Diaphragm rescue alone prevents heart dysfunction in dystrophic mice

Alastair Crisp1,†, HaiFang Yin2,3,*,†, Aurelie Goyenvalle1, Corinne Betts3, Hong M. Moulton4, Yiqi Seow3, Arran Babbs1, Thomas Merritt3, Amer F. Saleh5, Michael J. Gait5, Daniel J. Stuckey3,‡, Kieran Clarke3, Kay E. Davies1 and Matthew J.A. Wood3,*

1MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, South Parks Road, Oxford OX1 3QX, UK, 2Tianjin Research Centre of Basic Medical Science, Tianjin Medical University, Qiangtai Road, Heping District, Tianjin 300070, China, 3Department of Physiology, Anatomy and Genetics, University of Oxford, South Parks Road, Oxford OX1 3QX, UK, 4Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR 97331, USA and 5Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

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Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused, in most cases, by the complete absence of the 427 kDa cytoskeletal protein, dystrophin. There is no effective treatment, and affected individuals die from respiratory failure and cardiomyopathy by age 30. Here, we investigated whether cardiomyopathy could be prevented in animal models of DMD by increasing diaphragm utrophin or dystrophin expression and thereby restoring diaphragm function. In a transgenic mdx mouse, where utrophin was over expressed in the skeletal muscle and the diaphragm, but not in the heart, we found cardiac function, specifically right and left ventricular ejection fraction as measured using in vivo magnetic resonance imaging, was restored to wild-type levels. In mdx mice treated with a peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) that resulted in high levels of dystrophin restoration in the skeletal muscle and the diaphragm only, cardiac function was also restored to wild-type levels. In dystrophin/utrophin-deficient double-knockout (dKO) mice, a more severely affected animal model of DMD, treatment with a PPMO again produced high levels of dystrophin only in the skeletal muscle and the diaphragm, and once more restored cardiac function to wild-type levels. In the dKO mouse, there was no difference in heart function between treatment of the diaphragm plus the heart and treatment of the diaphragm alone. Restoration of diaphragm and other respiratory muscle function, irrespective of the method used, was sufficient to prevent cardiomyopathy in dystrophic mice. This novel mechanism of treating respiratory muscles to prevent cardiomyopathy in dystrophic mice warrants further investigation for its implications on the need to directly treat the heart in DMD.
expression. Utrophin is highly homologous to dystrophin and can functionally compensate for its loss when upregulated. Alternatively, for a subset of DMD patients, skipping of non-essential exons, induced by antisense oligonucleotides (AOs), can partially correct disease pathology by restoring a functional, although shortened, dystrophin protein. AO-mediated exon skipping therapies have shown promise in animal models after systemic delivery and in human subjects after local intramuscular delivery.

DMD is a systemic disease affecting the heart and other muscles, with 95% of patients exhibiting clinically relevant cardiomyopathy, making it necessary for any therapy to restore, or compensate for, the function of dystrophin in the heart. Most therapies are initially evaluated in the mdx mouse, an animal model of DMD, which lacks dystrophin. It displays a less severe phenotype than DMD due to compensation for the loss of dystrophin by its functional homolog, utrophin. The mdx mouse has abnormal ECGs and severely affected diaphragm at 6 months and left ventricular (LV) dysfunction by 11 months. Right ventricular (RV) dysfunction precedes the LV dysfunction, with a significant reduction in RV ejection fraction apparent at 8 months. We hypothesized that the RV dysfunction is due to the severely affected diaphragm and not the heart itself, and therefore that it would be possible to improve cardiac function by improving diaphragm function, without directly restoring or compensating for dystrophin loss in the heart.

To test this hypothesis, we studied various mouse models, with differently affected hearts and diaphragms. First, we looked at the Fiona mouse, a transgenic mdx mouse in which utrophin is upregulated in the skeletal muscles and the diaphragm but not in the heart. Secondly, we studied the effects of AOs on both the mdx mouse and the dystrophin/utrophin-deficient double-knockout (dKO) mouse, which is a more severe animal model of DMD than the mdx mouse. Both mdx and dKO mice were treated with phosphorodiamidate morpholino oligomers (PMOs) conjugated with cell-penetrating peptides (PMPMs). PMPMs are targeted to muscle cells where they induce exon skipping, restoring functional dystrophin with high efficiency. Several different PMPMs were used in this study, differing in their peptide or mode of conjugation (Table 1). This, and variations in route of administration and dose, resulted in differences in levels of dystrophin restoration in the skeletal muscles, the diaphragm and the heart.

This is the first study to investigate the novel implications of the finding that RV dysfunction precedes LV dysfunction in the mdx mouse. A number of groups are currently working on ways to treat cardiomyopathy in DMD animal models and, by extension, DMD patients, so a proper understanding of its mechanism is vital. We demonstrate that utrophin upregulation in the diaphragm, but not in the heart, is sufficient to restore cardiac function to wild-type levels in the mdx mouse; that dystrophin restoration in the diaphragm of the mdx and dKO mouse can do likewise; and that there is no difference in heart function between dKO mice with dystrophin restoration in both the diaphragm and the heart compared with restoration in the diaphragm alone. Thus, three separate lines of evidence from our studies in dystrophic mice support the hypothesis that restoring levels of diaphragm dystrophin or replacement with its homolog utrophin is sufficient to prevent the onset of cardiomyopathic changes. This novel mechanism for preventing cardiomyopathy by restoring diaphragm function in dystrophic mice warrants further investigation for its implications on the need to directly treat the heart in DMD.

RESULTS
Utrophin upregulation in the skeletal muscle, but not in the heart, of the Fiona mouse corrects heart function
We first studied the Fiona mouse, a transgenic mdx mouse in which utrophin is upregulated to three to four times normal levels in the hind limb muscles and approximately twice the normal level in the diaphragm, but not upregulated in the heart. The mouse has restored localization of DAPC components, a reduction in the numbers of centrally nucleated fibers and normalized force generation in both hind limb muscles and the diaphragm. To confirm this was still the case, 9-month-old Fiona, mdx and C57/Bl10 mice were sacrificed and western blot analysis of the quadriceps, the diaphragm and the heart found similar levels of upregulation in the quadriceps and the diaphragm, but no upregulation in the heart.

To determine whether restoring normal function to the diaphragm was sufficient to correct heart function, Fiona mice were imaged using cine-magnetic resonance imaging (MRI) and their cardiac function compared with age-matched mdx and C57/Bl10 control mice at both 6 and 9 months (Supplementary Material, Tables S1 and S2). At 6 months, the mdx mouse had a significant reduction in RV ejection fraction compared with age-matched wild-type C57/Bl10 mice. In age-matched Fiona mice, this was corrected and heart function was restored to near wild-type levels. At 9 months, the mdx mouse had a significant reduction in LV and RV ejection fraction compared with age-matched C57/Bl10 mice. This was again corrected in the age-matched Fiona mouse.

Restoration of dystrophin in the skeletal muscle and the diaphragm of mdx mice treated with B-PMO1 corrects heart function
As a significant reduction in RV ejection fraction was found in the 6-month-old mdx mouse, we investigated the extent to which cardiac function could be ameliorated by diaphragm rescue in mdx mice treated by AO-mediated exon skipping. We and others have previously shown that PPMO AOs conjugated to cell-penetrating peptides can restore cardiac dystrophin expression in the mdx mouse. Previous work has suggested that PPMO-induced cardiac dystrophin restoration in mdx mice can improve LV function. We hypothesized that the previously observed improvements in heart function were a result of diaphragm dystrophin restoration and not directly due to dystrophin restoration in the heart itself.
Six-month-old mdx mice were treated with a single dose of 18.75 mg/kg of B-PMO1 administered intravenously. Subsequent western blot analysis (Fig. 3A) showed that effective dystrophin restoration was observed in the skeletal muscle and the diaphragm but not in the heart. Two weeks later, mice were imaged using cine-MRI and their cardiac function compared with untreated mdx littermates and age-matched C57/Bl10 mice (Supplementary Material, Table S3). The treated mdx mice showed a significant increase in RV ejection fraction and LV and RV cardiac output compared with their untreated littermates, restoring cardiac function to wild-type levels (Fig. 3B). This supports our hypothesis and corroborates the results found with the Fiona mice despite the different method used for restoring diaphragm function.

Restoration of dystrophin in skeletal muscles after long-term P007-PMO administration to dKO mice corrects heart function

Next, we tested our hypothesis in a more severe model of DMD—the dystrophin/utrophin-deficient dKO mouse. The untreated dKO mouse has an average lifespan of 8–10 weeks (23) and although there is an increase in cardiac inflammation and fibrosis at 10 weeks (26), there is little concurrent reduction in heart function (A.C., unpublished data). We recently demonstrated that PPMO treatment could prevent the onset of dystrophic pathology in, and increase the lifespan of, the dKO mouse (27). Interestingly, although these dKO mice appeared almost completely rescued, there was no dystrophin restoration in the heart, making it possible that they would develop cardiomyopathy as they aged. The mice showed relocalization of DAPC components, reduction in serum creatine kinase (CK) levels and normalized force generation in both fore- and hindlimb muscles. Ten-day-old dKO mice were injected intraperitoneally with 25 mg/kg/week of P007-PMO for 6 weeks, as previously described (27), then subsequently re-injected monthly at the same dose. This treatment resulted in an improvement of their dystrophic pathology and an increase in lifespan. Of four mice studied, two were sacrificed at 13 months to confirm levels of dystrophin restoration in the heart, the diaphragm and other muscles, and two remain alive at 18 months. RNA and protein analysis of the treated muscles at 13 months confirmed the previous results obtained at 13 weeks (27). Dystrophin expression was present in all the tested muscles except the heart, with particularly high levels of restoration in the diaphragm (Fig. 4A), consistent with the immunohistochemical staining (data not shown). Age-matched untreated mdx mice were used as a negative control due to the limited lifespan of untreated dKO mice. Although age-matched controls cannot be used after 10 weeks, it is likely that older untreated dKO mice would have a more severe phenotype than age-matched mdx mice since the dKO mouse does have a worse phenotype until its death. Treated dKO mice were imaged using cine-MRI at 9 months and their cardiac function compared with age-matched wild-type C57/Bl6 mice (Supplementary Material, Table S4). A significant increase in LV ejection fraction (to wild-type levels) and an increase in RV ejection fraction (to near wild-type levels) were found compared with untreated age-matched mdx mice.
controls (Fig. 4B). This supports the data found in both previous experiments.

Near-complete restoration of dystrophin in skeletal muscles and the diaphragm after intraperitoneal delivery and also in the heart after intravenous delivery of B-PMO2 results in cardiac function improvement in dKO mice

Finally, we directly compared heart function in dKO mice in which dystrophin had also been restored in the heart with those in which it had not. This would allow us to determine what, if any, contribution restoration of cardiac dystrophin would make to cardiac function. B-PMO2 administration has previously been shown to restore dystrophin effectively in mdx mice (10,11,24,25). We found that treatment of dKO mice with B-PMO2 via intravenous (IV) injection restored dystrophin throughout all skeletal muscles, the diaphragm and the heart, whereas treatment via intraperitoneal (IP) injection only restored dystrophin in the skeletal muscles and the diaphragm. Long-term, repeated treatment with B-PMO2 via both these injection routes allowed us to directly assess the contribution of cardiac dystrophin restoration to cardiac function. Ten-day-old dKO mice were injected either intravenously or intraperitoneally with B-PMO2 at a 25 mg/kg dose weekly for 23 weeks and sacrificed 2 weeks after the last injection. Both the survival rate and health conditions were significantly improved following treatment and all treated dKO mice were observed to be as healthy as their mdx littermate controls to the end-point of the experiment (at 6 months of age; data not shown). Immunohistochemical staining showed that, in IV and IP injected mice, a near-normal level of dystrophin expression was present in all the tested skeletal muscles, and in IV injected mice, dystrophin restoration was also present in the heart (Fig. 5A). Western blotting showed that ~100% of normal levels of dystrophin was restored in all skeletal muscles and ~50% of normal levels in the heart following IV administration of B-PMO2. In the samples treated via IP injection, 100% dystrophin restoration could only be seen in the diaphragm, with less dystrophin (~30–50%) restored in other skeletal muscles and no dystrophin detectable in the heart (Fig. 5B).

Both administration routes resulted in the successful relocalization of multiple DAPC component proteins as shown by serial immunostaining (Supplementary Material, Fig. S1A), and significant force recovery when compared with age-matched untreated mdx controls as shown by grip strength tests (Supplementary Material, Fig. S1B). Centrally nucleated myofiber counts showed a significant reduction in both IV and IP injected mice compared with untreated age-matched mdx littermate controls, a finding which is corroborated by a significant decrease in the percentage of regenerating fibers detectable in treated muscles (Supplementary Material, Fig. S1C). Other measurements, for example of serum CK, also indicated phenotypic improvement in treated dKO mice (data not shown).

Treated dKO mice were imaged using cine-MRI immediately before sacrifice and compared with age-matched mdx and wild-type C57/BL6 mice (Supplementary Material, Table S5). A significant increase in RV ejection fraction was found in dKO mice treated via either route compared with untreated age-matched mdx controls (Fig. 5C), indicating that cardiac function was improved irrespective of treatment route. Cardiac function for both groups of dKO mice was statistically indistinguishable from that of the age-matched wild-type C57BL6 mice controls. No additional benefit was observed in heart function in treated dKO mice, with dystrophin restored in both the diaphragm and the heart compared with restoration in the diaphragm alone. These data further confirmed our hypothesis that diaphragm rescue is sufficient to restore heart function to wild-type levels in dystrophic mice.

DISCUSSION

Two promising therapeutic candidates for the treatment of DMD are utrophin upregulation and AO-mediated exon skipping. Work on skeletal muscle treatment has been ongoing for some time but studies have recently started to focus on rescuing the heart. In this study, using mdx and dKO animal models and evaluating both therapeutic approaches, we demonstrate that compensation for, or restoration of, diaphragm dystrophin is sufficient to rescue cardiac function without direct treatment of the heart itself.

Work on utrophin upregulation has been carried out in the mdx mouse (3–7,28), dKO mouse (29,30) and dystrophic dog (31); however, these studies have largely focussed on the skeletal muscle, with many of them using skeletal muscle-specific expression systems. Our study shows that complete rescue of the diaphragm by utrophin upregulation was sufficient to restore the mdx heart to wild-type levels of cardiac function to at least 9 months of age, as measured by significant
improvements in RV ejection fraction at 6 months and both LV and RV ejection fraction at 9 months.

Recently, exon-skipping AOs have been studied in clinical trials in the Netherlands (16) and the UK (17). However, based on the data from pre-clinical animal models, high doses of unmodified AOs are likely to be required for correction of dystrophin expression in skeletal muscles, and the amount of dystrophin restoration in the heart muscle remains very low (10,11). Conjugation of cell-penetrating peptides to PMOs has resulted in remarkable and sustained dystrophin restoration in skeletal muscles and also, at lower levels, in the heart (12,13,24,25). A number of these studies (12,25) have shown improvement in cardiac function following treatment of the \textit{mdx} mouse with these PPMOs. Our results show that in \textit{mdx} mice following single injections of B-PMO1, leading to restoration of high levels of dystrophin in the diaphragm but none in the heart, normal levels of cardiac function are found. This suggests that the previous studies may have incorrectly attributed the observed improvements in cardiac function to dystrophin restoration in the heart. These data support the results found following utrophin upregulation in the diaphragm and suggest that restoration of normal diaphragm function, irrespective of method, is sufficient to restore \textit{mdx} mouse heart function to wild-type levels.

In the more severely affected \textit{dKO} model, we found that long-term IP administration of P007-PMO restored high levels of dystrophin to the skeletal muscles and the diaphragm but not to the heart, resulting in a greatly increased lifespan, with treated mice continuing to live beyond 18 months. These mice showed essentially normal cardiac function at 9 months, further supporting the result in the \textit{mdx} mice. Furthermore, in \textit{dKO} mice treated with B-PMO2, despite the difference in restoration of dystrophin expression in the heart between IP and IV administration routes, there was no significant difference in terms of phenotypic, functional and morphological parameters and both groups displayed normalized cardiac function at 6 months. This suggests that, at least up to 9 months of age, cardiac function in dystrophic mice can be normalized without directly treating the heart. Certain mutations in the dystrophin gene seem to mainly affect the heart, causing X-linked dilated cardiomyopathy (XLDC) (32,33). This would appear to directly contradict our hypothesis. A separate pathway may be involved in the pathogenesis of XLDC, or this pathway may only become active in older mice than we studied. As such, an understanding of the pathogenesis of the heart dysfunction in XLDC would be instructive.

Previous work has suggested that functional improvement in skeletal muscles due to dystrophin restoration may be
with age-matched in LV and RV ejection fraction was found in untreated treated with P007-PMO at 25 mg/kg repeated doses. A significant decrease pressure, suggesting the presence of RV failure (36). This pulmonary hypertension increases right atrial hypertension). Furthermore, it has been shown in DMD causes an increase in pulmonary vascular pressure (pulmonary hypertension could also be due to the absence of dystrophin in vascular smooth muscle cells, but dystrophin has been shown to be non-essential for the maintenance of normal vascular tone or for acute changes in tone (37), suggesting that this is probably not the primary cause. Therefore, it is likely that utrophin upregulation or dystrophin restoration in the respiratory muscles, most importantly in the diaphragm, prevents a decline in lung function, leading to our assertion that it is the improvement in diaphragm function that is responsible for the improvement in heart function.

In summary, this study indicates that, in both the mdx and dKO models of DMD, treatment of the heart is not necessary in order to improve heart function. Restoration of diaphragm (and other respiratory muscle) function, irrespective of method and AO chemistry, is sufficient to restore cardiac function to near-normal levels in mice up to 9 months of age. This suggests that, in dystrophic mice, the use of PPMOs with heart activity may be unnecessary. Further work is necessary to establish whether normal diaphragm function remains sufficient to preserve cardiac function in older mice and to determine whether stressing the heart, for example by forced running or high-dose dobutamine infusion, may give a measure of cardiac function that is independent of diaphragm function. These observations provide a novel mechanism for dystrophic cardiomyopathy and its correction and suggest that treatments that maintain or restore diaphragm function are likely to have consequent benefits for heart function in DMD patients.

**MATERIALS AND METHODS**

**Animals and injections**

Six-month-old *Fiona, mdx* and C57/Bl10 mice were used for the utrophin upregulation experiments (*n* = 9 in each group). Six-month-old *mdx* mice with their *mdx* littermates and C57/Bl10 mice as controls were used in the single-dose B-PMO1 IV administration experiments at 18.75 mg/kg dose (*n* = 6 for both *mdx* groups and *n* = 8 for wild-type controls).

Ten-day-old *dKO* mice were used in the long-term P007 administration experiments (*n* = 4). They were injected intraperitoneally 25 mg/kg/week for 6 weeks, as previously described (27), then subsequently re-injected monthly.

Ten-day-old *dKO* mice and *mdx* littermates as controls were used in the long-term B-PMO2 administration experiments (*n* = 3 in the test groups and *n* = 6 in control groups) after genotyping. For systemic IV and IP injections, B-PMO2 in 80 μl of saline buffer was injected into *dKO* mice at the final dose of 25 mg/kg.

Mice were killed by CO₂ inhalation or cervical dislocation at the desired time points, and muscles and other tissues were snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C.

All of the experiments were carried out in the Animal Unit of the Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK, according to procedures authorized by the UK Home Office.

**Peptide–PMO conjugates**

Three different conjugates were used in the study. Their sequences are summarized in Table 1.

PMO AO M23D, a 25mer PMO targeting the boundary sequences of exon and intron 23 of the mouse dystrophin gene (11), was conjugated to various peptides by a stable amide linker (38) as synthesized by Gait’s Laboratory (Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK) or by AVI Biopharma, Inc. (Corvallis, OR, USA).

**RNA extraction and nested RT–PCR analysis**

Total RNA was extracted with TRIzol (Invitrogen, UK), and 200 ng of RNA template was used for a 20 μl RT–PCR.
Dystrophin restoration and cardiac function improvement following repeated systemic administration of B-PMO2 to dKO mice. dKO mice were treated with 23 repeated weekly injections of B-PMO2 at 25 mg/kg dose either intravenously (IV) or intraperitoneally (IP). (A) Immunostaining of muscle tissue cross-sections to detect dystrophin protein expression and localization in C57/BL6 normal control (top panel), untreated dKO mouse (middle panel), IV-treated (third panel) and IP-treated dKO mice (bottom panel). Muscle tissues analyzed were from tibialis anterior, gastrocnemius, quadriceps, biceps, diaphragm, heart and abdominal wall muscles (scale bar: 200 μm). (B) Western blot for dystrophin expression in IV- and IP-treated dKO mice. Equal loading of 10 μg of protein is shown for each sample. α-Actinin was used as the loading control. (C) In vivo cardiac cine-MRI of dKO mice treated with B-PMO2 at 25 mg/kg repeated doses. A significant decrease in RV ejection fraction was found in untreated mdx mice compared with age-matched wild-type C57/Bl6 mice (P < 0.05), but no difference in LV ejection fraction. Heart function was restored to normal levels in dKO mice treated with B-PMO2 both IV and IP. An asterisk indicates measurements significantly different to B-PMO2 treated mice (P < 0.05).

Table 1. Oligonucleotide and peptide nomenclature and sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Abbreviation</th>
<th>Length</th>
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<tr>
<td>M23D</td>
<td>5'-GGCCAAACCTCGGCTTACCTGAAAT-3'</td>
<td>PMO</td>
<td>25</td>
</tr>
<tr>
<td>P007</td>
<td>N-RXRRXRRXRXXRXXB-C</td>
<td>(RXRR)XB</td>
<td>14</td>
</tr>
<tr>
<td>B peptide</td>
<td>N-RXRRBRXXRXXBRXXB-C</td>
<td>(RXRRBRB)XB</td>
<td>14</td>
</tr>
</tbody>
</table>

R, α-arginine; X, 6-aminohexanoic acid; B, β-alanine; B-PMO1 and B-PMO2 were from different suppliers. B-PMO1 was provided by Gait’s Laboratory (Medical Research Council, Laboratory of Molecular Biology, Cambridge, UK) using the synthesis and conjugation protocols as reported (41) (H.Y. and A.F.S., manuscript submitted). B-PMO2 is from AVI Biopharma (OR, USA) using published methods (35).
with a OneStep RT–PCR kit (Qiagen, UK). The primer sequences were used as previously reported (13). The products were examined by electrophoresis on a 2% agarose gel.

Protein extraction and western blot
For the utrophin upregulation experiments, total muscle extracts were prepared by homogenization in extraction buffer (125 mM Tris, pH 8, 4% SDS, 40% glycerol, 0.05% PMSF, 0.1 mM DTT), and then heating at 95°C for 5 min. Samples were loaded onto 6% polyacrylamide gels and transferred to nitrocellulose. Urophisin expression was detected using a 1:50 dilution of MANCHO3 (39) and made visible with anti-mouse IgG linked to horseradish peroxidase and the ECL western blotting analysis system (Amersham Pharmacia Biosciences, UK). α-Aktinin was detected using monoclonal antibody with a 1:200 dilution (Sigma, UK). The intensity of the bands obtained from Fiona, mdx and C57/Bl10 muscles was measured by ImageJ software; the quantification is based on band intensity and area.

For the exon skipping experiments with B-PMO1 and 2, protein extraction and western blot were carried out as previously described (13); with P007-PMO, protein extraction and western blot were carried out as previously described (27). Various amounts of protein from normal C57/BL6 mice were used as a positive control, and corresponding amounts of protein from muscles of treated or untreated mice were used. The membrane was probed with DYS1 (NovoCastra, UK) for the detection of dystrophin protein. The bound primary antibody was detected by horseradish peroxidase-conjugated rabbit anti-mouse IgGs and the ECL western blotting analysis system (Amersham Pharmacia Biosciences, UK). The intensity of the bands obtained from treated muscles was measured by ImageJ software; the quantification is based on band intensity and area, and is compared with that from normal muscles of C57BL6 mice.

Measurement of heart function
High-resolution cine-MRI was carried out using a 40 mm 31P-imaging probe on an 11.7 T (500 MHz) system comprising a vertical magnet (Magnex Scientific, Oxon, UK), a Bruker Avance console (Bruker Medical, Ettingen, Germany) and a shielded gradient system (Magnex Scientific, Oxon, UK), as previously described (40). Mice were anesthetized with isoflurane at a rate of 4 l/min and maintained at 2% isoflurane as previously described (40). Mice were anesthetized using 4% isoflurane at a rate of 4 l/min and maintained at 2% isoflurane at 1.5 l/min oxygen flow throughout the MRI experiments. Short-axis slices were acquired to cover the whole heart using a cardiac-triggered fast low-angle shot sequence. For each slice, end-diastolic and end-systolic frames were selected according to maximal and minimal ventricular volumes. LV mass was calculated by multiplying the LV wall volume by the specific gravity of the myocardium (1.05 g/cm³). The epicardial and endocardial borders were outlined in end-diastolic and end-systolic frames, using the free-hand drawing function of ImageJ. The number of voxels in each compartment multiplied by the pixel size of 0.015 mm³ yielded the respective volumes. Stroke volume (SV = EDL – ESL), ejection fraction (EF = SV/EDL) and cardiac output (CO = SV × heart rate) were calculated from the end-diastolic and end-systolic lumen volumes. Peak ejection and filling rates were calculated from the difference in cavity volume over time from a single cine-MRI image acquired at the mid-papillary level.

Statistical analysis
All data are reported as mean values ± SEM. Statistical differences between treatment groups and control groups were evaluated by two-tailed Student’s t-test.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES


