

Emergent Dilated Cardiomyopathy Caused by Targeted Repair of Dystrophic Skeletal Muscle

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Duchenne muscular dystrophy (DMD) is a fatal disease characterized by deterioration of striated muscle, affecting skeletal and cardiac muscles. Recently, several therapeutic approaches have shown promise for repairing dystrophic skeletal muscles. However, these methods often leave the dystrophic heart untreated. Here we show that, in comparison to fully dystrophin-deficient animals, targeted transgenic repair of skeletal muscle, but not cardiac muscle, in otherwise dystrophin-deficient (*mdx*) mice paradoxically elicited a fivefold increase in cardiac injury and dilated cardiomyopathy in these animals *in vivo*. Skeletal muscle repair was shown to increase the voluntary activity of the *mdx* mice as quantified by voluntary running on the exercise wheel. Because the dystrophin-deficient heart is highly sensitive to increased stress, we hypothesize that increased activity (enabled by the repaired skeletal muscle) provided the stimulus for heightened cardiac injury and heart remodeling. In support of this hypothesis, the primary cellular compliance defect in dystrophin-deficient cardiac myocytes was found to be unchanged by skeletal muscle repair in the *mdx* mice. These findings provide new information on the evolution of cardiac disease in dystrophin-deficient animals and underscore the importance of implementing global striated muscle therapies for muscular dystrophy.

Received 25 January 2008; accepted 25 February 2008; published online 15 April 2008. doi:10.1038/mt.2008.52

INTRODUCTION

Recent therapeutic approaches to Duchenne muscular dystrophy (DMD), featuring stem cells, direct intramuscular injection of DNA vectors, and oligonucleotide-mediated exon skipping, have shown potential for restoring dystrophin expression in skeletal muscles.^{1–6} These strategies often leave the dystrophic cardiac muscle essentially untreated, a potentially significant omission given the great clinical relevance of cardiac function in DMD. The consequences of skeletal muscle-centric therapies for DMD patients are unknown and important to ascertain. Insights can be inferred from patients with X-linked cardiomyopathy, who have normal skeletal muscles but have marked dystrophic cardiomyopathy. These patients present with significant cardiac dysfunction

and advanced heart failure in the second decade of life, a clinical course that is accelerated relative to that in DMD patients.^{7–9} This observation raises the question of whether therapies restricted to skeletal muscles alone in patients with DMD would accelerate heart disease in these patients.

We addressed this important question by implementing skeletal muscle-restricted transgene expression of a highly functional mini-dystrophin protein in the dystrophin-deficient *mdx* mouse. Transgenic dystrophin-deficient mice (*mdx^{4cv}*) were generated, using a skeletal muscle actin promoter to induce expression of a highly functional mini-dystrophin gene exclusively in the skeletal muscles of the mice.¹⁰ Skeletal muscles of transgenic *mdx^{4cv}* (TG-*mdx^{4cv}*) mice have normal histology and full correction of the contraction-mediated force deficits characteristic of dystrophin deficiency.¹⁰ The normal skeletal muscle cytoskeletal structure and function make this an excellent model for evaluating the consequences of skeletal repair on untreated dystrophin-deficient myocardium.

RESULTS

The skeletal muscle-restricted mini-dystrophin expression in the TG-*mdx^{4cv}* mouse is shown in **Figure 1**. The hearts of TG-*mdx^{4cv}* as well as NTG-*mdx^{4cv}* mice are fully devoid of dystrophin. The hearts removed from 4- to 5-month-old TG-*mdx^{4cv}* or NTG-*mdx^{4cv}* animals displayed normal histology with no evidence of significant chronic fibrosis (data not shown). However, an examination of intracellular immunoglobulin G accumulation revealed that TG-*mdx^{4cv}* mice have a fivefold increase in acute cardiac injury as compared to their nontransgenic (NTG-*mdx^{4cv}*) littermates which are devoid of dystrophin in both skeletal and cardiac muscles (**Figure 1**). Importantly, the left ventricles of TG-*mdx^{4cv}* mice displayed significant dilation in comparison with those of their NTG-*mdx^{4cv}* littermates (**Figure 2a** and **b**). This left ventricular dilation was accompanied by a significant decline in contractile function as measured by ejection fraction (**Figure 2c**) and by preload recruitable stroke work, a load-independent measure of cardiac function (**Figure 2d**). Furthermore, the passive tension-extension properties of single cardiac myocytes from NTG-*mdx^{4cv}* and TG-*mdx^{4cv}* mice were similar, and both demonstrated severe reduction of compliance compared to myocytes from dystrophin-replete mice (**Figure 3**). The marked left ventricular dilation and contractile dysfunction found in

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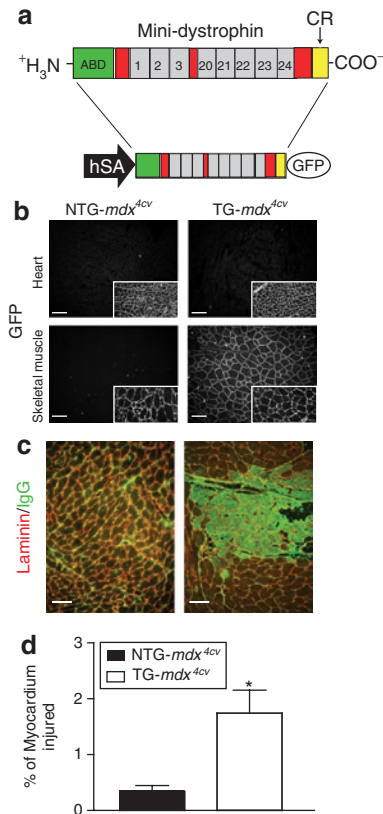


Figure 1 Increased cardiac injury in TG-*mdx*^{4cv} mice. **(a)** Schematic representation of the skeletal muscle-directed mini-dystrophin-GFP fusion protein transgene construct (top). Actin-binding domain (ABD; green), hinge regions (red), spectrin repeats (grey, number corresponds to the position in the full-length dystrophin), cysteine-rich domain (CR; yellow), and human skeletal actin promoter (hSA). **(b)** The expression of mini-dystrophin is localized to skeletal muscle, with no expression evident in the heart (upper images). Insets show laminin staining; (Bar = 100 μ m). GFP, green fluorescent protein. **(c)** Representative images of myocardial lesions. Laminin (red) and immunoglobulin (IgG; green) immunoreactivity outline individual myocytes. In regions of cardiac injury, immunoglobulin has entered the center of the cardiac myocytes (right). Bar = 25 μ m. **(d)** Quantification of the area of myocardium with lesions. Values are mean \pm SEM. The asterisks indicate that NTG-*mdx*^{4cv} is significantly different from TG-*mdx*^{4cv} ($P < 0.05$).

TG-*mdx*^{4cv} mice constitute direct hemodynamic evidence of an emergent (e.g., newly formed) dilated cardiomyopathy in these animals *in vivo*.

In order to address potential factors that may elicit increased cardiac injury and dilated cardiomyopathy in TG-*mdx*^{4cv} mice, we quantified their activity by giving them access to a running wheel. We tested the hypothesis that full correction of skeletal muscle and respiratory muscle functions in TG-*mdx*^{4cv} mice¹⁰ would permit an increase in their physical activity that would, in turn, place additional stress on the dystrophin-deficient myocardium in these mice. The results revealed that TG-*mdx*^{4cv} mice showed a significant increase in the magnitude and speed of their voluntary running activity (Figure 4). Presumably, this increased activity is independent of the presence of a running wheel, the wheel simply allows the quantification of this increased activity. Because the dystrophic myocardium is highly sensitive to increased workloads and stresses,^{11–13} the additional activity exhibited by the TG-*mdx*^{4cv}

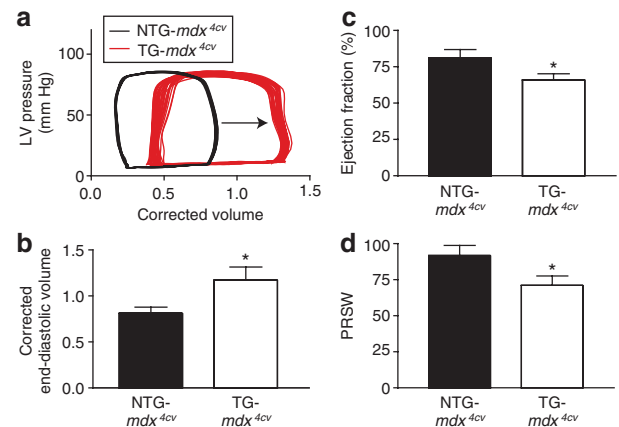


Figure 2 *In vivo* hemodynamic assessment of cardiac function. **(a)** Representative *in vivo* pressure-volume loops in TG-*mdx*^{4cv} and NTG-*mdx*^{4cv} mice. Summary of **(b)** left ventricular (LV) end-diastolic volumes, **(c)** ejection fraction, and **(d)** preload recruitable stroke work (PRSW). The values are mean \pm SEM ($n = 15$ for NTG-*mdx*^{4cv} and $n = 13$ for TG-*mdx*^{4cv}). The asterisks indicate that TG-*mdx*^{4cv} is significantly different from NTG-*mdx*^{4cv} ($P < 0.05$). The daggers indicate ventricular volumes were corrected for body weight.

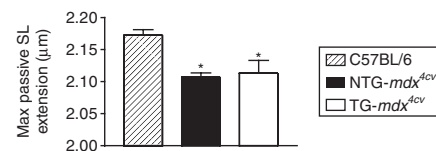


Figure 3 Summary of relationship between single adult cardiac myocyte passive tension and sarcomere length extension. Adult cardiac myocytes isolated from both NTG-*mdx*^{4cv} and TG-*mdx*^{4cv} mice show significant reduction in tolerance to passive length extension as compared to control myocytes. Values are mean \pm SEM ($n = 8$ for C57BL/6, $n = 7$ for NTG-*mdx*^{4cv}, and $n = 9$ for TG-*mdx*^{4cv}). The asterisks indicate a significant difference from C57BL/6 ($P < 0.05$).

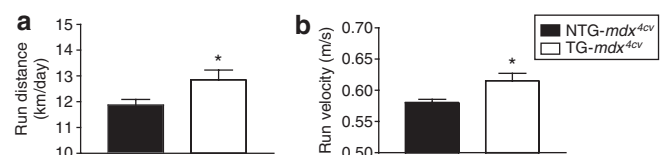


Figure 4 Quantification of activity on running wheel. **(a)** Quantification by daily distance and **(b)** quantification by velocity. The data are determined from a period of 40–43 days of exercise from 8 to 11 mice. The asterisks indicate that the result for TG-*mdx*^{4cv} mice is significantly different from that for NTG-*mdx*^{4cv} mice ($P < 0.05$).

mice could have provided the stimulus for the increase in cardiac injury and left ventricular dilation demonstrated in this study. We noted that the running activity in TG-*mdx*^{4cv} mice, while increased, was not fully corrected back to control values (16.1 \pm 0.2 km/day for control versus 12.9 \pm 0.4 km/day and 11.9 \pm 0.2 km/day for TG-*mdx*^{4cv} and NTG-*mdx*^{4cv}, respectively). This can be accounted for by the role the heart plays in aerobic activity and is in keeping with the increased cardiac disease in TG-*mdx*^{4cv} that likely limited their full return to normal exercise behavior.

DISCUSSION

To our knowledge, this is the first study to demonstrate a direct link between correction of skeletal and respiratory muscles

and heightened cardiac disease in an animal model of DMD. This finding suggests that, in the context of patients with DMD, caution needs to be exercised when considering potential therapeutic approaches that may have efficacy in skeletal and respiratory muscles but very little activity in the heart. Stem cells, myostatin inhibition, intraskeletal muscle injection of gene therapy vectors, and oligonucleotide-based exon skipping, all have shown promise in improving DMD skeletal muscle, but these therapeutic strategies have not been effective in treating the heart.^{1–5,14} One of the most dramatic examples of skeletal muscle-centric therapies has been the partial return to normal mobility in dystrophic dogs receiving mesoangioblast stem cells to their limb musculature.¹ The overall laudable goal of these approaches is to return mobility to DMD patients, and to do so would certainly be of great benefit to these patients; however, the present data suggest that a potential tragic consequence of these treatments could be to accelerate cardiac disease and progression to overt heart failure.

The results presented in this study are in accordance with the clinical picture of patients with X-linked dilated cardiomyopathy, who have a specific loss of dystrophin in the myocardium.^{7–9} Because of a variety of genetic mechanisms, the hearts of these patients are devoid of dystrophin expression, while dystrophin protein is present in their skeletal muscles, thereby preventing the development of skeletal muscle disease.^{15–23} In X-linked dilated cardiomyopathy patients, heart disease develops earlier and progresses much faster than in DMD patients, who lack dystrophin in both cardiac and skeletal muscles.^{9,16,24,25} The normal levels of skeletal muscle dystrophin present in X-linked dilated cardiomyopathy patients, in contrast to DMD patients, result in normal mobility in their childhood and adolescent years, as demonstrated by their ability to participate in competitive sports.^{16,17,19,20}

The mechanism underlying the worsening of heart disease in X-linked dilated cardiomyopathy as compared to DMD patients is unknown. However, the data presented here suggest that increases in activity level may play a role. Many studies have demonstrated the fragile nature of dystrophin-deficient hearts in both mice and humans, particularly in the face of heightened stress.^{11,13,26–28} It is therefore possible that even small increases in activity, as seen here in increased voluntary running activity (Figure 4), may provide sufficient additional stress to cause the heightened cardiac injury and cardiac hemodynamic dysfunction observed in these dystrophin-deficient hearts.

The results of this study underscore the complexity of treating a global disease such as DMD, wherein the alleviation of disease in skeletal muscle may in fact hasten disease progression in the heart. As new experimental therapies become more effective, it will be increasingly important to understand the capacity of these therapies to treat both skeletal and cardiac muscles in muscular dystrophy.

MATERIALS AND METHODS

Animals. Control (C57 BL/6J) mice were obtained from Jackson Laboratories (Bar Harbor, ME), NTG-*mdx*^{4cv} and mini-dystrophin TG-*mdx*^{4cv} were generated as described earlier.¹⁰ The *mdx*^{4cv} mice were generated through mutagenesis screening of C57 BL/6 mice, and found to

contain a premature stop codon in exon 53 of the dystrophin gene.²⁹ All the mice were maintained in barrier isolation facilities at the University of Michigan. The mice were 4–5 months old, and the TG-*mdx*^{4cv} and NTG-*mdx*^{4cv} mice were littermates, all of which were heterozygous for the transgene. The procedures used in this study were approved by the University of Michigan Committee on the Use and Care of Animals.

Immunofluorescence. Mouse immunoglobulin was detected using an anti-mouse immunoglobulin G conjugated to alexia 488. Laminin was detected using the rabbit polyclonal antibody from Sigma (St Louis, MO; L9393). Lesion quantification was performed without the knowledge of genotype. Lesions were defined as areas that had positive IgG staining and retained normal myocardial morphology; areas of vessels and other fibrous regions were not included in the final analysis. The analysis was carried out on 7–14 heart sections from four to seven animals.

Functional assays. Cardiac catheterization was performed as described elsewhere.^{11,12} Because of significant differences in total body weight between age-matched TG-*mdx*^{4cv} and NTG-*mdx*^{4cv}, all the absolute left ventricular volumes are expressed per gram of body weight. Passive extension-tension assays were performed using carbon fibers attached to acutely isolated membrane-intact cardiac myocytes, as described elsewhere.¹¹

Wheels to quantify voluntary running. In order to quantify activity levels in these mice, running wheels with a diameter of 10 cm and a textured plastic surface were placed in each mouse cage. Wheel rotation was monitored by optical scanning, and recorded every 10 seconds (Data Science International, St. Paul, MN). After a seven day acclimatization period, running data were analyzed by converting the number of full rotations into distance. These distances were totaled for each 24-hour period. The average velocity was calculated by dividing the total distance run by the total time spent on the running wheel. The data are derived from 26 to 53 days of running from 8 to 16 mice.

Statistical methods. All comparisons utilized the *t*-test, except when multiple comparisons were required. For the analysis of multiple comparisons an analysis of variance with a Dunnett's post test was utilized.

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