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Lack of MG53 in human heart precludes utility as a biomarker of myocardial injury or endogenous cardioprotective factor

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Aims Mitsugumin-53 (MG53/TRIM72) is an E3-ubiquitin ligase that rapidly accumulates at sites of membrane injury and plays an important role in membrane repair of skeletal and cardiac muscle. MG53 has been implicated in cardiac ischaemia–reperfusion injury, and serum MG53 provides a biomarker of skeletal muscle injury in the *mdx* mouse model of Duchenne muscular dystrophy. We evaluated the clinical utility of MG53 as a biomarker of myocardial injury.

Methods and results We performed Langendorff ischaemia–reperfusion injury on wild-type and dysferlin-null murine hearts, using dysferlin deficiency to effectively model more severe outcomes from cardiac ischaemia–reperfusion injury. MG53 released into the coronary effluent correlated strongly and significantly ($r = 0.79–0.85$, $P < 0.0001$) with functional impairment after ischaemic injury. We initiated a clinical trial in paediatric patients undergoing corrective heart surgery, the first study of MG53 release with myocardial injury in humans. Unexpectedly, we reveal although MG53 is robustly expressed in rat and mouse hearts, MG53 is scant to absent in human, ovine, or porcine hearts. Absence of MG53 in 11 human heart specimens was confirmed using three separate antibodies to MG53, each subject to epitope mapping and confirmed immunospecificity using MG53-deficient muscle cells.

Conclusion MG53 is an effective biomarker of myocardial injury and dysfunction in murine hearts. However, MG53 is not expressed in human heart and therefore does not hold utility as a clinical biomarker of myocardial injury. Although cardioprotective roles for endogenous myocardial MG53 cannot be extrapolated from rodents to humans, potential therapeutic application of recombinant MG53 for myocardial membrane injury prevails.

Keywords MG53 • TRIM72 • Ischaemia–reperfusion injury • Ischaemic preconditioning/postconditioning • Biomarker of myocardial injury

1. Introduction

Mitsugumin-53 (MG53/TRIM72) is a member of the tripartite motif (TRIM) family of E3 ubiquitin ligases. MG53 is rapidly recruited to sites of membrane damage, binding phosphatidylserine exposed at the damaged lipid bilayer.[1] MG53 knockout mice display a mild, progressive muscular dystrophy characterized by defective membrane resealing of skeletal myofibres.[1] MG53 oligomerizes in response to the oxidative extracellular environment at sites of membrane injury,[1] forming an intricate lattice with the muscular dystrophy membrane repair protein dysferlin during repair.[2] As yet, genetic abnormalities in MG53 have not been identified as a cause of disease in humans.[3]

MG53 expression in mice is highest in skeletal and cardiac muscle, and heart tissue of MG53 knockout mice damaged by laser irradiation displays a membrane resealing defect.[4] In a Langendorff ischaemia/reperfusion (I/R) model, MG53 knockout hearts suffered increased infarct size, TUNEL-positive nuclei and lactate dehydrogenase (LDH) release compared with WT hearts.[4] MG53 also plays an important

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role in ischaemic preconditioning[5] and postconditioning[6] via the reperfusion injury salvage kinase (RISK) pathway.

MG53 is released from injured skeletal muscle into the serum.[7] Studies in the *mdx* mouse model of Duchenne muscular dystrophy revealed that greater levels of injury suffered by dystrophic *mdx* mice correlated with greater levels of serum MG53.[7] Interestingly, this study revealed that recombinant human MG53 applied to the extracellular media bathing isolated myofibres binds to injury sites, improving membrane resealing outcomes.[7] These data suggest that MG53 can act effectively from the extracellular surface of an injured cell or enter via membrane disruptions and perform a membrane repair role. Indeed, systemic administration of recombinant human MG53 improves dystrophic pathologies in the *mdx* mouse[7] and reduces infarct sizes in murine and porcine models of cardiac ischaemia–reperfusion injury.[8]

We sought to study the efficacy of MG53 as a biomarker of myocardial injury. Promising results in a murine Langendorff I/R model led us to extend our studies to an ovine model of cardiopulmonary bypass and aortic cross-clamping, and a clinical trial in paediatric patients undergoing corrective heart surgery. However, during laboratory optimization of methods to assay serum MG53, we revealed using three separate validated antibodies that MG53 protein was minimal to undetectable in human, ovine, or porcine heart samples. Thus, serum MG53 in humans will predominantly be derived from the skeletal muscle pool and does not hold utility as a biomarker of myocardial injury in humans.

2. Materials and methods

2.1 Human studies

All human studies were performed in accordance with the principles of the Declaration of Helsinki. Human heart and muscle samples were obtained via protocols approved by the Human Research Ethics Committee of the Children's Hospital at Westmead (INMR Biospecimen Bank approval CHW/10/45); all study participants or their families provided informed consent. Human heart samples were obtained from paediatric cardiac surgery procedures, where removal of the tissue was a planned part of the operation (e.g. resection of outflow tract obstruction in tetralogy of Fallot) and were from right or left ventricles (as indicated in legend). Samples were obtained on cardiopulmonary bypass, with the heart arrested in diastole with cold blood cardioplegia and were immediately snap frozen in liquid nitrogen and stored at −80°C. The donor heart was removed via cold cardioplegia and stored for 12 h on ice awaiting transplant. A small sample dissected from the left ventricle was snap frozen in liquid nitrogen and stored at −80°C.

2.2 Animal studies

All animal studies were performed in accordance with The Australian Code of Practise for the Care and Use of Animals Used for Scientific Purposes and were approved by the CMRI-CHW Animal Care and Ethics Committee (protocols K286 & K301). Lambs (*Ovis aries*) used for cardiopulmonary bypass and aortic cross-clamping are as described in Egan *et al.*[9] Dysferlin-null A/J mice (*Mus musculus*) were sourced from the Jackson Laboratory (JAX, stock no. 000646) and wild-type A/J mice were purchased from the Animal Resources Centre, Perth, for both Langendorff and isolated cardiomyocyte studies. Langendorff experiments were also performed in the B6.A-*Dysf*^{prmd}/GeneJ (Bla/J) line bearing the A/J mutation (a retrotransposon insertion within intron 4 of the dysferlin gene) backcrossed onto the C57BL/6 strain. Bla/J mice were purchased from the Animal Resources Centre, Perth, while wild-type (WT) C57BL/6 mice were obtained from the Transgenic Facility at CHW. Hearts from 8- to 12-week male mice were used in all experiments. Porcine (*Sus scrofa*) and rat (*Rattus norvegicus*) samples were obtained through tissue sharing via Professor David Little, The Children's Hospital at Westmead.

2.3 Langendorff-perfused heart experiments

Murine hearts were excised from anaesthetized (4% isofluorane in O_2) and heparinized (300 U sodium heparin) mice and the aorta cannulated. Constant pressure (90 mmHg) perfusion was commenced with a modified Krebs/Heinseleit buffer [containing (mmol/L); NaCl 119, $NaHCO_3$ 22, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.0, Glucose 11, EDTA 0.5, Na-Pyruvate 2] warmed to 37°C and bubbled with 5% CO_2 in O_2 to obtain physiological pH of 7.4. A left ventricular balloon attached via water-filled tubing to a pressure transducer was inflated to set the diastolic left ventricular end pressure to 5 mmHg. Hearts were externally paced at 8 Hz, allowed to stabilize then subjected to an I/R protocol (see legend for details). Pacing was ceased during ischaemia and recommenced 4 min into reperfusion. Data were collected to a PowerLab (ADInstruments, Castle Hill, Australia).

2.4 Cardiomyocyte isolation

Cannulated hearts were perfused for 4 min at 3 mL/min using calcium-free isolation buffer to arrest contraction [containing (mmol/L); NaCl 113, KCl 4.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgSO_4$ 1.2, $NaHCO_3$ 12, $KHCO_3$ 10, HEPES 10, Taurine 30, 2,3-butanedionemonoxime (2,3-BDM) 10, glucose 5.5, pH 7.46], then perfused for 10 min with digestion buffer [Liberase-DL (Roche) 0.25 mg/mL, trypsin 1.4 mg/mL, $CaCl_2$ 12.5 μmol/L in isolation buffer]. The left ventricle was minced into 1–2 mm pieces and diluted with an equal volume of stopping buffer no. 1 [10% heat inactivated FCS (HiFCS), 12.5 μmol/L $CaCl_2$ in IonOptix buffer (isolation buffer without 2,3-BDM)]. The suspension was triturated with decreasing aperture pasteur pipettes into a homogeneous cell suspension, diluted with an equal volume of stopping buffer no. 2 (5% HiFCS, 12.5 μmol/L $CaCl_2$ in IonOptix buffer) and filtered through 180 μm nylon mesh. Cardiomyocytes were counted and viability determined by morphology, then Ca^{2+} re-introduced over 20 min incrementally, doubling the calcium concentration every 4 min, to a final working concentration of 1 mmol/L calcium.

2.5 Video microscopy (IonOptix) studies

Cardiomyocytes were incubated with 5 μmol/L fura-2 acetoxymethyl ester (fura-2AM) at room temperature for 20 min in the dark, followed by exchange to fresh IonOptix+Ca^{2+} ($CaCl_2$ 1 mmol/L) buffer for 10 min to allow hydrolysis of the AM ester. Cells were loaded into a Warner chamber and allowed to settle for 5 min before superfusion at 1 mL/min with IonOptix+Ca^{2+} buffer gassed with 5% CO_2 in O_2 and pacing at 1 Hz, 35 V. Individual contracting cells were selected for video analysis (IonOptix LLC, MA, USA) based on suitable morphology and paced-only contractions. After 10 min stable contraction at 1 Hz, baseline measurements of 10–20 contractions were recorded. Measurements were repeated at 10 and 20 min post-baseline. A fresh cell suspension was loaded into the chamber for each cell measurement. All studies presented were performed on 16 cells of each genotype, isolated from 7 animals and used within 3 h of isolation.

2.6 LDH analysis

Effluent samples (500 μl) were collected from the Langendorff-perfused hearts, snap frozen, and stored at −80°C for later analysis. LDH content was determined using a CytoTox96 assay kit (Promega, Australia).

2.7 Western blot

Muscle cryosections or coronary effluent were solubilized in SDS lysis buffer [2% SDS, 50 mM Tris pH 7.4, 10% glycerol, 50 mM DTT, 1 : 500 protease inhibitor cocktail (Sigma P8340)] as previously described.[3] Proteins were separated by SDS–PAGE and probed with rabbit polyclonal anti-mouse pAb-mMG53$_{144}$ (1:5000, generously provided by Prof Jianjie Ma,

University of Medicine and Dentistry New Jersey, USA), pAb-hMG53$_{288}$ (1:3000 Aviva Systems Biology ARP42971_P050), and pAb-hMG53$_{108}$ (1:3000 Aviva Systems Biology ARP42970_P050). Other antibodies tested but shown not to specifically recognize MG53 by western blot were anti-TRIM72 (N-12) (Santa Cruz sc-165789), anti-TRIM72 (Aviva Systems Biology OAEB00972); anti-TRIM72 (Abnova clone 2B8, H00493829-M04), and anti-TRIM72 (Sigma HPA023122).

2.8 Epitope mapping

A series of MG53 deletion constructs was generated to confirm the antigenic region recognized by each antibody. The pEGFP-mMG53 plasmid (provided by Prof. Jianjie Ma) was digested with PstI, XmnI/SmaI, EcoRI, ApaI, SacII, and SmaI then re-ligated to generate pEGFP-MG53Δ143–477, Δ198–477, Δ238–477, Δ360–477, Δ403–477, and Δ449–477, utilizing the stop codons in three frames within the pEGFPN1 backbone for synthesis of truncated proteins.

2.9 CRISPR/Cas9 gene editing

A sgRNA/Cas9 expression plasmid [pSpCas9(BB)-2A-Puro (PX459)] was a gift from Feng Zhang (Addgene no. 48139)[10] with guides targeting exon-2 (TGCACCCGGCCTTCTGCGTC) or exon-7 (GCACTCCACTCGGC GACCAG) of MG53 used to knock-out murine MG53 in cultured C2C12 myoblasts. Targeting and disruption of the MG53 locus in CRISPR/Cas9 lines were confirmed by sequencing and western blot.

2.10 Statistical analysis

Comparison of functional performance of Langendorff-perfused mouse hearts used mixed models linear analysis. Isolated cardiomyocyte studies were analysed using the Kruskal–Wallis test. LDH release in coronary effluent was compared using a two-tailed Mann–Whitney *U* test. Correlation of coronary effluent MG53 and LDH content or LVDP was performed using the method of Pearson. Statistical analyses were performed using Prism (GraphPad Software, CA, USA) or IBM SPSS Statistics v20 software. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1 Murine MG53 is a rapid and abundant biomarker of murine cardiac ischaemic injury and correlates with functional impairment from I/R injury

We performed Langendorff I/R injury protocols on wild-type and dysferlin-null A/J mice, using dysferlin deficiency to model more severe outcomes from cardiac I/R injury.[11,12] Accordingly, dysferlin-null mice showed a more profound impairment in cardiac functional performance following I/R challenge, with significantly higher diastolic left ventricular pressure in reperfusion (D-LVP, *Figure 1A*) and poorer resolution of ventricular contractility (left ventricular developed pressure, LVDP, the pressure differential between systole and diastole, *Figure 1B*). Indeed, dysferlin-null hearts achieved less than half the functional recovery in LVDP of their wild-type controls following I/R injury. Consistent with previous reports,[11,12] dysferlin-null hearts suffer greater damage with I/R, resulting in a stiffer myocardium during diastole, reducing the ability of dysferlin-null hearts to relax for filling, as well as slower and less forceful contraction during systole following I/R injury. These findings mirror clinical features of cardiac injury following myocardial infarction or surgery involving cardiopulmonary bypass.

To study the efficacy of MG53 as a biomarker of myocardial membrane injury and functional performance following I/R injury, we compared levels of MG53 released into the coronary effluent (*Figure 1C*, detected using anti-mMG53$_{144-477}$) with that of released LDH (*Figure 1D* and *E*), a classical biomarker of membrane permeability induced by the challenge of ischaemia–reperfusion.[13] In parallel, we compared levels of MG53 with the functional parameter LVDP (*Figure 1F*).

MG53 was robustly detected in coronary effluent as soon as 2 min into reperfusion, with higher levels released from dysferlin-null hearts (*Figure 1C*) that suffered a greater degree of functional impairment with I/R injury (*Figure 1B*). Higher levels of LDH were also released from dysferlin-null hearts relative to wild-type controls, consistent with greater membrane permeability resulting from the I/R insult (*Figure 1D*). A strong positive correlation between released LDH and MG53 was observed for both wild-type and dysferlin-deficient A/J hearts (*Figure 1D*, Pearson $r = 0.85$, $P < 0.0001$), demonstrating the potential utility of MG53 as a novel biomarker of cardiac I/R injury across a wide range of ischaemic outcomes. Similarly, there was a strong inverse correlation between the levels of MG53 in the coronary effluent and the functional parameter LVDP (*Figure 1F*) for both WT and dysferlin-null hearts ($r = -0.77$, $P < 0.0001$). MG53 release thus accurately reflects the ischaemic burden experienced by murine hearts in the Langendorff I/R model.

Similar results were obtained in a Langendorff I/R challenge performed on hearts from C57BL/6 WT or dysferlin-null (Bla/J) mice. Dysferlin-null hearts demonstrated higher diastolic-LVP (*Figure 2A*), recovery of LVDP less than half that of WT hearts (*Figure 2B*), increased MG53 release to the coronary effluent on commencement of reperfusion (*Figure 2C*, $t = 32$, $t = 62$), and significantly elevated coronary effluent LDH early in reperfusion (*Figure 2D*). Similarly, there was a strong positive correlation between released LDH and MG53 (*Figure 2E*, Pearson $r = 0.79$, $P < 0.0001$) and a strong inverse correlation between MG53 and LVDP (*Figure 2F*, Pearson $r = -0.71$, $P < 0.0001$). We confirmed equivalent levels of MG53 and LDH in naive wild-type dysferlin-null and wild-type hearts on both the A/J (*Figure 2G* and *I*) and C57BL/6 (*Figure 2H*) genetic backgrounds, establishing that higher levels of released MG53 and LDH were not related to compensatory up-regulation due to dysferlin deficiency.

Dysferlin deficiency did not exert functional deficits on baseline cardiomyocyte contractility or calcium handling (*Figure 3*), suggesting no impairment to intrinsic sarcolemmal membrane integrity. *In vitro* analyses of contractility using IonOptix video microscopy of isolated A/J cardiomyocytes loaded with the calcium indicator fura-2AM revealed no difference between wild-type and dysferlin-null cardiomyocytes for any physical parameters of sarcomere shortening or relaxation (*Figure 3A*), nor dynamics of calcium release and re-uptake (*Figure 3B*). These data support the notion that the impact of dysferlin deficiency on functional outcomes from I/R injury is likely to relate primarily to its role in membrane repair, consistent with the ~2.5-fold increase in peak LDH released into the coronary effluent in dysferlin-null vs. wild-type hearts reflecting more profound membrane injury.

Further studies in wild-type A/J and C57BL/6 hearts demonstrated the levels of MG53 released into 40 μL of coronary effluent equated to that detected in 1.25–2.5 μg total naive heart lysate (*Figure 4A*) and thus presented a viable candidate for standard immunoassay approaches. Cumulative analysis of myocardial MG53 released to the coronary effluent over 30 min of reperfusion estimates loss of 10–15% of the total pool of myocardial MG53 (*Figure 4B* and *C*). Conversely, analysis of myocardial MG53 (*Figure 4D*) remaining in mouse hearts subjected to an I/R protocol, an equivalent period of baseline perfusion, or to no perfusion showed no significant difference in MG53 levels, supporting the calculated values for MG53 release and suggesting

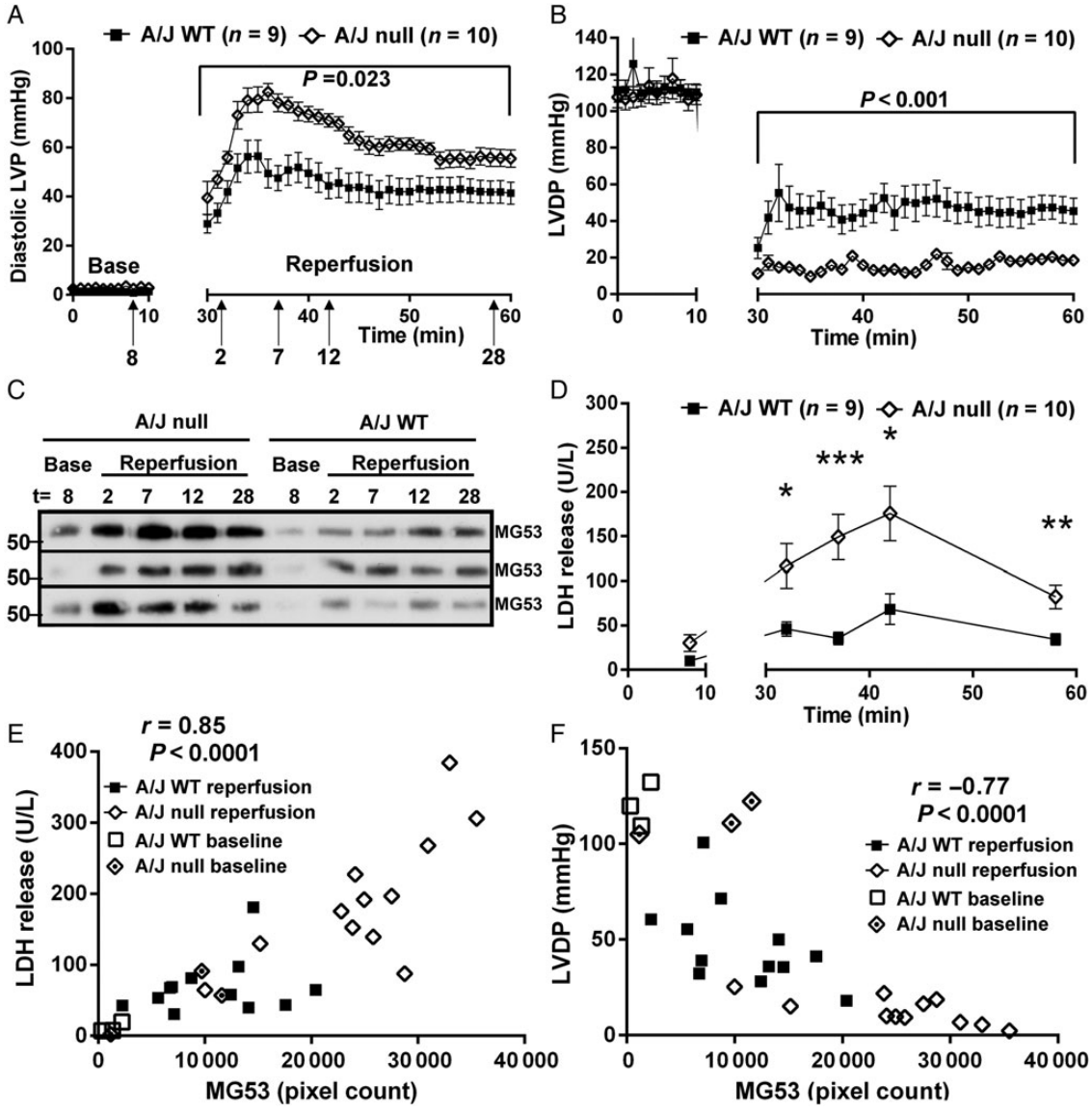

Figure 1 MG53 accurately reflects the degree of ischaemia–reperfusion injury to murine A/J hearts. A/J WT and dysferlin-null hearts were subjected to a Langendorff I/R protocol comprising 10 min baseline perfusion, 20 min global no-flow ischaemia, and 30 min reperfusion. (*A*) Following equivalent baseline function, on reperfusion A/J dysferlin-null hearts demonstrated higher diastolic left ventricular pressure than WT hearts (Diastolic-LVP), and (*B*) lower left ventricular developed pressure (LVDP), indicating poorer functional recovery from ischaemic injury. (*C*) Levels of MG53 detected in the coronary effluent increases following ischaemia–reperfusion injury, with higher levels released from dysferlin-null hearts, consistent with increased membrane permeability in this model and (*D*) supported by a corresponding increase in LDH content of coronary effluent (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$). (*E*) Comparison of levels of released MG53 and LDH gave a strong positive correlation (Pearson $r = 0.85$, $P < 0.0001$), while (*F*) shows a strong inverse correlation of MG53 with LVDP (Pearson $r = -0.77$, $P < 0.0001$), demonstrating hearts with a poorer LVDP released more MG53 ($n = 3$). These data highlight the potential of MG53 as a serum biomarker of cardiac ischaemic burden.

that MG53 expression is not induced in the murine myocardium by ischaemic challenge. Therefore, measures of serum MG53, which is rapidly released from injured hearts, may have specific utility for assessment of myocardial ischaemic injury.

3.2 MG53 expression is low to undetectable in human heart samples

We sought to assess the clinical efficacy of serum MG53 as a prognostic index of ischaemic burden. We obtained serum and tissue samples from a cohort of paediatric patients undergoing corrective heart surgery for structural heart defects and utilized serum and tissue samples collected from a cohort of lambs subjected to 2 h of cardiopulmonary bypass and aortic cross-clamping, followed by 9 h of monitoring.[9] Initial studies of human serum samples obtained pre- and post-operatively in patients with the highest rise in serum troponin T did not show evidence of serum MG53 (*Figure 6A*, troponin levels for both patients pre-surgery <50 pg/mL, post-surgery Patient 1 = 2000 pg/mL and Patient 2 = 2294 pg/mL). However, western blot of serum is extremely challenging due to the extremely high levels

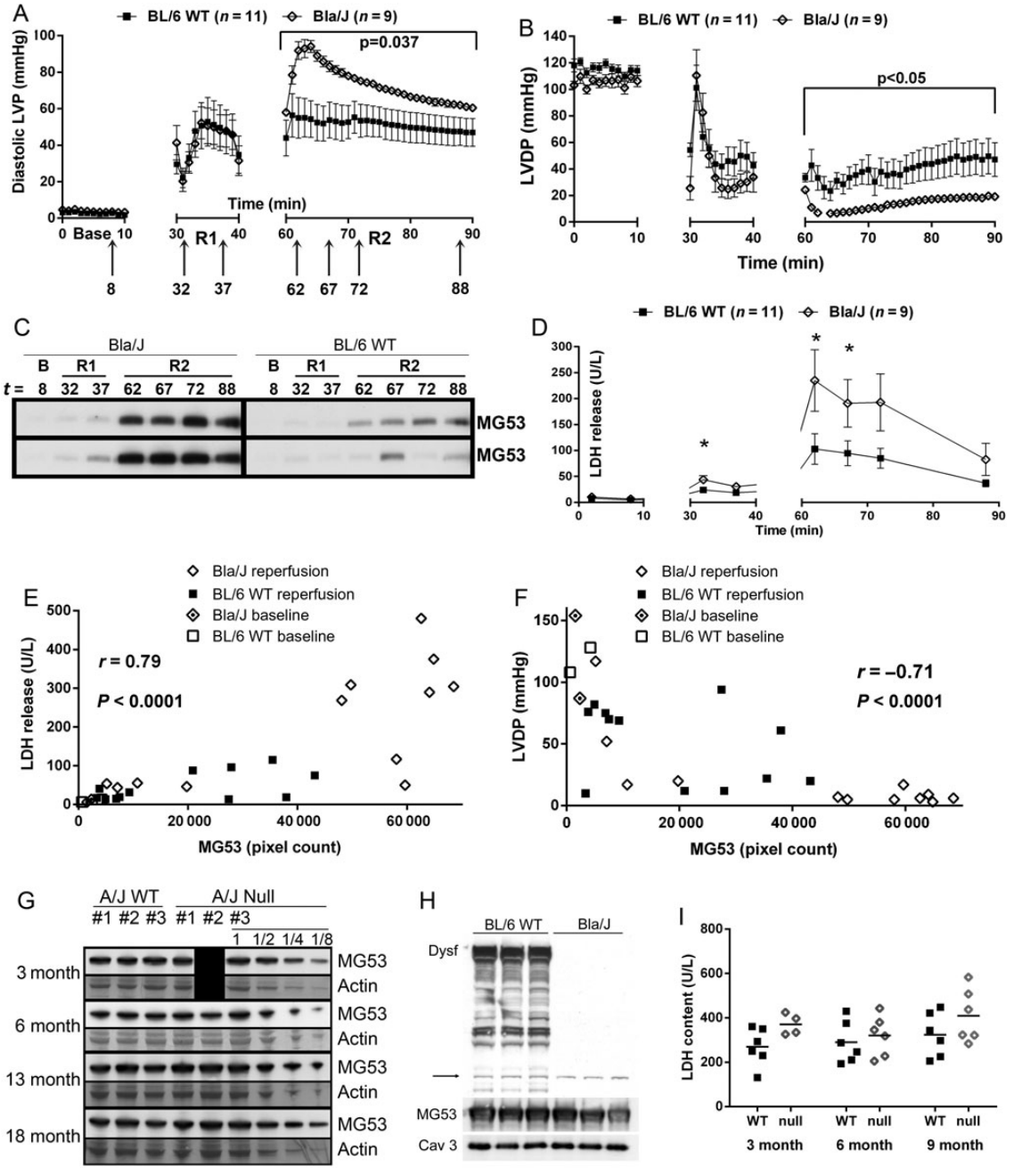

Figure 2 Murine MG53 is an accurate biomarker of cardiac ischaemic burden in the C57BL/6 heart. The C57BL/6 mouse line shows greater intrinsic resistance to cardiac I/R challenge,[14] displaying little functional impairment following 20 min of global ischaemia. We therefore utilized two consecutive 20 min ischaemic challenges to obtain a consistent and measureable functional deficit in both WT and dysferlin-null hearts following ischaemic challenge. C57BL/6 dysferlin-null hearts subjected to an extended I/R challenge [10 min baseline perfusion (b), 20 min global no-flow ischaemia (I1), 10 min reperfusion (R1), 20 min global no-flow ischaemia (I2), 30 min final reperfusion (R2)] showed a deficit in functional recovery in agreement with hearts on the A/J background. (*A*) Dysferlin-null hearts showed significantly higher diastolic left ventricular pressure throughout the second reperfusion period when the ischaemic insult primarily manifests (R2, 60–90 min) and showed much poorer recovery of baseline LVDP compared with WT hearts (*B*). Accordingly, dysferlin-null Bla/J hearts released more MG53 to the coronary effluent than WT hearts as detected by western blot (*C*) in parallel with greater LDH release (*D*) (*$P < 0.05$). Levels of released MG53 showed a strong and highly significant positive correlation with LDH release (*E*) and a strong and highly significant inverse correlation with LVDP (*F*). Dysferlin-null hearts on either the A/J (*G*) or C57BL/6 genetic background (*H*) express equivalent MG53 to WT hearts, and LDH content of WT and dysferlin-null hearts is not different in the A/J background (*I*).

of serum albumin (*Figure 6B*). During optimization of methods to assay serum MG53 after extraction of albumin and IgG, or via ELISA, we unexpectedly revealed that MG53 was expressed in the skeletal muscle of all species examined, and in mouse and rat hearts, but was not expressed in cardiac muscle of lambs, pigs, or humans (*Figure 5A*, upper panel, rabbit anti-mMG53$_{144-477}$).

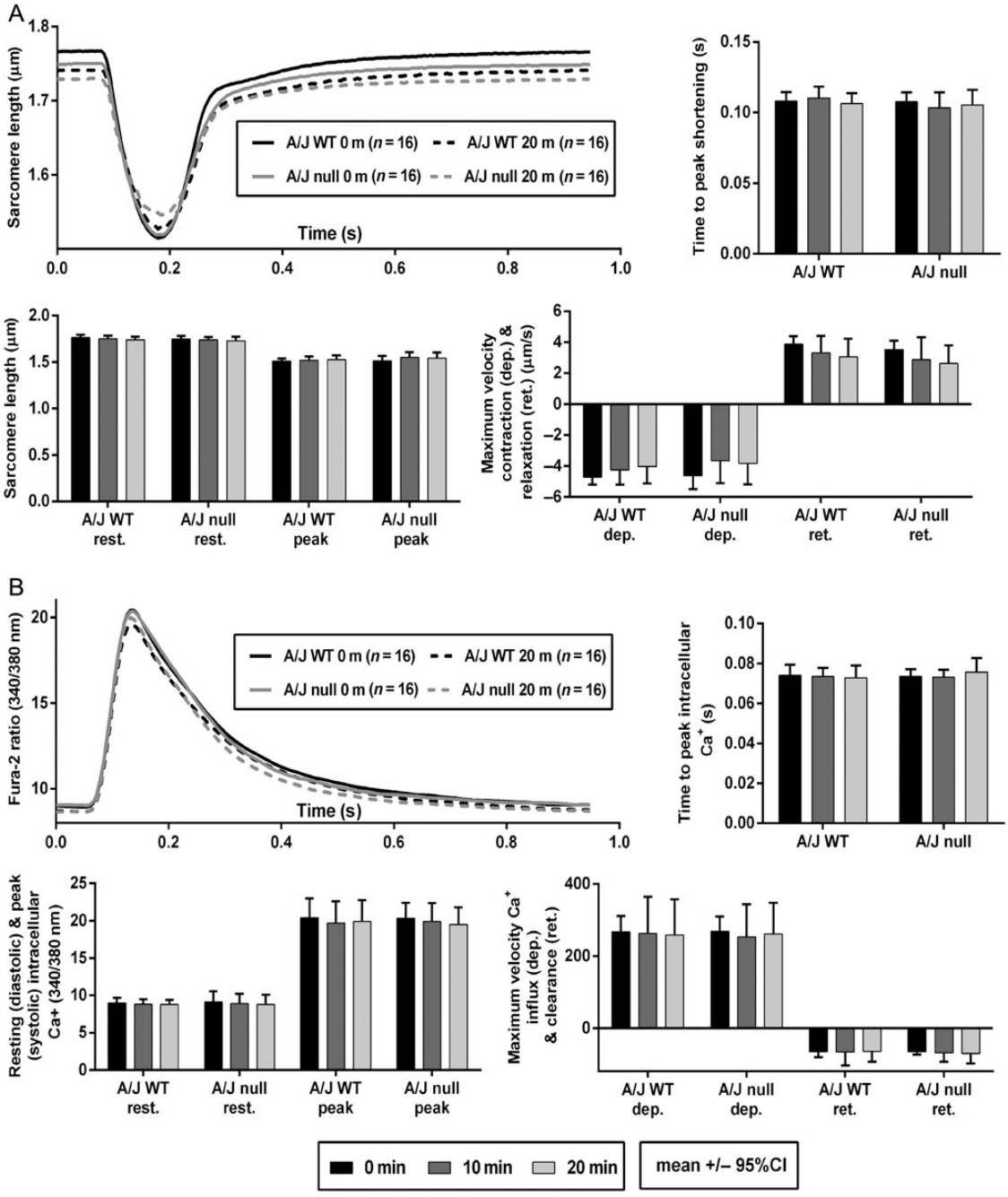

Figure 3 Wild-type and dysferlin-null cardiomyocytes show identical contractile and calcium handling properties under conditions of baseline superfusion. (*A*) Sarcomeric contractile properties of A/J WT (black lines) and A/J dysferlin-null (grey lines) cardiac myocytes were measured at baseline (solid) and after 20 min (dashed) of pacing at 1 Hz. All parameters of sarcomeric contractility were equivalent in wild-type and dysferlin-null myocytes; time to peak shortening (or 50% peak shortening, not shown), resting and peak sarcomere length, and maximum velocities of contraction and relaxation. (*B*) Calcium transients initiating contraction in A/J WT (black lines) and A/J dysferlin-null (grey lines) cardiac myocytes were measured at baseline (solid) and after 20 min (dashed) of pacing at 1 Hz. All parameters of calcium transients were equivalent in wild-type and dysferlin-null myocytes; time to peak calcium (or 50% peak calcium, not shown), resting and peak intracellular calcium, maximum velocity of calcium release, and clearance (A/J null $n = 16$, A/J WT $n = 16$, mean $\pm$ 95% CI).

To confirm this unexpected result, we purchased five anti-MG53 antibodies from commercial suppliers. We derived a series of MG53 deletion constructs to confirm the antigenic region within MG53 for each antibody (*Figure 5B* and *C*) and established MG53-deficient C2C12 cell lines via CRISPR/Cas9 gene-editing to confirm the specificity of each antibody for MG53 (*Figure 5D*). Three antibodies were pursued that recognized a 53 kDa band in murine and human skeletal muscle samples (Prof. Ma rabbit polyclonal anti-mouse 144–477, Aviva Systems Biology pAb-hMG53$_{108}$ and pAb-hMG53$_{288}$). Each antibody showed specific immunoreactivity to the appropriate antigenic region (*Figure 5C*; pAb-mMG53$_{144-477}$ was shown to be immunoreactive to sequences between amino acids 144–198) and showed specificity

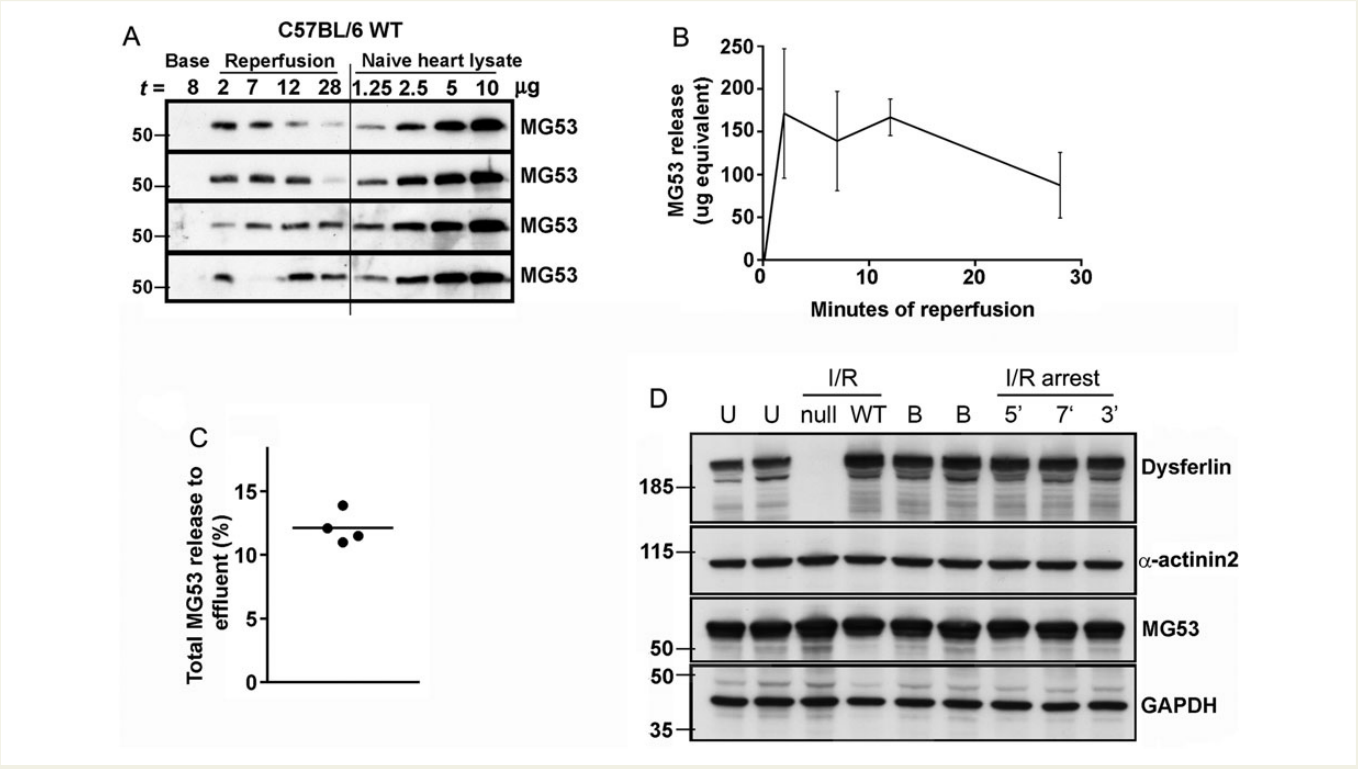

Figure 4 Wild-type murine hearts subjected to a Langendorff ischaemia/reperfusion protocol release ~10–15% of their total MG53 to the coronary effluent. (*A*) Comparative western analyses of coronary effluent vs. naive murine heart lysate as a loading standard. Forty microlitres of coronary effluent collected during reperfusion from C57BL/6 WT hearts contained as much MG53 as up to 2 µg of total protein lysate from naive hearts and was readily detectable by immunoblot methodology. The temporal MG53 release profile (*A, B*) for each heart varied, but the total amount of MG53 released over 30 min of reperfusion was remarkably consistent (*C*), in the order of 10–15% of the total MG53 contained in a naive WT heart (calculated by extrapolation of the MG53 content in 40 µL effluent to the total volume of effluent flowing through the heart during the 30 min reperfusion) ($n = 4$). (*D*) Wild-type hearts subjected to no perfusion (U), an I/R protocol (I/R, dysferlin-null and WT hearts both shown), baseline perfusion (B) for an equivalent period as the I/R protocol, or hearts harvested early in the reperfusion phase of an I/R protocol (I/R arrest, time into reperfusion shown) displayed similar levels of MG53 protein, suggesting MG53 expression did not vary in response to an ischaemic insult; MG53 was neither induced nor substantially degraded in this period of time.

for the MG53 gene product in three separate CRISPR/Cas9 MG53-null lines (*Figure 5D*).

As reported, MG53 is highly expressed in skeletal and cardiac muscle of mice and rats[1,5,6] (*Figure 5A, E* and *F*). However, all three antibodies demonstrated an absence of MG53 protein in human, porcine, and ovine heart despite effectively recognizing MG53 protein expressed in skeletal muscle (*Figure 5A, E* and *F*). Cardiac MG53 was minimal to undetectable in 10 human heart surgical samples and 1 adult donor heart (*Figure 5E*), and was also undetectable in ventricles and atria of sheep and pig heart (*Figure 5F*).

To address whether myocardial MG53 is highly susceptible to proteolysis, we performed a lability test by harvesting skeletal and cardiac muscle samples from mice, then leaving the specimens cool (in the fridge, ~4–8°C) for 2, 4, 6, 24, and 48 h to mimic the conditions with which our human samples are handled (*Figure 6C*). There was no evidence for overt lability of MG53 in these samples.

4. Discussion

Using murine Langendorff models of IR injury in two different genetic backgrounds, dysferlin-null hearts consistently showed poor functional recovery following IR challenge, achieving less than half the recovered baseline LVDP of their wild-type controls, comprising both higher diastolic-LVP and lower systolic-LVP. These results confirm a role for dysferlin for recovery from IR injury and provided an effective means to reliably model poor functional outcomes from IR injury. During pursuit of indicators to confirm impaired membrane repair as the underlying mechanism contributing to poor outcomes in dysferlin-deficient hearts, we revealed that MG53 is rapidly and abundantly released into the coronary effluent following ischaemic challenge. MG53 release showed a highly significant and strong positive correlation with the established biomarker LDH across all samples assessed ($P < 0.0001$), from both WT and dysferlin-null hearts at baseline and throughout reperfusion.

MG53 satisfied a number of criteria for cardiac biomarkers of I/R injury: low levels at baseline, release is induced rapidly following injury and correlates significantly with functional outcomes. These promising indications led us to extend our studies to an ovine model of cardiopulmonary bypass and a clinical trial of 30 paediatric patients undergoing corrective heart surgery. We sought to determine levels of MG53 in pre- and post-operative serum samples and correlate these with functional outcomes and levels of other routinely assayed biomarkers (LDH, troponin T, creatine kinase).

Our study is the first to examine endogenous MG53 protein expression in the human heart. Unexpectedly, in 11 human heart samples we

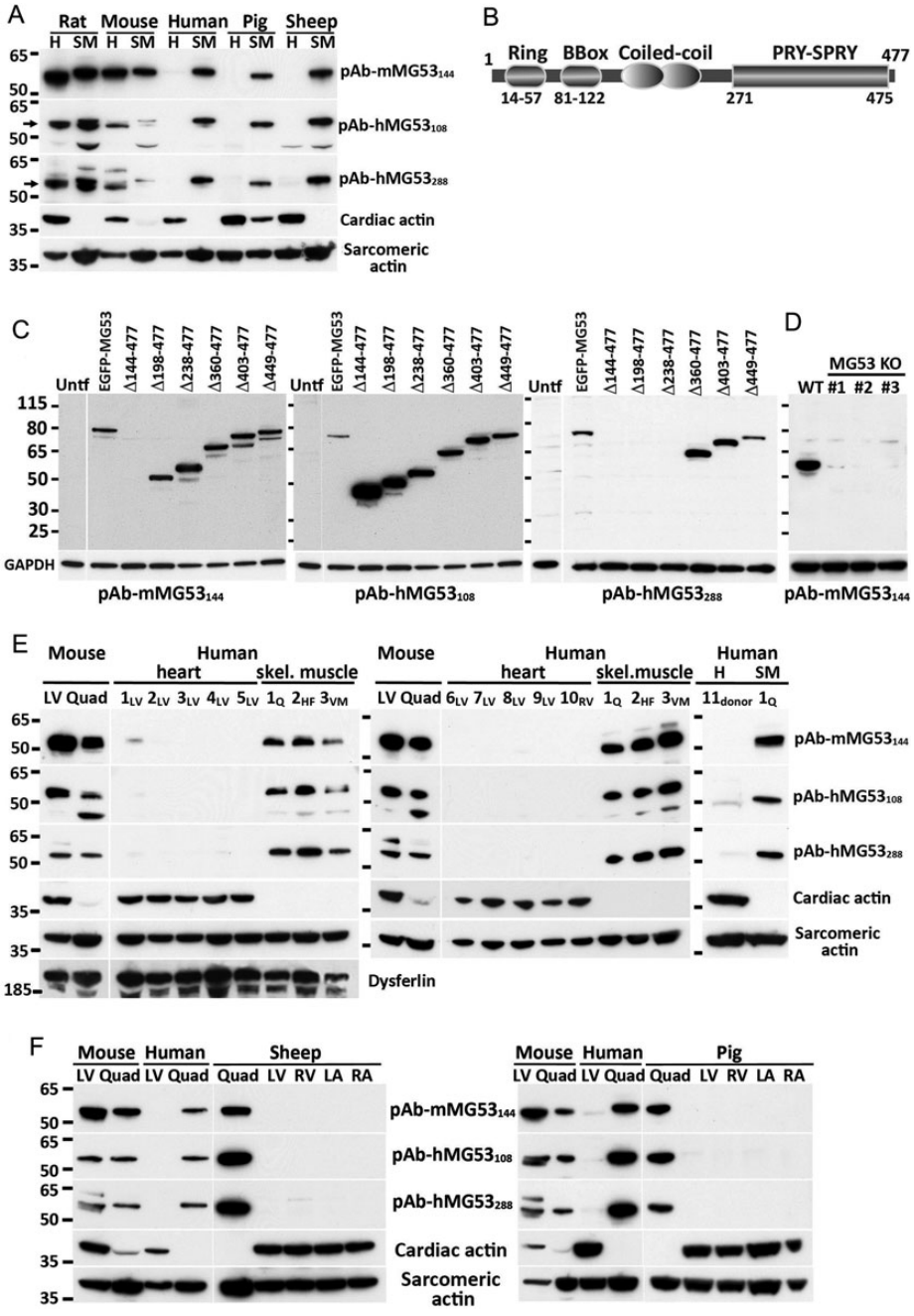

Figure 5 MG53 is not expressed in human, pig, or sheep myocardium using three validated antibodies. (*A*) Western Blotting of 10 μg total protein from heart (H) and skeletal muscle (SM) samples from mouse, rat, human, pig, and sheep. MG53 (arrow) is present in mouse and rat skeletal muscle and heart, though pAbs 108 and 288 show lower affinity for mouse than rat. In contrast, although MG53 is readily detected in skeletal muscle of human, pig, and sheep, MG53 is absent in cardiac muscle of these species (Lanes 5, 7, and 9). (*B*) Schematic representation of MG53 protein domain structure. (*C*) The antigenic regions of antibodies pAb-mMG53$_{144}$, pAb-hMG53$_{108}$, and pAb-hMG53$_{288}$ were confirmed using deletion constructs expressed in 3T3 cells. Untf = untransfected control 3T3 cells (*D*) The specificity of pAb-mMG53$_{144}$, pAb-hMG53$_{108}$, and pAb-hMG53$_{288}$ was confirmed via western blotting of wild-type C2C12 and three separate CRISPR/Cas9 gene-edited MG53-null lines. (*E*) MG53 was found at very low levels or not detected in 10 human heart samples from patients with congenital heart defects [1$_{LV}$—ventricular septal defect (VSD), patent ductus arteriosus (PDA), male 5 years; 2$_{LV}$—Tetralogy of Fallot (TOF), male 2 years; 3$_{LV}$—levo-transoposition of the great arteries, pulmonary atresia (PA), VSD, PDA, male 3 years; 4$_{LV}$—double outlet right ventricle, VSD, pulmonary stenosis, PDA, patent foramen ovale, female 4 years; 5$_{LV}$—PA, VSD, male 4 years; 6$_{LV}$—truncus arteriosus type 1, VSD, atrial septal defect, Dandy–Walker Syndrome, female 5 years; 7$_{LV}$—VSD, male 11 years; 8$_{LV}$—TOF, male 9 months; 9$_{LV}$—PDA, Shone's complex, Noonan Syndrome, female 8 years; 10$_{RV}$—PA, VSD, PDA, male 2 years), and a control donor heart (11$_{donor}$, female 23 years) but was detected in three human skeletal muscle samples (Q = quadricep, female, 5 years; HF = hip flexor, female, 5 years; VM = vastus medialis, male, 18 years) as well as mouse cardiac (LV) and skeletal muscle (Quad, 10 weeks, 10 μg total protein). In contrast, dysferlin is strongly expressed in both human skeletal muscle and heart (left bottom panel, Dysferlin). (*F*) MG53 was absent in left and right atria and ventricles of porcine (38 days) and ovine (12 weeks) hearts (10 μg total protein). LV, left ventricle, RV, right ventricle, LA, left atrium, RA, right atrium.

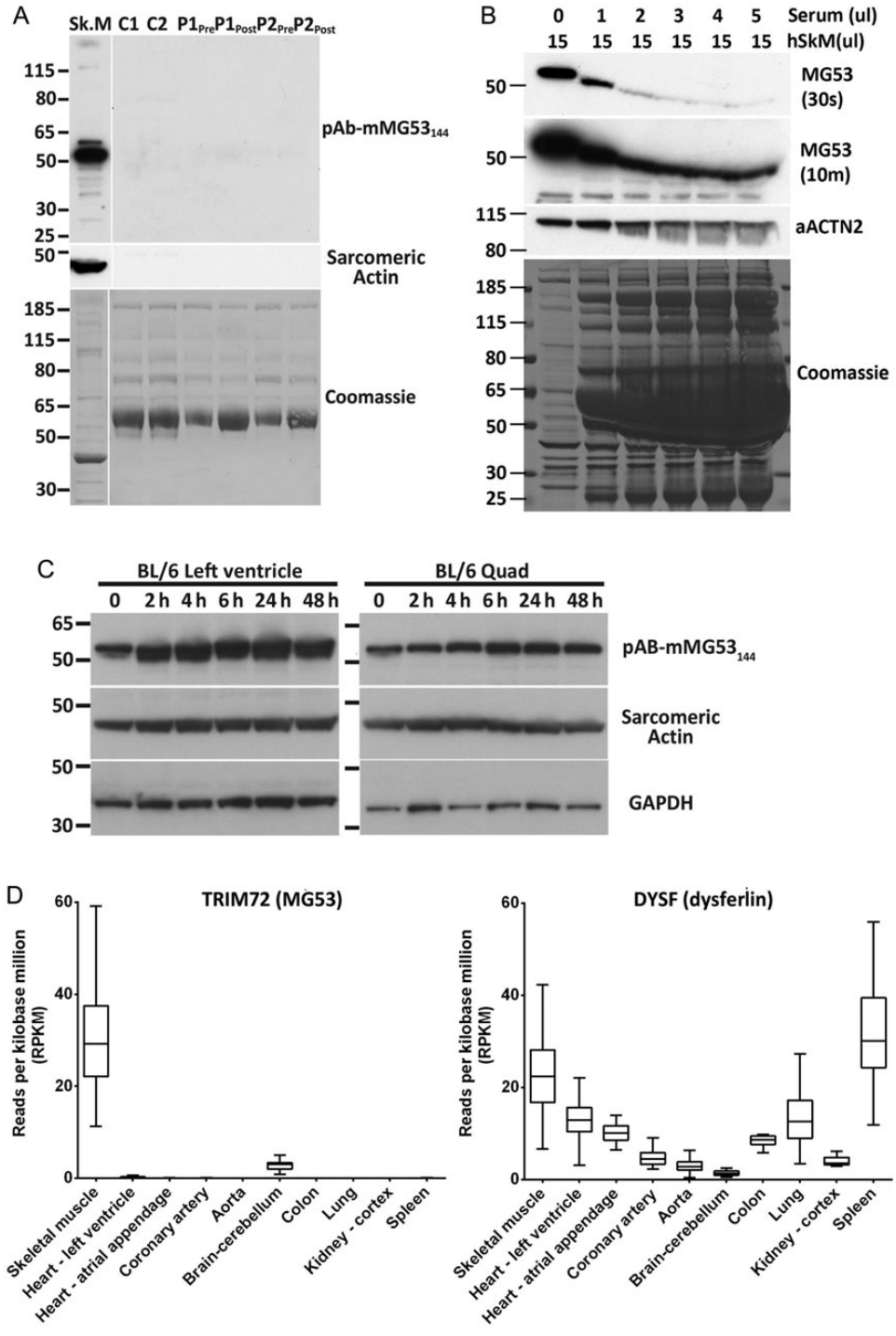

Figure 6 MG53 is not detected in serum from paediatric patients undergoing heart surgery, demonstrates no overt lability over 48 h, and protein expression in human heart correlates with mRNA expression levels according to the GTEx Portal. (*A*) Western blot analysis of 0.25 μL serum (containing ∼15–20 μg total protein) from two controls (C1, C2) and two patients ($P1_{pre}$, $P1_{post}$, $P2_{pre}$, $P2_{post}$) undergoing corrective surgery for structural hearts defects, and with significantly elevated troponin T levels post-surgery failed to detect serum MG53 in both pre- and post-surgical samples. (*B*) Western blot of equivalent amounts of human skeletal muscle lysate with increasing volumes of human serum demonstrate the difficulty of detecting MG53 in serum against the huge background levels of serum proteins, in particular albumin (MW∼65 kDa) which physically deforms the gel matrix as seen in the Coomassie panel at bottom. (*C*) Murine MG53 from both heart and skeletal muscle tissue left at ∼4°C for up to 48 h displays no overt lability, consistent with MG53 being absent from our human heart samples rather than lost due to degradation of the protein. (*D*) Genotype Tissue Expression (GTEx) Project RNA-Seq data from a range of human tissues shows MG53 (TRIM72) expression is greatest in skeletal muscle, and >100-fold greater than in heart [median value for skeletal muscle 29.21 vs. left ventricle 0.157 (>180-fold) vs. right atrial appendage 0.032 (>900-fold)], while for dysferlin, mRNA is highly expressed in both skeletal muscle and heart [median value for skeletal muscle 22.44 vs. left ventricle 12.89 (<2-fold) vs. right atrial appendage 10.06 (<2.5-fold)], consistent with our western blot analyses of MG53 and dysferlin in these two tissues.

had available for study, MG53 expression was extremely low or absent using three different validated antibodies recognizing distinct epitopes within MG53. Ten heart samples were derived from paediatric patients undergoing surgery to correct congenital structural heart defects. The 11th sample was from a potential donor heart for transplant (female, 23 years) for which a recipient was not available, and we are confident this sample represents a bonafide healthy control heart. Thus, although MG53 is abundantly expressed in human skeletal muscle, it is not expressed at significant levels in human heart.

MG53 is also absent in the left and right atria and ventricles of sheep and pig hearts. In all species, our three antibodies readily detect MG53 in skeletal muscle samples, confirming effective recognition of their respective epitopes. Importantly, our human western blot results are consistent with comprehensive mRNA sequencing data provided by the Genotype Tissue Expression (GTEx) Project that analysed 43 tissues from 175 donors (http://www.gtexportal.org/home/gene/TRIM72, 2 February 2016, date last accessed). The GTEx portal establishes that levels of TRIM72 mRNA in human skeletal muscle (*Figure 6D*, TRIM72) are >100-fold higher than levels detected in human heart, where levels are baseline and comparable to the levels detected in other tissues where MG53 is not reported to be expressed (e.g. lung, colon). In contrast, levels of dysferlin mRNA are high in both skeletal muscle and heart, in concordance with human protein expression levels (*Figure 5E*, dysferlin).

Based on previous rodent studies in skeletal[7] and cardiac muscle,[8] it has been proposed that MG53 may provide a novel biomarker for cardiac injury.[15] Indeed, our murine Langendorff study confirms the utility of released MG53 as a rapid and accurate biomarker of the degree of cardiac injury across a wide range of ischaemic outcomes. As a biomarker of cardiac ischaemic injury in rodent studies, MG53 holds tremendous promise. However, the absence of MG53 in human heart precludes clinical translation into surgical and emergency room settings. Importantly, our results emphasize the potential of MG53 as a unique biomarker of skeletal muscle injury[7] and disease progression in disorders such as Duchenne muscular dystrophy.

Our results are also relevant to roles proposed for MG53 in ischaemic preconditioning[5] and postconditioning[6] based on studies in rodent heart. Endogenous cardioprotective roles for myocardial MG53 cannot be extrapolated from rodents to humans. However, our results do not detract from recent studies in which delivery of exogenous MG53 shows promise as a therapeutic modulator of membrane injury in skeletal and cardiac muscle.[7,8] Indeed, future studies in large porcine and ovine animal models may be strengthened with the understanding that recombinant MG53 is delivered into a clean background without endogenous myocardial MG53.

In conclusion, MG53 is not expressed at significant levels in human heart and thus holds no utility as a clinical biomarker of myocardial injury in humans, nor as an endogenous cardioprotective agent in ischaemic pre- or postconditioning.

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References

1. Cai C, Masumiya H, Weisleder N, Matsuda N, Nishi M, Hwang M, Ko J-K, Lin P, Thornton A, Zhao X, Pan Z, Komazaki S, Brotto M, Takeshima H, Ma J. MG53 nucleates assembly of cell membrane repair machinery. *Nat Cell Biol* 2009;**11**:56–64.
2. Lek A, Evesson FJ, Lemckert FA, Redpath GMI, Lueders A-K, Turnbull L, Whitchurch CB, North KN, Cooper ST. Calpains, cleaved mini-dysferlinC72, and L-type channels underpin calcium-dependent muscle membrane repair. *J Neurosci* 2013;**33**:5085–5094.
3. Waddell LB, Lemckert FA, Zheng XF, Tran J, Evesson FJ, Hawkes JM, Lek A, Street NE, Lin P, Clarke NF, Landstrom AP, Ackerman MJ, Weisleder N, Ma J, North KN, Cooper ST. Dysferlin, annexin A1, and mitsugumin 53 are upregulated in muscular dystrophy and localize to longitudinal tubules of the T-system with stretch. *J Neuropath Exp Neur* 2011;**70**:302–313.
4. Wang X, Xie W, Zhang Y, Lin P, Han L, Han P, Wang Y, Chen Z, Ji G, Zheng M, Weisleder N, Xiao R-P, Takeshima H, Ma J, Cheng H. Cardioprotection of ischemia/reperfusion injury by cholesterol-dependent MG53-mediated membrane repair. *Circ Res* 2010;**107**:76–83.
5. Cao C-M, Zhang Y, Weisleder N, Ferrante C, Wang X, Lv F, Zhang Y, Song R, Hwang M, Jin L, Guo J, Peng W, Li G, Nishi M, Takeshima H, Ma J, Xiao R-P. MG53 constitutes a primary determinant of cardiac ischemic preconditioning. *Circulation* 2010;**121**:2565–2574.
6. Zhang Y, Lv F, Jin L, Peng W, Song R, Ma J, Cao C-M, Xiao R-P. MG53 participates in ischaemic postconditioning through the RISK signalling pathway. *Cardiovasc Res* 2011;**91**:108–115.
7. Weisleder N, Takizawa N, Lin P, Wang X, Cao C, Zhang Y, Tan T, Ferrante C, Zhu H, Chen P-J, Yan R, Sterling M, Zhao X, Hwang M, Takeshima M, Cai C, Cheng H, Takeshima H, Xiao R-P, Ma J. Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. *Sci Transl Med* 2012;**4**:139ra85.
8. Liu J, Zhu H, Zheng Y, Xu Z, Li L, Tan T, Park KH, Hou J, Zhang C, Li D, Li R, Liu Z, Weisleder N, Zhu D, Lin P, Ma J. Cardioprotection of recombinant human MG53 protein in a porcine model of ischemia and reperfusion injury. *J Mol Cell Cardiol* 2015;**80**:10–19.
9. Egan JR, Butler TL, Cole AD, Abraham S, Murala JS, Baines D, Street N, Thompson L, Biecker O, Dittmer J, Cooper S, Au CG, North KN, Winlaw DS. Myocardial membrane injury in pediatric cardiac surgery: an animal model. *J Thorac Cardiov Surg* 2009;**137**:1154–1162.
10. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 2013;**8**:2281–2308.
11. Martindale JJ, Metzger JM. Uncoupling of increased cellular oxidative stress and myocardial ischemia reperfusion injury by directed sarcolemma stabilization. *J Mol Cell Cardiol* 2014;**67**:26–37.
12. Tzeng H-P, Evans S, Gao F, Chambers K, Topkara VK, Sivasubramanian N, Barger PM, Mann DL. Dysferlin mediates the cytoprotective effects of TRAF2 following myocardial ischemia reperfusion injury. *J Am Heart Assoc* 2014;**3**:000662.
13. Askenasy N, Vivi A, Tassini M, Navon G, Farkas DL. NMR spectroscopic characterization of sarcolemmal permeability during myocardial ischemia and reperfusion. *J Mol Cell Cardiol* 2001;**33**:1421–1433.
14. Barnabei MS, Palpant NJ, Metzger JM. Influence of genetic background on ex vivo and *in vivo* cardiac function in several commonly used inbred mouse strains. *Physiol Genomics* 2010;**42A**:103–113.
15. Kohr MJ. Mitsugumin-53: potential biomarker and therapuetic for myocardial ischaemic injury? *J Mol Cell Cardiol* 2015;**81**:46–48.