG53 Is Sufficient to Trigger Systemic Insulin Resistance and Metabolic Syndrome

To further investigate a possible causal relationship between long-term elevation of MG53 in the blood and the development of metabolic disorders, we used the

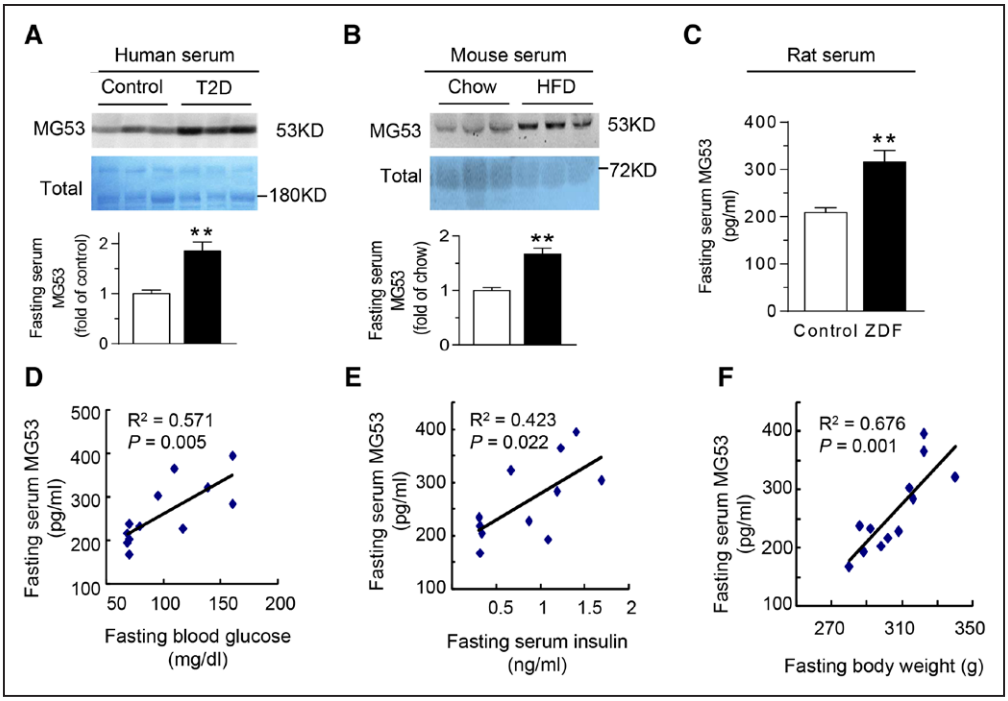

Figure 3. Diabetic hyperglycemia is associated with increased serum mitsugumin 53 (MG53) levels.
A and **B**, Representative Western blots and averaged data showing an increase in serum MG53 levels in humans with or without type 2 diabetes mellitus (T2DM; control, n=21; T2DM, n=37; **A**) or in mice fed standard chow (n=15) or a high-fat diet (HFD; n=18) for 35 weeks (**B**). Data are normalized to the nonspecific (Total) bands. **C**, Serum MG53 levels in control and Zucker diabetic fatty (ZDF) rats at 12 weeks of age (n=6 per group), assessed by ELISA assay. **D** through **F**, Correlation between serum MG53 level and fasting blood glucose (**D**), fasting serum insulin levels (**E**), and fasting body weight (**F**) in control and ZDF rats at 12 weeks of age. All parameters were measured under fasting conditions. For **A** and **B**, data are normalized to the corresponding nonspecific bands (Total), which were obtained through Brilliant Green staining. Data are mean±SEM. ***P*<0.01, Student *t* test (**A** through **C**) or Pearson correlation coefficient (**D** through **F**).

transgenic mice with heart-specific overexpression of MG53 (*mg53 h-TG*), as described previously.[5] We reckoned that the myocardial MG53 secretion would provide a constant supply of circulating MG53, whereas the MG53 expression would be unperturbed in skeletal muscle (Figure VA in the online-only Data Supplement). We found that compared with wt animals, the blood glucose and insulin levels of *mg53 h-TG* mice, along with MG53 abundance in the heart and serum, were increased as early as 1 week of age (Figure VB–VE in the online-only Data Supplement), in the absence of change in body weight at the early time point. At 14 weeks of age, the basal serum MG53 level was further increased in *mg53 h-TG* mice relative to wt littermates (Figure 4A). Moreover, the young *mg53 h-TG* mice exhibited moderate obesity (Figure 4B), glucose intolerance, and insulin intolerance (Figure 4C and 4D), although the heart did not show significant morphological or contractile abnormalities at this age (Figure VF–VM in the online-only Data Supplement). At 30 weeks of age, cardiac-specific overexpression of MG53 triggered full-blown metabolic syndrome, as manifested by hyperglycemia, hyperinsulinemia, and dyslipidemia (Figure 4E–4G), abdominal fat accumulation, and increased white fat, brown fat, and fat-to-lean ratio (Figure 4H–4L), together with hepatosteatosis and pancreatic islet hypertrophy (Figure 4M–4O and Figure VN in the online-only Data Supplement), without marked change of skeletal muscle morphology (Figure 4P). As systemic insulin resistance evolved, the *mg53 h-TG* mice displayed more severe obesity, glucose intolerance, and insulin intolerance at this time point (Figure 4B–4D). Meanwhile, the daily energy expenditure of *mg53 h-TG* mice was significantly lower than that of the wt counterpart (Figure 4Q–4S), but there were no differences in their daily food intake, core body temperature, or physical activity (Figure VO–VQ in the online-only Data Supplement). At the later time point, *mg53 h-TG* mice also developed diabetic cardiomyopathy.[5] Taken together, we have shown that increased circulating MG53 precedes the development of systemic insulin resistance, metabolic syndrome and cardiomyopathy in cardiac-specific MG53 transgenic mice, suggesting that long-term elevation of circulating MG53 impairs the whole-body insulin response and secondarily leads to obesity, diabetes mellitus, and various cardiovascular complications.

Administration of Recombinant MG53 Protein Attenuates the Multiorgan Insulin Response in Mice

The above data strongly suggest that circulating MG53 directly affects the insulin responsiveness of multiple organs. To further validate this possibility, recombinant

human MG53 (rhMG53) protein from *Escherichia coli* (Figure VIA in the online-only Data Supplement) or BSA (6 mg/kg IV) was administered to fasting male C57 mice 10 minutes before insulin injection (1 U/kg IP), and its direct effect on multiorgan insulin sensitivity, as indexed by insulin-induced Akt phosphorylation, was assessed 10 minutes after insulin stimulation (Figure VIB in the online-only Data Supplement). Notably, recombinant human rhMG53 attenuated the insulin-induced Akt phosphorylation in all tissues examined, including skeletal muscle, liver, visceral fat, and the heart (Figure 5A). These results also suggest that both intracellular MG53 and extracellular MG53 converge on the insulin signaling pathway and synergistically blunt the insulin response in striated muscle.

A Monoclonal Antibody Neutralizing Blood MG53 Improves Hyperglycemia and Enhances Insulin Sensitivity in Diabetic *db/db* Mice

The identification of MG53 as a novel hyperglycemia-inducible myokine/cardiokine implies that targeting serum MG53 may provide an effective means of blocking MG53-mediated interorgan communication, thereby alleviating the progression of cardiometabolic diseases. In particular, the application of neutralizing monoclonal antibodies against MG53 might offer an immunotherapeutic strategy. In diabetic *db/db* mice at 8 to 10 weeks of age, elevated blood MG53 was accompanied by profound obesity, dyslipidemia, hyperglycemia, and hyperinsulinemia (Figure 5B and Figure VIC–VIG in the online-only Data Supplement). Treatment with anti-MG53 No. 90 (1.5 mg IV per animal), but not a control immunoglobulin (IgG), significantly ameliorated the diabetic hyperglycemia and improved the insulin sensitivity (Figure 5C and 5D). The single treatment of the anti-MG53 did not alter the body weight of the diabetic *db/db* mice during 1 week (Figure VIH in the online-only Data Supplement). The blood glucose–lowering effect of the anti-MG53 was maintained for at least a week (Figure 5C). Concomitantly, insulin tolerance tests performed at day 7 revealed enhanced insulin action after the anti-MG53 treatment (Figure 5D). Similarly, another custom-made monoclonal antibody (anti-MG53 No. 3) also exhibited glucose-lowering effect without altering body weight in diabetic *db/db* mice (Figure VI, part I and VIJ in the online-only Data Supplement). In wt mice, MG53 antibody No. 90 increased insulin sensitivity (Figure 5F) without altering the blood glucose or body weight (Figure 5E and Figure VIK in the online-only Data Supplement), suggesting that, even in the nondiabetic background, blocking MG53 in the blood enhances systemic insulin responsiveness. These results not only underscore a causal relationship between an elevation of serum MG53 and the development of systemic insulin resistance and T2DM but also provide the first proof-of-principle efficacy of immunotherapy treating T2DM based on the neutralization of serum MG53.

MG53 Binds to the ECD of the IR to Block Insulin Signaling

In search of the mechanism underlying the myokine/cardiokine action of MG53 to inhibit insulin signaling, we gathered multiple lines of evidence to demonstrate that MG53 can bind to the IR ECD. First, coimmunoprecipitation assays revealed a physical interaction between purified rhMG53 protein and the human IR ECD, which contains 28 to 956 amino acid residues of the IR[29] (Figure 6A). To further analyze the binding of MG53 to the IR ECD, we used surface plasmon resonance assays. The human IR ECD was immobilized on a CM5 sensor chip; the natural ligand, insulin, was used as a positive control; and BSA served as a negative control (Figure 6B and Figure VIIA in the online-only Data Supplement). Purified rhMG53 bound to the IR ECD (Figure 6C) with an average K_D of 8.0±2.8 nmol/L (n=5), which was even lower than the K_D of insulin (28±4 nmol/L; n=4). To map out the interface domain(s) of MG53 with the IR ECD, we produced recombinant MG53 fragments (Figure 6D) and found that the C-terminal domain (amino acids 58–477) and the SPRY domain (amino acids 271–477), but not the TRIM (amino acids 1–270) or the coiled-coil (amino acids 122–270) domain, could bind to the IR ECD (Figure 6E–6H), suggesting that the SPRY domain is required for MG53 binding to the IR. However, the C-terminal domain and the SPRY domain bound to the IR ECD with lower affinities relative to that of the full-length MG53, indicating that the intact conformation of MG53 is essential for its high-affinity binding to the IR ECD. Thus, the binding of MG53 to the IR ECD is direct and specific with a physiologically and pathologically relevant affinity.

In addition, we found that MG53, but not insulin or the IR ECD, bound to the neutralizing antibody (anti-MG53) with a high affinity (Figure VIIB–VIID in the online-only Data Supplement). The antibody markedly attenuated the MG53 interaction with the IR ECD in a dose-dependent manner (Figure 6I). In contrast, the control IgG could not bind to MG53 and thus did not affect MG53 binding to the IR ECD (Figure VIIE and VIIF in the online-only Data Supplement). Although extracellular MG53 was able to attenuate insulin signaling in cultured human hepatoma cells (Figure 7A), it did not alter the binding of insulin to the IR, as manifested by radioligand binding assay in C2C12 cells, HEK293 cells, and liver tissue (Figure 7B–7D). These results suggest that extracellular MG53 acts as an allosteric, rather than a competitive, blocker of the IR.

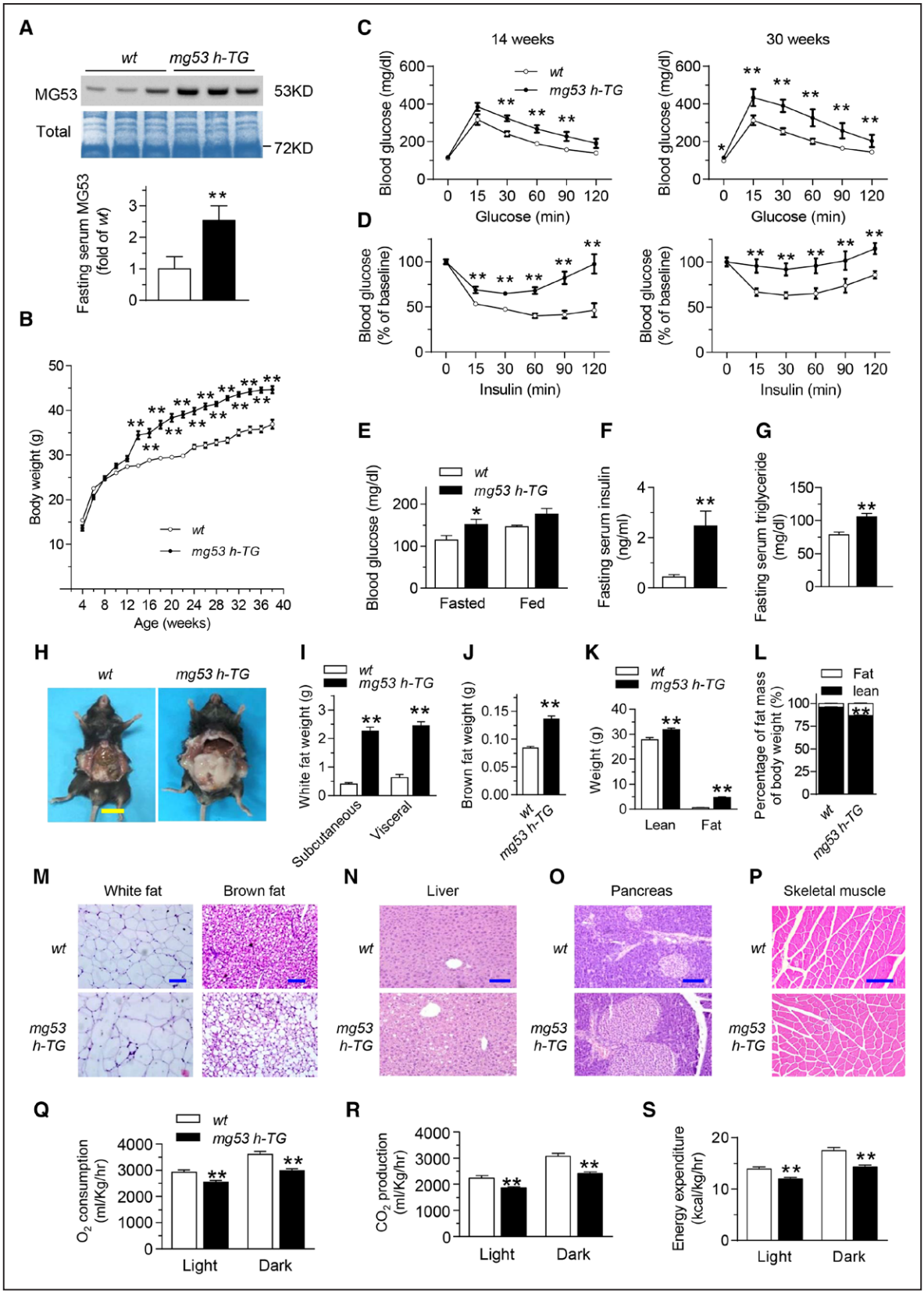

Figure 4. Cardiac-specific overexpression of mitsugumin 53 (MG53) leads to systemic insulin resistance and metabolic syndrome.
A, Representative Western blots and averaged data showing serum MG53 levels in wild-type (wt) and transgenic mice with cardiac-specific overexpression of MG53 (*mg53 h-TG*) at 14 weeks of age (n=12 for wt and 14 for *mg53 h-TG*). **B**, Fasting body weights of wt and *mg53 h-TG* mice from 3 to 38 weeks of age (n=28 for wt and 22 for *mg53 h-TG*). **C** and **D**, Glucose tolerance tests (**C**) and insulin tolerance tests (**D**) in wt and *mg53 h-TG* mice at the indicated ages (**C**: 14 weeks, n=9 for both WT and *mg53 h-TG*; 30 weeks, n=7 for wt and 6 for *mg53 h-TG*; **D**: 14 weeks, n=9 for wt and 10 for *mg53 h-TG*; 30 weeks, (*Continued*)

DISCUSSION

In the present study, we provide multiple lines of evidence that MG53 is a previously unappreciated glucose- and insulin-inducible myokine/cardiokine secreted from striated muscle. First, MG53 is secreted from striated muscle in rodents and humans in response to high glucose or insulin via a canonical secretory pathway. Second, serum MG53 levels are elevated in rodents and humans with obesity and T2DM. Third, intravenous administration of recombinant MG53 inhibits insulin response in multiple organs. Neutralizing blood MG53 with monoclonal antibodies ameliorates hyperglycemia and enhances insulin sensitivity in diabetic *db/db* mice, providing the proof of principle for MG53-based immunotherapeutic treatment of T2DM and related complications. Finally, we have demonstrated that circulating MG53 binds to the ECD of the IR without competitively inhibiting insulin binding. These in vitro and in vivo data indicate that MG53 secreted by striated muscle suppresses whole-body insulin sensitivity via its endocrine action. These findings not only unveil MG53 as a glucose- and insulin-regulated myokine/cardiokine, in addition to its intracellular E3 ligase activity for ubiquitin-dependent degradation of IR and IRS1,[6,7] but also establish a causal relationship between elevated serum MG53 and the development of systemic insulin resistance and T2DM. Similar to the situation of proprotein convertase subtilisin/kexin type 9–mediated regulation of low-density lipoprotein receptors and lipid metabolism,[30,31] intracellular and extracellular MG53 may exert mechanistically distinct yet biologically synergistic effects on IR signaling and glucose homeostasis. Targeting extracellular MG53 by immunotherapy, as demonstrated in this study, or by chemical blockade opens an exciting new avenue for the treatment of T2DM and its complications.

MG53 Suppresses Insulin Signaling Without Altering Skeletal Muscle Mass

Over the past decade, a plethora of proteins secreted from mouse and human muscle have been identified by proteomic approaches and referred to as myokines. For instance, myostatin, a muscle-specific member of the transforming growth factor-β superfamily,[32] is a versatile protein that has been implicated in the pathogenesis of insulin resistance and muscle wasting. Inhibition or deficiency of myostatin results in a phenotype of doubling muscle mass in humans and animal models.[33] In contrast, upregulation of myostatin leads to muscle atrophy,[34] resulting in muscle wasting and insulin resistance. As a myokine, myostatin also acts in an endocrine mode to modulate bone and adipose tissue remodeling and indirectly regulate metabolism.[35,36]

Similar to myostatin, MG53 is a multifunctional protein. However, upregulation of MG53 directly triggers insulin resistance and metabolic diseases, including obesity and T2DM, via its dual functions as a myokine/cardiokine and an E3 ligase, without altering muscle mass. Previous studies have shown that there are few or no abnormalities in skeletal muscle in genetic mouse models with MG53 deficiency or overexpression.[6,7] This conclusion is supported by the present findings that cardiac-specific overexpression of MG53 in mice elevates serum MG53 levels along with increased blood glucose and insulin without altering body weight as early as 1 week of age. The transgenic mice at 14 weeks of age develop systemic insulin resistance and metabolic disorders, in the absence of morphological or functional defects in cardiac and skeletal muscle, suggesting that increased circulating MG53 directly suppresses whole-body insulin response and secondarily induces metabolic disorders and cardiovascular complications. Indeed, short-term administration of recombinant MG53 protein leads to systemic insulin resistance. Thus, we conclude that MG53 directly targets the insulin signaling pathway without significantly affecting muscle mass under the present experimental conditions.

MG53 Expression and Secretion Are Subjected to Metabolic Control

While regulating multiorgan metabolic homeostasis, MG53 secretion is regulated by metabolic status. High glucose, high insulin, or their combination effectively induces MG53 secretion, whereas L-glucose, which cannot be utilized by organisms, has no such effect. Moreover, increases in circulating MG53 correlate with the progression of metabolic disorders such as hyperglycemia, hyperinsulinemia, and obesity in rodents and humans with T2DM. Because MG53 is more abundant in skeletal muscle than in the heart (especially in humans),[22] skeletal muscle is largely responsible for the secretion of MG53 into circulation and may play a more important role in the regulation of systemic metabolism than the heart. Under disease conditions, there is likely a vicious cycle from nutrient overloading, to sequential increases in MG53 expression and secretion, then to suppression of multiorgan insulin signaling, to impair-

Figure 4 Continued. n=8 for wt and 7 for *mg53 h-TG*). **E** through **G**, Blood glucose (**E**), fasting serum insulin (**F**), and fasting serum triglyceride levels (**G**) at 30 weeks of age (n=11 for wt and 13 for *mg53 h-TG*). **H** through **O**, Photographs taken during necropsy reveal fat deposit in the abdominal cavity (**H**), subcutaneous and visceral fat weight (**I**), brown fat weight (**J**), body lean and fat mass (**K**), body lean and fat mass as a percentage of body weight (**L**), and hematoxylin and eosin staining of white and brown fat (**M**), liver (**N**), pancreas (**O**), and skeletal muscle (**P**) of wt and *mg53 h-TG* mice at 30 weeks of age (n=11 for wt and 14 for *mg53 h-TG*). Scale bars for white fat, 50 μm; brown fat, 25 μm; liver, pancreas, and skeletal muscle, 100 μm. **Q** through **S**, Oxygen consumption (**Q**), CO_2 production (**R**), and daily energy expenditure (**S**) of wt and *mg53 h-TG* mice at 30 weeks of age (n=12). Data are mean±SEM. **P*<0.05, ***P*<0.01 vs wt, Student *t* test.

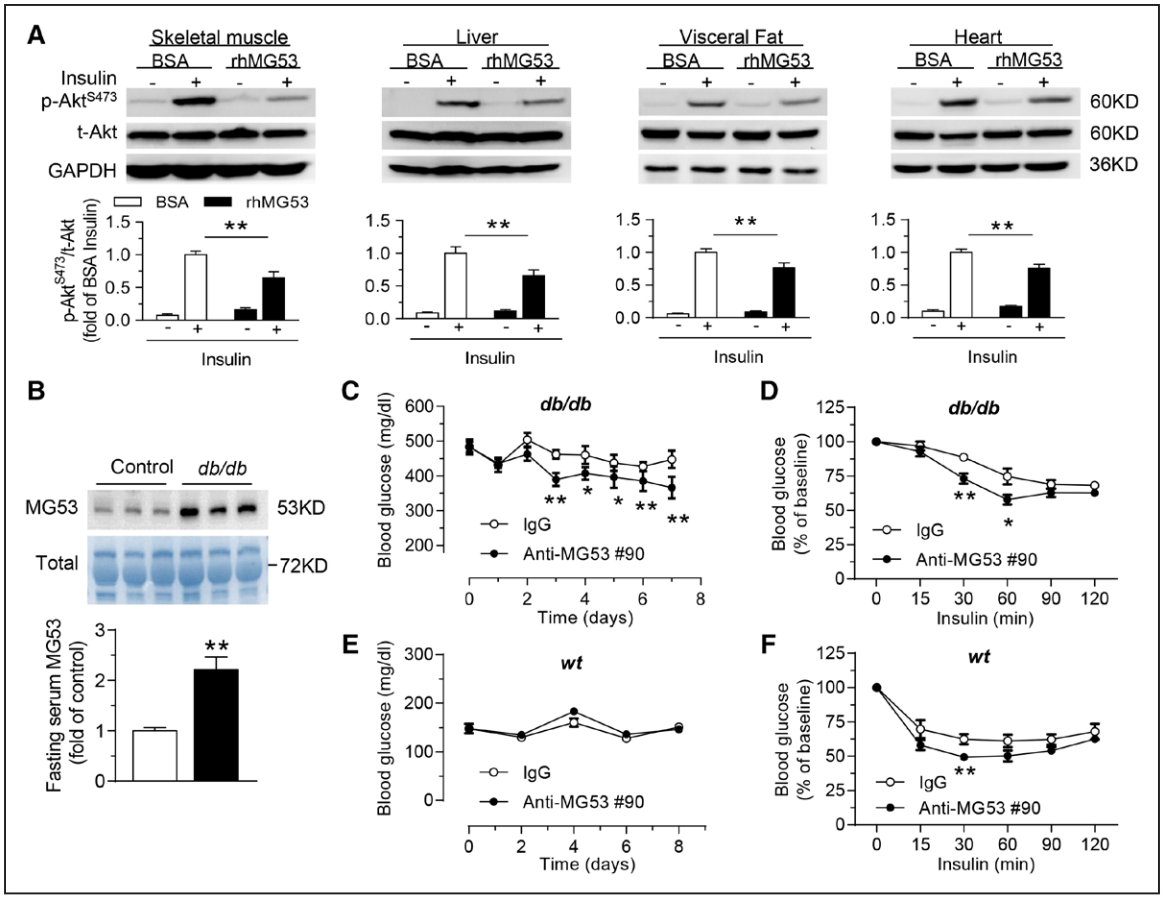

Figure 5. Recombinant mitsugumin 53 (MG53) blocks insulin action in normal mice, whereas neutralizing blood MG53 with the anti-MG53 has therapeutic effects in mice.

A, Recombinant human MG53 protein (rhMG53) blocked insulin-induced Akt phosphorylation (Ser473) in skeletal muscle, liver, visceral fat, and the heart in C57 mice at 8 to 10 weeks of age. Mice were first treated with BSA or rhMG53 protein (6 mg/kg IV) and, 10 minutes later, subjected to insulin stimulation (1 U/kg IP). Tissue samples were collected 10 minutes after insulin stimulation (n=8 per group; ***P*<0.01, 1-way ANOVA; see Figure VIB in the online-only Data Supplement for protocol). **B**, Representative Western blots and averaged data of serum MG53 protein in normal controls and diabetic *db/db* mice at 8 to 10 weeks of age (n=10 per group; ***P*<0.01, Student *t* test). **C**, Blood glucose in the diabetic *db/db* mice treated with an MG53 monoclonal antibody (anti-MG53) or immunoglobulin G (IgG; 1.5 mg IV per animal; n=9 per group; **P*<0.05, ***P*<0.01 vs corresponding baseline, Student *t* test). **D**, Insulin tolerance tests (insulin 2 U/kg IP) were performed 7 days after the anti-MG53 or IgG treatment (n=9 per group; **P*<0.05, ** *P*<0.01 vs corresponding value in IgG group, Student *t* test). **E**, Blood glucose in the wt mice treated with anti-MG53 or IgG (1.5 mg IV per animal; n=9 per group; Student *t* test). **F**, Insulin tolerance tests (insulin 2 U/kg IP) were performed 7 days after anti-MG53 or IgG treatment (1.5 mg IV per animal) in the wt mice (n=9 per group; ** *P*<0.01 vs corresponding value in IgG group, Student *t* test). Data are mean±SEM.

ment of whole-body metabolic homeostasis, eventually leading to metabolic diseases, in particular obesity and T2DM, which, in turn, further elevates the MG53 levels in muscle and circulation.

It is noteworthy that some previous studies have failed to show increases in MG53 abundance in animals with metabolic disorders.[7,37,38] The discrepancy between those reports and the present study might be attributable to the differences in experimental settings and the quality of antibodies used, although it is currently unclear why those previous studies have shown counter observations.[7,37,38] In our previous study,[6] we used freshly prepared skeletal muscle proteins and an antibody from Abcam (catalog No. ab83302) and observed significant increase of MG53 in mice on a high-fat diet. Using multiple commercially available antibodies and real-time polymerase chain reaction, here we have validated high-fat diet–induced upregulation of MG53 at the protein (Figure VIIIA–VIIID in the online-only Data Supplement) and mRNA (Figure VIIIE in the online-only Data Supplement) levels, consistent with previous reports.[39,40] More important, we have demonstrated that circulating MG53 is elevated in animals and humans with obesity and T2DM and that increases in serum MG53 correlate with body weight and blood glucose and insulin levels.

To determine whether circulating MG53 modulates insulin sensitivity and metabolism or indirectly via regulating the secretion of other myokines/cardiokines, we have measured the concentrations of 3 well-studied, metabolism-related myokines/cardiokines, including follistatin like 1,[41] adiponectin,[42] and interleukin 6,[9] in *mg53 h-TG* and MG53-deficient mice and their wt

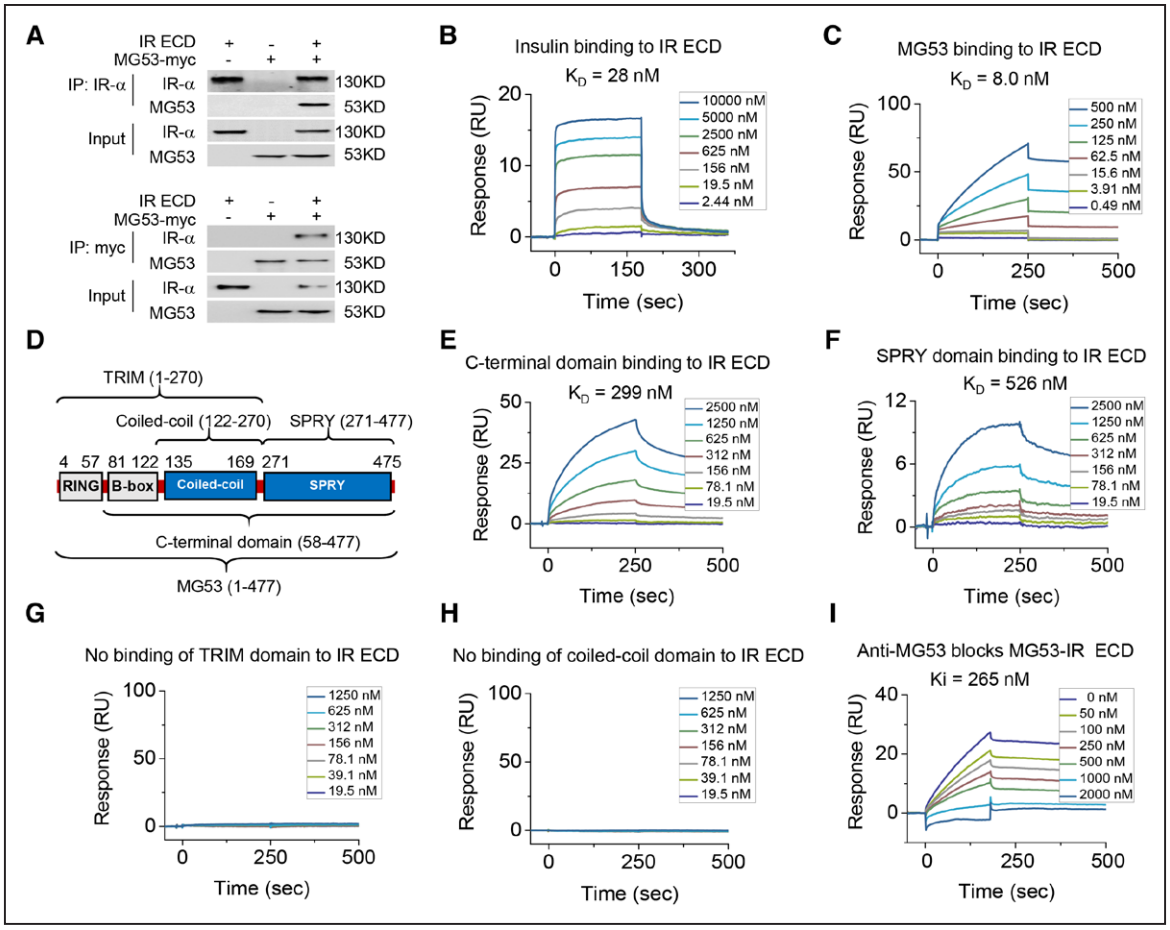

Figure 6. Mitsugumin 53 (MG53) binds to the insulin receptor extracellular domain (IR ECD).
A, Coimmunoprecipitation of purified recombinant human MG53 (rhMG53) tagged with myc at its C terminus (MG53-myc) and the IR ECD (n=3). The input represents 5% of the proteins used for each immunoprecipitation. Purified recombined mouse MG53-myc (2 μg) and 2 μg human IR ECD protein were mixed with 20 μL Protein A Sepharose 4 Fast Flow (GE Healthcare) and 0.5 μg antibody in 500 μL cold 1×PBS buffer and incubated at 4°C for 3 hours. The resins were then washed 5 times with cold PBS buffer, and the bound proteins were detected by Western blot. **B**, Surface plasmon resonance (SPR) measurements illustrating binding of insulin to the IR ECD (n=4). **C**, SPR measurements illustrating binding of full-length MG53 with the IR ECD (n=5). **D**, Schematic map of MG53 domains. **E** through **H**, SPR measurements illustrating binding of the C-terminal (**E**), SPRY (**F**), TRIM (**G**), and coiled-coil (**H**) domains of MG53 to the IR ECD (n=3 for each group). **I**, The anti-MG53 blocks binding of rhMG53 to the IR ECD (n=3). In all SPR experiments, the IR ECD was immobilized on the chip, with indicated concentrations of insulin (**B**), rhMG53 (**C**), or its truncations (**E** through **H**). In **I**, a mixture of rhMG53 at a fixed concentration (0.5 μmol/L) and the anti-MG53 at various concentrations (0–2 μmol/L) was injected over the surface of the chip with the IR ECD immobilized.

littermates. High-glucose and -insulin treatment elicits distinctly different effects on these cytokines and MG53. Moreover, the secretion of these well-established myokines is independent of MG53 (Figure IX in the online-only Data Supplement). Thus, MG53 secretion is a direct cellular response of high glucose and insulin stimulation rather than a bystander associated with the release of other secretory proteins into the extracellular space.

Both Extracellular and Intracellular MG53 Species Converge at the Insulin Signaling Pathway

Previous studies have shown that systemic blockade of insulin action in mice or humans is not sufficient to reduce energy expenditure or to cause obesity,[43] and, in fact, fat-specific deletion of the IR induces apoptosis of adipocytes.[44] Now, the question is how upregulation of MG53, as an IR-blocking protein, triggers full-blown metabolic syndrome. The reason is that extracellular MG53, in conjunction with its intracellular E3 ligase function, blocks not only IR but also IRS1 function. Genetic evidence has shown that mice with 50% reduction of both IR and IRS1, but not either of them, exhibit severe metabolic syndrome.[45] Furthermore, MG53 promotes lipid uptake in myocardium by enhancing the expression levels of peroxisome proliferator-activated receptor-α and its target genes, resulting in lipid accumulation and toxicity, which further disrupt insulin signaling.[5] Thus, MG53-induced metabolic disorders are mediated by multiple mechanisms, including extracellular blockade of IR, intracellular degradation of IR and IRS1 proteins, and lipid toxicity.

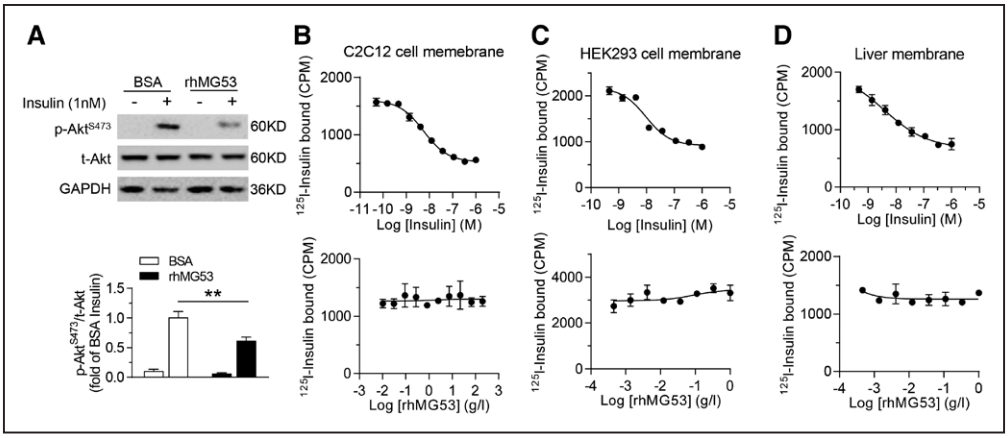

Figure 7. Extracellular mitsugumin 53 (MG53) inhibits insulin signaling but does not competitively block insulin binding to the insulin receptor (IR). **A**, Representative Western blots and averaged data showing that rhMG53, but not BSA (20 μg/mL), blocks insulin-stimulated (1 nmol/L for 10 minutes) phosphorylated (p-) AKT^{S473} levels in Bel7404 cells. **B**, Radioactive ligand binding assays showing that nonradioactive insulin, but not MG53, inhibited ^{125}I-insulin binding to the IR in membrane proteins prepared from C2C12 cells transfected with an IR-expressing plasmid (n=3). **C**, Radioactive ligand binding assays showing that nonradioactive insulin, but not MG53, inhibited ^{125}I-insulin binding to the IR in membrane proteins prepared from HEK293 cells transfected with an IR-expressing plasmid (n=3). **D**, Radioactive ligand binding assays showing that nonradioactive insulin, but not MG53, inhibited ^{125}I-insulin binding to the IR in membrane proteins prepared from mouse liver tissue (n=3). Data are mean±SEM (** P<0.01, 1-way ANOVA; n=7 without insulin, n=8 with insulin treatment).

The paradigm of the systemic regulation of glucose metabolism by MG53 resembles the regulation of lipid metabolism by proprotein convertase subtilisin/kexin type 9.[30,31] In addition to its intracellular protein convertase action, secreted proprotein convertase subtilisin/kexin type 9 binds to low-density lipoprotein cholesterol receptors and promotes their intracellular degradation, resulting in dyslipidemia.[46] Indeed, anti–proprotein convertase subtilisin/kexin type 9 monoclonal antibodies produce beneficial effects.[47,48] Likewise, neutralizing blood MG53 with monoclonal antibodies displays therapeutic effects in diabetic mice. Thus, targeting circulating MG53 by immunotherapy or other means, in addition to inhibition of its intracellular E3 ligase activity, may represent an exciting new opportunity for the treatment of T2DM and its complications.

Potential Involvements of MG53 in Physiological Alterations of Insulin Sensitivity

In addition to its role in the pathogenesis of obesity and T2DM, glucose/insulin-sensitive MG53 secretion may contribute to the physiological insulin resistance. Previous studies have demonstrated that insulin-stimulated glucose uptake is reduced in sedentary versus exercised rats[49] and humans.[50] Emerging evidence shows that physical training downregulates muscle MG53 expression,[39] contributing to the exercise-induced beneficial effect. However, intensive exercise may lead to MG53 leakage from muscle, resulting in an elevation of serum MG53. For instance, short-term intensive exercise increases serum MG53 levels in the *mdx* dystrophic mice (but not in the wt mice) as a result of muscle injury and subsequent leakage of MG53.[21] Nevertheless, understanding the potential role of MG53 in physiological regulation of insulin sensitivity by physical activity and nutrients requires future study.

Conclusions

The present study demonstrates that MG53 secreted by striated muscle suppresses whole-body insulin sensitivity via its endocrine action. These findings not only unveil MG53 as a glucose- and insulin-regulated myokine/cardiokine but also establish a causal relationship between elevated serum MG53 and the development of systemic insulin resistance and resultant T2DM. Thus, intracellular and extracellular MG53 may exert mechanistically distinct yet biologically synergistic effects on insulin signaling and metabolic homeostasis. Targeting extracellular MG53 provides a novel therapeutic approach for the treatment of T2DM and related complications.

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Authors

Hong-Kun Wu, BS*; Yan Zhang, MD, PhD*; Chun-Mei Cao, MD, PhD*; Xinli Hu, PhD; Meng Fang, BS; Yuan Yao, BS; Li Jin, BS; Gengjia Chen, BS; Peng Jiang, BS; Shuo Zhang, BS; Ruisheng Song, MD, PhD; Wei Peng, PhD; Fenghua Liu, PhD; Jiaojiao Guo, PhD; Lifei Tang, PhD; Yanyun He, BS; Dan Shan, BS; Jin Huang, MD, PhD; Zhuan Zhou, PhD; Dao Wen Wang, MD, PhD; Fengxiang Lv, PhD; Rui-Ping Xiao, MD, PhD

Correspondence

Rui-Ping Xiao, MD, PhD, or Fengxiang Lv, MD, PhD, Institute of Molecular Medicine, Room 236, New Life Science Bldg, Peking University, 5th Yiheyuan Rd, Beijing, China 100871. Email xiaor@pku.edu or lfx@pku.edu.cn

Affiliations

State Key Laboratory of Membrane Biology, Institute of Molecular Medicine (H.-K.W., Y.Z., C.-M.C., X.H., M.F., Y.Y., L.J., G.C., P.J., S.Z., R.S., W.P., F.L., J.G., L.T., Y.H., D.S., Z.Z., F.L., R.-P.X.), and Beijing City Key Laboratory of Cardiometabolic Molecular Medicine (Z.Z., R.-P.X.), Peking University, China. Peking-Tsinghua Center for Life Sciences, Beijing, China (Z.Z., R.-P.X.). Department of Internal Medicine, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China (J.H., D.W.).

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Disclosures

None.

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