Prevention of Dystrophin-Deficient Cardiomyopathy in Twenty-One-Month-Old Carrier Mice by Mosaic Dystrophin Expression or Complementary Dystrophin/Utrophin Expression

Brian Bostick,* Yongping Yue,* Chun Long, Dongsheng Duan

Abstract—A cure for dystrophin-deficient muscular dystrophy requires treating both skeletal muscle and the heart. Whereas mosaic dystrophin expression has been shown to protect skeletal muscle, controversy exists over whether mosaic expression is protective in the heart. We have shown recently that mosaic dystrophin expression prevents stress-induced heart damage in young carrier mice. Although an interesting finding, the clinical relevance remains to be established because young dystrophin-null mdx mice do not have heart disease. On the other hand, heart failure has been reported in human carriers. To resolve this mouse/human discrepancy, we evaluated the cardiac phenotype in 21-month-old mdx, carrier, and normal mice. We found dilated cardiomyopathy in old mdx mice but not in age-matched carrier mice. All anatomical parameters and physiological assay results (ECG and closed-chest Millar catheter) were within the normal range in old carrier mice. Focal myocardial inflammation was found in a small fraction of old carrier mice, but it had no major impact on heart function. Dobutamine stress revealed a near normal hemodynamic profile except for a marginal reduction in systolic pressure in old carrier mice. Immunostaining and Western blot showed dystrophin expression in 50% cardiomyocytes in old mdx carrier mice. Interestingly, utrophin was upregulated in dystrophin-negative heart cells in carrier mice. In summary, we have provided the first clear-cut evidence that dilated cardiomyopathy in old mdx mice was prevented by mosaic dystrophin expression or complementary dystrophin/utrophin expression. Our results raise the hope for ameliorating dystrophic cardiomyopathy through partial gene and/or cell therapy. (Circ Res. 2008;102:121-130.)

Key Words: cardiomyopathy ■ gene therapy ■ myocardium ■ genetics ■ heart disease

Heart disease profoundly affects the life quality of Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) patients.1,2 DMD and BMD are caused by mutations in the dystrophin gene. Dystrophin is a long rod-shaped cytoskeletal protein located at the cytosolic surface of the sarcolemma. It glues the cytoskeleton, sarcolemma, and extracellular matrix together to prevent cell membrane damage during muscle contraction. Absence of dystrophin or abnormal dystrophin expression weakens the physical link between the extracellular matrix and the cytoskeleton. As a consequence, the affected muscle cells undergo degeneration and necrosis. Eventually, muscle tissue is replaced by fibrous, bony and/or fatty tissue and loses function.

Pathology in the heart and the diaphragm determines the life span in DMD/BMD patients. Until recently, approximately 80% to 90% of DMD patients died from respiratory failure because of a weak diaphragm. With improved respiratory care, heart-related death has become more frequent, even approaching 40% in some studies.3 Currently, symptomatic management is the only treatment option. The advent of gene and cell therapies brings the hope of a cure for DMD/BMD.2 In gene therapy, the mutated gene is replaced and/or repaired.4 In cell therapy, a population of functional stem cells is introduced to regenerate muscle.5 The fundamental idea behind these novel therapies is to produce enough dystrophin-expressing muscle cells to halt or reverse the dystrophic process.

To completely transduce and/or regenerate every single muscle cell may not be realistic. It is thus critical to determine whether mosaic dystrophin expression in a subpopulation of cells can fulfill the physiologic need. Fifty-percent mosaic expression has been shown to ameliorate severe skeletal muscle disease in human patients.6,7 We recently observed similar findings in mice with adenosassociated virus–mediated expression of a highly abbreviated microdystrophin gene.8 Despite the importance and need for treating dystrophic cardiomyopathy, very few studies have been performed to determine whether mosaic dystrophin expression benefits the heart.

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To address this issue, we previously examined heart function in 3-month-old mdx mice and carrier mice. mdx mice are naturally occurring dystrophin-null mice found in the C57Bl/10 (BL10) background but they do not display classic dystrophic cardiomyopathy such as prominent fibrosis at young age. However under positive inotropic stress, the young mdx hearts show obvious sarcolemma damage as well as reduced hemodynamic performance. Carrier mice express dystrophin in only half of the heart cells. Surprisingly, stress-induced heart damage was completely normalized in young carrier mice. Although this is an informative result, it may not be applicable to patients who, unlike young mdx mice, do experience dilated cardiomyopathy. Whether mosaic expression can indeed prevent cardiomyopathy remains an unanswered question.

Partial/mosaic correction is the most likely outcome of gene and/or cell therapy for DMD/BMD heart disease. It is therefore important to determine whether an incomplete therapy can lead to clinically meaningful improvement in the heart. To answer this question, we first established a phenotypic cardiomyopathy model in 21-month-old mdx mice. We then evaluated heart structure and function in age-matched carrier mice. Mosaic dystrophin expression in half cardiomyocytes prevented the development of cardiomyopathy in old carrier mice. Additional studies showed utrophin upregulation in dystrophin-negative cardiomyocytes. Taken together, we have demonstrated, for the first time, that mosaic dystrophin expression was sufficient to maintain healthy hearts in a phenotypic model. Our data strongly support further pursuing gene and/or cell therapy for dystrophin-deficient cardiomyopathy in DMD/BMD patients.

Materials and Methods

Animals
All animal experiments were approved by the Animal Care and Use Committee of the University of Missouri and were in accordance with NIH guidelines. All experimental mice were housed in a specific pathogen-free facility. BL10 (C57Bl/10SnJ) and mdx (C57Bl/10ScSn-Dmdmdx/J) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). Female carrier mice were generated by crossing BL10 and mdx mice. Locomotor activity was measured in 3-month-old BL10, mdx, and carrier, respectively. Values in Table are significantly different from that in BL10 or carrier. Sample sizes are 14, 9, and 10 for BL10, mdx, and carrier, respectively. Values in Table are significantly different from that in BL10 but not carrier.

Histopathology Studies
Standard hematoxylin/eosin staining was used to reveal general histology. Fibrosis was evaluated by Masson trichrome staining. Standard hematoxylin/eosin staining was used to reveal general structure and function in age-matched carrier mice. Mosaic dystrophin expression in half cardiomyocytes prevented the development of cardiomyopathy in old carrier mice. Additional studies showed utrophin upregulation in dystrophin-negative cardiomyocytes. Taken together, we have demonstrated, for the first time, that mosaic dystrophin expression was sufficient to maintain healthy hearts in a phenotypic model. Our data strongly support further pursuing gene and/or cell therapy for dystrophin-deficient cardiomyopathy in DMD/BMD patients.

Immunostaining
Dystrophin was examined with three antibodies including a mouse monoclonal antibody against the C-terminal domain (Dys-2, 1:30; clone Dy8/6CS, IgG1; Novocastra, Newcastle, UK), a mouse monoclonal antibody against the rod domain (Manex50, 1:20; clone DRP3/20C5, IgG1; Vector Laboratories, Burlingame, Calif); β-Sarcoglycan was revealed with a mouse monoclonal antibody (NCL-b-SARC, 1:50; clone 5B1, IgG1; Novocastra). Dystrobrevin was revealed with a mouse monoclonal antibody (1:200; clone 23, IgG1; no. 610766, BD Biosciences, San Diego, Calif). Syntrophin was revealed with a pan-syntrophin mouse monoclonal antibody that recognized the PDZ domain (Manex50, 1:200; clone 6A9, IgG1; a Dy8/6C5, IgG1; Novocastra, Newcastle, UK), a mouse monoclonal antibody against spectrin-like repeat 4 to 6 (1:400; Santa Cruz Biotechnology). Dystrophin was examined with three antibodies including a mouse monoclonal antibody against the utrophin N-terminal domain (VP-U579, 1:20; clone DRP3/20C5, IgG1; Vector Laboratories, Burlingame, Calif), β-Sarcoglycan was revealed with a mouse monoclonal antibody against the C terminus (NCL-b-DG, 1:50; clone 43DAG1/8DS, IgG2a; Novocastra). β-Sarcoglycan was revealed with a mouse monoclonal antibody (NCL-b-SARC, 1:50; clone 5B1, IgG1; Novocastra). Dystrobrevin was revealed with a mouse monoclonal antibody (1:200; clone 23, IgG1; no. 610766, BD Biosciences, San Diego, Calif). Syntrophin was revealed with a pan-syntrophin mouse monoclonal antibody that recognized the PDZ domain (ab11425, 1:200; clone 1351, IgG1; Abcam, Cambridge, Mass). Immunostaining was performed essentially as we described previously. 12,13

Western Blot
Microsomal membrane preparation was prepared according to our published protocol. 13 Protein (50 μg) was loaded in each lane on a 6% SDS–polyacrylamide gel. After electrophoresis, protein was transferred to a polyvinylidene difluoride membrane. Immunoblot was performed with the Dys-2 antibody (1:100). 9 Protein loading was confirmed by Rapid blue staining of a duplicate gel (Geno Technology, St Louis, Mo).

Noninvasive Twelve-Lead ECG in Mouse
Mice were first anesthetized with isoflurane (4% induction, 1.5% maintenance) using an Isotón 3 vaporizer (Summit Medical Equipment Co, Bend, Ore). Mice were then placed on a thermally-controlled plate (37°C) and acclimated for 10 minutes before ECG recording. Five 29G needle electrodes (including 4 limb leads and 1 movable chest lead) and an ECG lead selector switch (Model MLA0112S) from AD Instruments (Colorado Springs) were used to sequentially record a full 12-lead ECG. Electrical signals from each lead were filtered and amplified by a single-channel BioAmp (Model ML132) from AD Instruments. Finally ECG tracings were analyzed using a PowerLab software–based ECG analysis module (AD Instruments).

Pressure–Volume Loop Analysis of Heart Hemodynamics
Mice were anesthetized with isoflurane as described above. A closed-chest hemodynamic assay was performed by placing a 1.4 F Millar micropoint pressure–volume (PV) catheter (SPR-839, Millar Instrument, Houston, Tex) into the left ventricle through the right carotid artery. 9 Hemodynamic parameters were collected with the Millar Aria-1

Table. Weight and Weight Ratios in Old Mice

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>BL10</th>
<th>mdx</th>
<th>Carrier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size, N</td>
<td>25</td>
<td>44</td>
<td>19</td>
</tr>
<tr>
<td>Age, day</td>
<td>647.8±21.50</td>
<td>635.36±10.20</td>
<td>631.35±9.42</td>
</tr>
<tr>
<td>BW, g</td>
<td>32.07±1.10</td>
<td>21.60±0.44*</td>
<td>29.78±1.09</td>
</tr>
<tr>
<td>TW, mg</td>
<td>37.05±0.94</td>
<td>34.74±1.35</td>
<td>34.62±0.85</td>
</tr>
<tr>
<td>TL, mm†</td>
<td>18.30±0.08</td>
<td>18.61±0.22</td>
<td>18.34±0.04</td>
</tr>
<tr>
<td>HW, mg</td>
<td>125.85±2.88</td>
<td>114.15±2.53†</td>
<td>123.28±5.12</td>
</tr>
<tr>
<td>HW, mg</td>
<td>117.80±2.58</td>
<td>105.89±2.49‡</td>
<td>114.93±4.65</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>1.17±0.03</td>
<td>1.61±0.05*</td>
<td>1.19±0.05</td>
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<tr>
<td>HW/BV, mg/g</td>
<td>4.00±0.12</td>
<td>5.44±0.14*</td>
<td>4.06±0.16</td>
</tr>
<tr>
<td>HW/TW, mg/g</td>
<td>3.46±0.13</td>
<td>3.60±0.18</td>
<td>3.66±0.21</td>
</tr>
<tr>
<td>HW/TL, mg/mm†</td>
<td>6.81±0.35</td>
<td>5.27±0.16‡</td>
<td>6.18±0.17</td>
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<tr>
<td>WW/BW, mg/g</td>
<td>3.74±0.11</td>
<td>5.04±0.13*</td>
<td>3.97±0.18</td>
</tr>
<tr>
<td>WW/TW, mg/g</td>
<td>3.23±0.12</td>
<td>3.30±0.19</td>
<td>3.41±0.19</td>
</tr>
<tr>
<td>WW/L, mg/mm†</td>
<td>6.41±0.33</td>
<td>4.97±0.16‡</td>
<td>5.83±0.17</td>
</tr>
</tbody>
</table>

BW indicates body weight; HW, heart weight; TW anterior tibialis muscle weight; TL, tibial length; VW, ventricular weight. Values in mdx are significantly different from that in BL10 or carrier. Sample sizes are 14, 9, and 10 for BL10, mdx, and carrier, respectively. Values in mdx are significantly different from that in BL10 but not carrier.
Figure 1. Myocardial fibrosis and calcification in old mdx mice are prevented in age-matched carrier mice. Representative photomicrographs of heart serial sections from old mdx (A), carrier (B), and BL10 (C) mice. Blue color in Masson trichrome staining reveals fibrosis. Red color in Alizarin red staining shows calcified tissues. High-power images are the boxed areas from the corresponding low-power photomicrographs. Irregular wall thickness, enlarged cavity, myocardial fibrosis, and calcification are only seen in mdx mouse hearts.
PV conductance system at a sampling rate of 1000 Hz and analyzed with the PVAN software (Millar Instrument) according to instructions of the manufacturer. Some mice were challenged with dobutamine after baseline measurement. Briefly, mice were injected with 5 μg/g body weight (BW) dobutamine hydrochloride IP (D0676, Sigma). At 5 minutes postinjection, PV loop data were collected. Dosage of dobutamine and timing of data collection were experimentally determined to achieve maximal adrenergic challenge.

**Statistical Analysis**

Data are presented as means±SEM. Statistical analysis was performed with the SPSS software (SPSS, Chicago, Ill). Statistical significance for multiple group comparison was determined by 1-way ANOVA followed by Bonferroni post hoc analysis. Student t test was used for 2-group comparison. Difference was considered significant when \( P<0.05 \).

**Results**

**Twenty-One-Month-Old mdx Mice Show Dilated Cardiomyopathy**

According to the known lifespan data for women (80 years) and female mice (27 months),14 21-month-old mice are equivalent to 62-year-old humans. Comparing with aged-matched normal and carrier mice, mdx mice were significantly emaciated. They lost one-third of their BW (Table). To determine whether old mdx mice undergo dilated cardiomyopathy, we first performed histopathology examination. We found obvious fibrosis and calcification (Figure 1). The ventricular chamber was also enlarged (Figure 1). Despite the morphological evidence of cardiomyopathy in old mdx mice, we did not see an increase in the heart weight (HW) to tibial length (TL) ratio or the ventricular weight (VW) to TL ratio (Table). As a matter of fact, the mdx mice had significantly lower HW/TL and VW/TL ratios than those of the normal mice (Table).

DMD/BMD patients often display characteristic ECG changes such as sinus tachycardia, shortened PR interval, prolonged QT interval, deep Q wave, and polyphasic R’ wave.15–19 The physiological foundation for these alterations remains to be elucidated, but it may at least relate to myocardial fibrosis.18 We observed similar ECG changes in old mdx mice (Figure 2). Furthermore, the cardiomyopathic index, a ratio of QT/PQ, was significantly higher in old mdx mice (Figure 2).20 Dilated cardiomyopathy is the classic clinical manifestation of dystrophin-deficient heart disease. It is characterized by ventricular chamber dilation and dysfunction. However, this has never been documented in mdx mice by catheter-based in vivo hemodynamic analysis. To further establish old mdx mice as a valid model for our study, we performed closed-chest hemodynamic measurement. Consistent with previous reports on dilated cardiomyopathy,21,22 we observed a marked rightward shift of the PV loop in aged mdx mice (Figure 3A). Chamber dilation also resulted in significant increase in both end-systolic and end-diastolic volume (Figure 3B and 3C). Impaired systolic function was reflected by a significant decrease in maximal pressure and the maximal rate of the left ventricular pressure development (dP/dtmax). Diastolic dysfunction was confirmed by 2 independent parameters of heart relaxation, a reduction in the minimal first derivative of left ventricular pressure (dP/dtmin) and an increase in the time constant of left ventricular isovolumetric pressure decay (Tau). Consequently, the overall heart function was compromised, as reflected by reduced stroke volume, ejection fraction, and cardiac output (Figure 3D).

**Mosaic Dystrophin Expression Normalizes Anatomical and Physiological Defects in Old mdx Heart**

Having established a clear diagnosis of dilated cardiomyopathy in 21-month-old mdx mice, we next asked whether heart
disease was prevented in age-matched carrier mice. On histopathology examination, the hearts of carrier mice were essentially indistinguishable from those of BL10 mice (Figure 1). There was no sign of fibrous tissue and/or calcium deposition in the myocardium nor was there abnormal ventricular chamber dilation. All of the anatomic parameters (such as HW, VW, HW/BW, VW/BW, HW/TL, and VW/TL ratios) were also normalized (Table). Besides structural analysis, we also characterized heart function in resting carrier mice. In a 12-lead ECG assay, we did not see any aberrant changes (Figure 2). The tracing was identical to that of the normal mice (Figure 2A). Quantitative evaluations of ECG parameters were also within the normal range (Figure 2B). In the closed-chest hemodynamic assay, we did not see any shift of the PV loops in carrier mice. The pattern overlapped perfectly with that from normal BL10 mice (Figure 3A). A detailed analysis revealed normal function during both systolic and diastolic phases of the cardiac cycle in carrier mice (Figure 3B through 3D). These results suggest that in contrast to old mdx mice, old carrier mice did not develop dilated cardiomyopathy.

To further evaluate cardiac health in old carrier mice, we searched for potential microscopic changes. Among 19 old carrier mouse hearts, we found 4 (21%) with focal myocardial inflammation (Figure 4A). Interestingly, normal resting hemodynamics was preserved in these mice (Figure 4B). Positive inotropic stimulation often unmarks hidden cardiac dysfunction. We therefore performed a dobutamine challenge test. In both BL10 and carrier mice, dobutamine administration significantly increased ejection fraction (Figure 4C). However, there was no difference in the majority of the poststress hemodynamic parameters between BL10 and carrier mice (Figure 4D). The only noticeable change was the maximal systolic pressure. It was significantly higher in BL10 mice (Figure 4D).

Taken together, old carrier mice were free from dilated cardiomyopathy despite a mild inflammation in some mice and minor systolic defect in stress test.

The Lack of Cardiomyopathy in Carrier Mice Is Not Attributable to Selective Preservation and/or Expansion of Dystrophin-Positive Cells

A preferential retention and/or expansion of dystrophin-positive cells could explain the apparently normal cardiac phenotype in aged carrier mice. The growth advantage of dystrophin-positive cells has been shown in mdx skeletal muscle. To delineate the mechanisms underlying heart protection in carrier mice, we quantified the number of dystrophin-positive cells in the heart.

We first counted revertant cardiomyocytes in young and old mdx mice. In these revertant cells, the open-reading frame is restored after the mutated exon is skipped during transcription processing. We observed single and clonal revertant cells in both young and old mdx hearts (Figure 5A). The total number of revertant cardiomyocytes was not significantly different between young and old mice (Figure 5B).

We next studied the carrier mouse heart. Mosaic dystrophin expression was seen in both young and old mice (Figure 5C and Figure I in the online data supplement at http://circres.ahajournals.org). On average, 53.15±% and
54.39±4.88% cardiomyocytes were dystrophin positive in young and old carrier mice, respectively (Figure 5D). Morphometric quantification was further confirmed by Western blot (Figure 5E). In summary, we did not see a selective preservation and/or expansion of dystrophin-positive cardiomyocytes in mdx and carrier mice.

**Upregulated Utrophin Fills in the Gap in the Heart of Carrier Mice**

To further understand myocardial protection in carrier mice, we examined utrophin expression in the heart. Utrophin is a structural and functional homolog of dystrophin. Utrophin upregulation has contributed, at least in part, to the mild skeletal muscle pathology in mdx mice. Consistent with previous reports (reviewed elsewhere), utrophin was absent in the BL10 mouse heart but was moderately upregulated in all cardiomyocytes in mdx mice (supplemental Figure II). In sharp contrast to the all-or-none pattern in the BL10 and mdx hearts, utrophin expression in the hearts of carrier mice showed a striking complementary profile. Utrophin was upregulated in dystrophin-negative areas but not in dystrophin-positive areas (Figure 6A and 6B). Utrophin upregulation also led to the reassembly of the dystrophin–glycoprotein complex (DGC) in dystrophin-negative cardiomyocytes in carrier mice. Although the DGC was restored in all cardiomyocytes in carrier mice, immunostaining intensity was much higher in dystrophin-positive cells than that in utrophin-positive cells (Figure 6C).

**Discussion**

A fundamental question in gene and/or cell therapy of dystrophin-deficient cardiomyopathy is whether one needs to correct every heart cell before organ level amelioration can be achieved. The presence of cardiomyopathy in DMD/BMD carriers seems to suggest that a mosaic expression is not sufficient to prevent heart disease. Because some cardiomyocytes will inevitably be missed in gene and/or cell therapy in patients, there is an urgent need to determine whether mosaic expression can benefit DMD/BMD patients.

The lack of a valid mouse model for dystrophin-deficient cardiomyopathy has hindered research in this field. The naturally occurring mdx mouse is the classic dystrophin-null model. Its
value in dystrophin-deficient cardiomyopathy study has been revisited recently.2,9,26–30 These studies have confirmed stress-induced cardiomyopathy in young \textit{mdx} mice (≤8 months old) and abnormal histopathology/echocardiography/ECG in moderately aged mice (≈12 to 15 months old). Comprehensive evaluation of old \textit{mdx} mice (≥18 months old, equivalent to ≥53 years of age in human) is lacking. Most importantly, none of the existing studies demonstrates the typical hemodynamic changes seen in dilated cardiomyopathy.9,27,29

To evaluate therapeutic implication of mosaic expression, we first established a phenotypic model of dilated cardiomyopathy. In the hearts of 21-month-old \textit{mdx} mice, we observed myocardial fibrosis, calcification, and an irregularly enlarged ventricular chamber (Figure 1 and Table). ECG examination also revealed patient-like changes, including the appearance of R' waves and deep Q waves, a reduction in PR interval, and a prolongation in QT interval (Figure 2).19 The most revealing findings are in the closed-chest hemodynamic assay. We detected the diagnostic PV loop right shift and ventricular dysfunction (Figure 3). This is the first definitive demonstration of dilated cardiomyopathy in \textit{mdx} mice.

The ultimate goal of this study was to determine whether mosaic expression can prevent cardiomyopathy. We have recently shown full hemodynamic protection in young carrier mice in an artificially stressed model.6 However, the absence of a true dilated cardiomyopathy in young \textit{mdx} mice has limited extrapolation of this finding to human patients. To truly evaluate the effect of mosaic expression, we have now studied old symptomatic mice. In contrast to the reported heart disease in human carriers, the aged carrier mice were exempted from dilated cardiomyopathy. Except for mild inflammation and a minor defect in a dobutamine stress test, the hearts of old carrier mice were essentially normal. They had normal weight and weight ratios and normal resting ECG and hemodynamics (Figures 1 through 3). These results strongly suggest that, at least in mice, mosaic dystrophin expression is sufficient to prevent dilated cardiomyopathy.

It has been well established that the skeletal muscle of human carriers and carrier mice is exempt from dystrophy. This is attributable to the syncytial nature of skeletal muscle structure. It allows the spreading of dystrophin from a single expressing nucleus to the entire fiber. Eventually, every fiber becomes dystrophin positive (supplemental Figures III and IV). A similar mechanism does not exist in the heart. The intercalated disks prevent dystrophin spreading between the singly nucleated cardiomyocytes. It has been shown in skeletal muscle that dystrophin-positive revertant cells are more resistant to degeneration than dystrophin-null myofibers.23 If the same principle

Figure 5. Quantification of dystrophin expression in the hearts of \textit{mdx} and carrier mice. A, Representative photomicrographs of single and clonal revertant cardiomyocytes in the \textit{mdx} hearts. Scale bar applies to all images. B, Morphometric quantification of dystrophin-positive revertant fibers in heart sections. There is no statistical difference between young and old \textit{mdx} mice. C, Representative dystrophin immunofluorescence staining in the hearts of carrier mice. Scale bar applies to both images. D, Representative dystrophin Western blots of old mouse hearts (from 7 independent experiments). Each lane represents 50 μg of microsomal preparation from the heart. The loading control is rapid blue staining of a duplicate gel. Dys indicates dystrophin; Pos, positive; young, 3 months old; old, 21 months old.
Figure 6. Immunofluorescence analysis of dystrophin, utrophin, and the dystrophin-associated glycoprotein complex in the hearts of carrier mice. A and B, Representative immunofluorescence staining photomicrographs of serial heart sections from carrier mice. One section was stained with a dystrophin antibody, and its adjacent section was stained with an utrophin antibody. High-power images are the boxed areas from the corresponding low-power photomicrographs. D indicates regions enriched with dystrophin-positive cardiomyocytes; U, regions enriched with utrophin-positive cardiomyocytes. Utrophin is upregulated in dystrophin-negative areas. C, Representative serial immunostaining in the hearts of 21-month-old carrier mice. Dys indicates dystrophin; Utr, utrophin; βDG, β-dystroglycan; βSG, β-sarcoglycan; Syn, syntrophin; Dbr, dystrobrevin.
works in the heart, we hypothesize that dystrophin-positive cardiomyocytes seen in young carrier mice would eventually dominate the heart. This relative increase in dystrophin-positive cells would explain the lack of cardiomyopathy in old carrier mice. To test this hypothesis, we quantified the number of dystrophin-positive fibers in the heart. Neither revertant cardiomyocytes in mdx mice nor dystrophin-positive cardiomyocytes in carrier mice showed an age-associated increase in number (Figure 5). This result excludes selective survival/expansion of dystrophin-positive cells as the underlying mechanism.

Utrophin shares structural similarity with dystrophin and has been actively pursued as a therapeutic molecule for DMD/BMD (reviewed elsewhere31). We next examined whether utrophin upregulation played a role. Similar to previous reports in young mdx mice and DMD/BMD patients,32,33 we found uniform utrophin upregulation in the hearts of old mdx mice (supplemental Figure II). Furthermore, we observed a minimal, albeit detectable, level of the DGC at the sarcolemma in the mdx heart (supplemental Figure IIB). Why does the uniformly expressed utrophin not confer protection in the mdx heart? One apparent explanation could be that the upregulated utrophin did not provide sufficient mechanical support. Several studies have shown that utrophin does not provide sufficient mechanical support. Studies have directly measured muscle force in mdx heart. Sapp et al have shown a contractile defect in the mdx atria.34 Janssen et al have measured ventricular muscle force.35 Using the developed force as an index, they show significantly lower developed force in mdx ventricle when compared with normal ventricle. Knocking out utrophin further depresses ventricular developed force in dystrophin/utrophin double knockout mice.36 Thus, utrophin upregulation offers only a weak mechanical support to the mdx heart, and it is insufficient to stop dilated cardiomyopathy (Figure 7).

Utrophin upregulation in the carrier mouse heart had a different pattern. It occurred only in dystrophin-negative cardiomyocytes (Figure 6). The remaining cardiomyocytes (~50%) expressed normal level dystrophin. Cells expressing dystrophin contained normal concentration of the DGC components, whereas cells expressing utrophin had a reduced yet intact DGC (Figure 6C). Based on these findings, we propose that the mosaic pattern of dystrophin-positive cells in carrier mice may act like steel bars in reinforced concrete and ultimately increase the overall strength of the heart (Figure 7). Taken together, our results have provided correlative evidence that in the background of moderate utrophin upregulation, 50% dystrophin-positive cardiomyocytes are sufficient to prevent dilated cardiomyopathy. Future studies in utrophin/dystrophin double knockout mice and/or cardiac-specific utrophin transgenic mice may further clarify the role utrophin plays in the heart.

Perhaps a more pressing issue is why human carriers are not protected. To answer this question, we first need to point out that severe dilated cardiomyopathy is actually a low incidence problem in DMD/BMD carriers. On average, only ~10% carriers develop severe heart disease.36,37 The majority have no cardiac problems, even at 55 years of age.38 The reason for the selective involvement of certain patients is not understood. It may involve a number of genetic, environmental, nutritional, and other yet undefined triggering factors. Future studies in this area may help us to better understand cardiac disease in DMD/BMD patients and carriers.

Taken together, we have demonstrated a complete prevention of cardiomyopathy through 50% mosaic dystrophin expression in old carrier mice. Because 100% correction is not feasible in gene and/or cell therapy, our results have provided a strong evidence for the potential of partial dystrophin correction for the prevention of dilated cardiomyopathy.
theoretical basis for the clinical usefulness of these novel treatments. Considering the fact that utrophin upregulation is a common finding in the hearts of DMD/BMD patients, achieving a full cardiac recovery may be an attainable goal in gene and/or cell therapy of dystrophin-deficient cardiomyopathy, especially when treatment is performed in utero or in neonatal patients before heart disease develops.

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Disclosures

None.

References

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Supplementary Figure Legends

Supplementary Figure 1. Mosaic dystrophin expression in the hearts of carrier mice. Panels A and B are representative dystrophin immunostaining from the hearts of two 3-m-old carrier mice. Panels C and D are representative dystrophin immunostaining from the hearts of two 21-m-old carrier mice. For each full heart section, high power images of one dystrophin sparse region and one dystrophin-enriched region are presented. These images correspond to the boxed regions in the corresponding low power photomicrographs.

Supplementary Figure 2. Immunofluorescence staining of dystrophin, utrophin and the representative DGC components in BL10 and mdx hearts. A, Representative dystrophin and utrophin immunostaining photomicrographs of 3-m-old (young) and 21-month-old (aged) BL10 and mdx mouse hearts. Dystrophin expression was only seen in BL10 mice and utrophin was up-regulated in both young and aged mdx hearts. B, Representative immunostaining photomicrographs of β-dystroglycan and syntrophin in old mdx and BL10 hearts.

Supplementary Figure 3. Uniform dystrophin expression in carrier mouse skeletal muscle prevents diaphragm muscular dystrophy. Representative photomicrographs of the diaphragm from 2-year-old mdx, carrier and BL10 mice,. Dys, immunofluorescence staining for dystrophin; MT, Masson trichrome staining for fibrotic tissue; HE staining shows general histology.

Supplementary Figure 4. A model for skeletal muscle protection in carrier mice. In BL10 skeletal muscle, dystrophin is expressed in every muscle fiber at 100% level and nuclei are located at the periphery. In mdx skeletal muscle, utrophin is moderately up-regulated but it cannot stop muscle degeneration and necrosis. In regenerated myofibers, nuclei are located at the center. In carrier mice, only 50% nuclei carry a functional dystrophin gene. However, the dystrophin protein spreads
throughout the entire myofiber. As a result, skeletal muscle is protected from muscular dystrophy in carrier mice.
B

Young (3-m-old)

1 mm

100 µm

Bostick et al 2007
Supplementary Figure 1B
Aged (21-m-old)
A

Young mice

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Loss of Dystrophin & Utrophin Up-regulation

Skeletal Muscle Disease

BL10

Mdx

Carrier