Microdystrophin Gene Therapy of Cardiomyopathy Restores Dystrophin-Glycoprotein Complex and Improves Sarcolemma Integrity in the *Mdx* Mouse Heart

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Background—More than 90% of Duchenne muscular dystrophy (DMD) patients develop cardiomyopathy, and many die of cardiac failure. Despite tremendous progress in skeletal muscle gene therapy, few attempts have been made to treat cardiomyopathy. Microdystrophin genes are shown to correct skeletal muscle pathological lesions in the *mdx* mouse model for DMD. Here, we tested the therapeutic potential of adeno-associated virus (AAV)–mediated microdystrophin gene therapy in the *mdx* mouse heart.

Methods and Results—AAV was delivered to the newborn *mdx* mouse cardiac cavity. The procedure was rapid and well tolerated. Efficient expression was achieved in the inner and the outer layers of the myocardium. The ubiquitous cytomegalovirus promoter resulted in substantially higher expression than the muscle-specific CK6 promoter. The therapeutic effects of microdystrophin were evaluated at 10 months after infection. Immunostaining demonstrated extensive microdystrophin expression and successful restoration of the dystrophin-glycoprotein complex. Importantly, AAV-mediated microdystrophin expression improved the sarcolemma integrity in the *mdx* heart.

Conclusions—We established a simple gene transfer method for efficient and persistent transduction of the *mdx* mouse heart. AAV-mediated microdystrophin expression restored the critical dystrophin-glycoprotein complex and improved sarcolemma integrity of the *mdx* heart. Our results revealed the promise of AAV-microdystrophin gene therapy for cardiomyopathy in DMD. (***Circulation***. 2003;108:1626-1632.)

Key Words: muscular dystrophy ■ genes ■ viruses ■ gene therapy ■ microdystrophin

Duchenne muscular dystrophy (DMD) is the most common lethal inherited muscle disease. Ninety-five percent of patients develop cardiac damage, and 10% to 15% of patients die of cardiac failure.[1] Furthermore, ≥90% of patients with the milder Becker muscular dystrophy (BMD) and female DMD carriers show cardiac involvement.[2] For the majority of patients, heart transplantation is the only available treatment for their end-stage cardiomyopathy.[3]

DMD and BMD are caused by mutations in the dystrophin gene. Replacement of the defective gene with a functional homologue holds great therapeutic promise. In the past several years, DMD gene therapy has focused on correcting skeletal muscle pathological lesions in murine (*mdx*) and canine (*cxmd*) models for DMD,[4] but very little effort has been made to treat the cardiomyopathy. The only reported study explored fetal cardiomyocyte engraftment in the *mdx* and the *cxmd* hearts, but expression was limited to the transplanted cells.[5]

The paucity of cardiac gene therapy attempts results primarily from 2 causes. First, unlike limb muscles that are easily accessible, the anatomic location and the physiological function of the heart present a formidable challenge to gene transfer. Second, the full-length dystrophin cDNA exceeds the packaging capacity of most currently available viral vectors. To circumvent the size limitation, microdystrophin genes that contained only ≈30% of the dystrophin cDNA coding sequence were developed (reviewed by Dickson et al[6]). Some microgenes were capable of providing functional improvement in the *mdx* mouse skeletal muscles, including the diaphragm.[7,7a]

Different heart gene transfer techniques have also been explored. Therapeutic genes were delivered to the heart by direct injection or coronary artery perfusion. However, most of the published studies have used larger animals, such as rat, hamster, and rabbit.[8,9] The small size of the mouse has made heart gene transfer a more difficult task.[10–12] Svensson et al[10] reported a direct myocardial injection method to deliver adeno-associated virus (AAV) to the adult mouse heart. However, expression was observed only in the injected area. Three methods have been explored to increase transduction

efficiency: multiple injections, coronary artery perfusion of the explanted heart, and intracavity gene delivery.[10–12] Christensen et al[12] achieved mouse heart gene transfer by delivering adenovirus directly to the cardiac cavity of embryonic and neonatal mice. This method resulted in up to 6 months of transgene expression without obvious disturbance of cardiomyocyte function.

In the present study, we further optimized the neonatal cardiac gene transfer technique described by Christensen et al[12] for the *mdx* heart gene therapy. More importantly, we examined the therapeutic potential of our recently described ΔR4-23/ΔCT microdystrophin (ΔR4) gene[7] in the *mdx* heart. Both transgenic and AAV-mediated gene transfer studies have suggested that the ΔR4 gene is the most functional microdystrophin construct for correcting skeletal muscle pathology in the *mdx* mouse.[7] Our results indicated that AAV-mediated neonatal cardiac gene transfer was an efficient method to stably deliver the ΔR4 gene to the *mdx* heart. Furthermore, ΔR4 gene expression was sufficient to restore the dystrophin-glycoprotein complex (DGC) and strengthen the sarcolemma integrity in the *mdx* heart.

Methods

Recombinant AAV Production

The viral stocks were generated as described previously.[13] The pCisCMV.LacZ and pCisCK6.LacZ were described previously.[14] The pCisRSV.Alkaphos was constructed by subcloning a 2.2-kb *Xba*I fragment containing heat-resistant alkaline phosphatase (alkaphos) gene into the pDD1.[14] The ΔR4 gene contains the N-terminal actin-binding domain, the first 3 and the last spectrin-like repeats, the hinges 1, 2, and 4, and the cysteine-rich domain. The pCisCMV.ΔR4 was generated by swapping in a 508-bp *Kpn*I/*Xba*I cytomegalovirus (CMV) promoter fragment to a previously described pCisCK6.ΔR4.[7]

Neonatal Cardiac Gene Transfer

All animal experiments were carried out in accordance with institutional guidelines. Heart gene transfer was performed according to a published protocol with modifications.[12] Briefly, neonatal *mdx* pups (<12 hours old) were placed in a custom-designed ice chamber to achieve hypothermic shock. Recombinant AAV (10 μL) (10^{10} virus particles in PBS) was injected directly into the cardiac cavity by puncturing through the chest wall. Injection micropipette was prepared with capillary tubing (OD, 1 mm; ID, 0.58 mm) on a pipette puller (David Kopf Instruments). A 1-mL rubber bulb was mounted to the micropipette to control virus delivery. Cardiac cavity injection was confirmed by visualization of blood flashback in the micropipette (Figure 1A). After injection, mice were placed on a 34°C warming pad to recover.

Reporter Gene Evaluation

LacZ expression was determined as described previously.[14] Alkaphos expression was detected on 8-μm tissue sections after heat-inactivation of the endogenous heat-labile alkaphos protein. Staining was performed at 37°C for 10 minutes in 75 mg/mL nitroblue tetrazolium chloride, 50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, 100 mmol/L Tris (pH 9.5), 50 mmol/L MgCl₂, and 100 mmol/L NaCl.

Immunofluorescence Staining

The ΔR4 microdystrophin was revealed by 2 anti–dystrophin N-terminal antibodies. The polyclonal anti–N-terminal antibody was used essentially as described previously.[7] The human specific monoclonal anti–N-terminal antibody (Dys-3, 1:10, Novocastra) was applied according to a published protocol.[15] Additional monoclonal antibodies, including anti–dystrophin-C-terminal Dys-2 (1:30), anti–
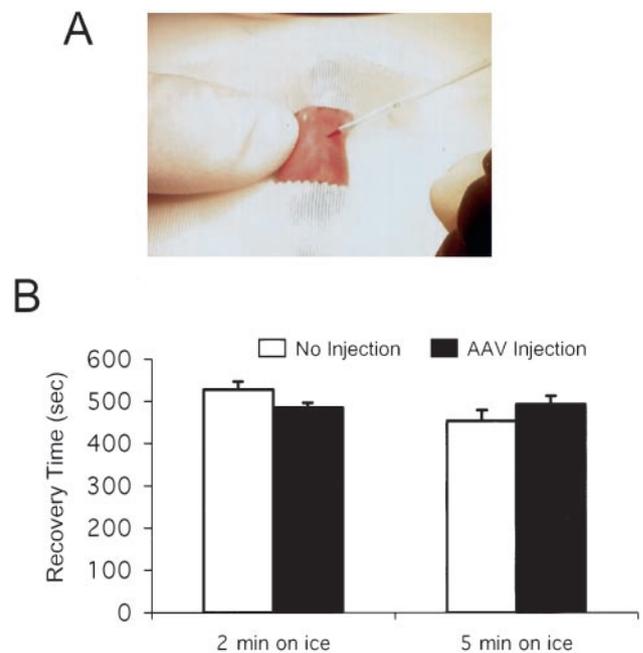

Figure 1. Neonatal *mdx* mouse heart gene transfer. A, AAV was delivered to neonatal *mdx* mouse heart by a micropipette. Flashback of blood in micropipette indicates correct location inside cardiac cavity. B, Effect of cold-shock time on recovery (mean±SEM).

β-sarcoglycan (1:50), and anti–β-dystroglycan (1:50) antibodies (Novocastra) were used the same way as for Dys-3.

In Vivo Cardiomyocyte Sarcolemma Integrity Assay

Evans blue dye (EBD) uptake in the heart was evaluated after β-isoproterenol challenge as described previously.[16] Briefly, β-isoproterenol and EBD were administered to 10-month-old AAV-infected *mdx* mice. The sarcolemma integrity was examined 4 hours later under the Texas Red channel by a Nikon E800 fluorescence microscope.

Results

Development of Neonatal Cardiac Gene Transfer in the *Mdx* Mouse

Christensen et al[12] recently reported a heart gene transfer method in hypothermia-shocked neonatal mouse. To apply this technique to the *mdx* mouse, we first optimized several critical surgery parameters. The anesthesia was achieved in the newborn *mdx* pups by placing them in a custom-designed ice chamber. We noticed a reflexive mouth opening in 80% of pups (n=143) when they reached deep anesthesia. On the basis of this observation, we found that on average, it took 46±1 seconds to anesthetize *mdx* pups.

A complicated micromanipulator gene delivery system was used by Christensen et al.[12] To simplify the procedure, we attached a 1-mL rubber bulb to the micropipette to control virus delivery. To facilitate penetration of the chest, a very small incision was made on the surface skin above the heart. Virus was injected into the heart when the micropipette reached the cardiac cavity (Figure 1A). On average, it took 93±2.7 seconds to complete the entire surgical procedure.

A longer cold-shock may help to extend the virus-heart contact time and increase the transduction efficiency. How-
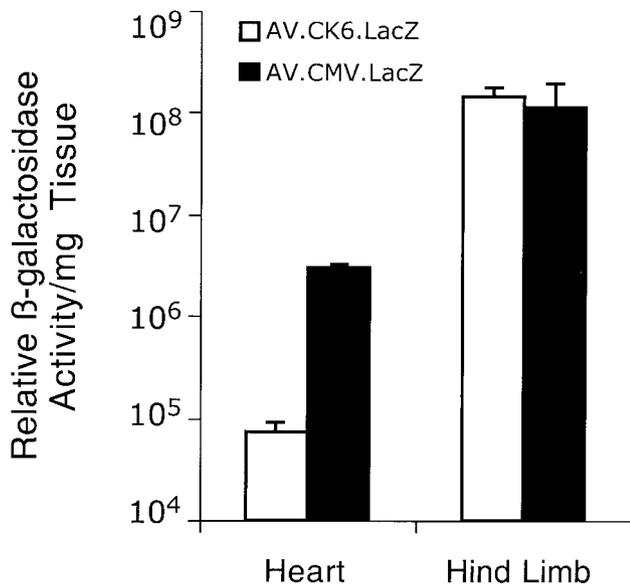
Figure 2. Effect of promoters on AAV-mediated neonatal *mdx* heart gene transfer. Transduction efficiency was compared between viruses carrying either CMV promoter or CK6 promoter. Similar levels of transduction were achieved in *mdx* skeletal muscle. However, CMV promoter resulted in much higher β-galactosidase expression in *mdx* mouse heart (n=10 for each group, mean±SEM).

ever, a longer cold-shock may also decrease the survival rate. We compared the recovery after a 2-minute or a 5-minute hypothermic shock. There was no substantial difference in the survival rate between the 2-minute (96%, n=84) and the 5-minute (95%, n=102) groups. The overall recovery time was also very similar in the 2-minute (488±11 seconds) and the 5-minute (486±18 seconds) groups. Therefore, the 5-minute cold-shock was chosen for AAV injection.

We also examined whether virus injection had a negative effect on mouse survival. In the absence of injection, the overall survival rate was 93% (n=23). In AAV-injected mice, the overall survival rate was 96% (n=163). No significant difference in recovery time was observed among different groups (Figure 1B). This result suggested that our modified heart gene transfer procedure was fairly safe.

Effect of the Promoter and Kinetics of Gene Expression

It has been suggested that virus-mediated gene therapy in dystrophic muscle may require tissue-specific promoters.[17]

The ubiquitous CMV promoter and the muscle-specific CK6 promoter have been used in AAV-mediated microdystrophin gene therapy.[7] To determine which promoter worked better for the heart, we compared the expression between AV.CMV.LacZ and AV.CK6.LacZ (n=10 for each group). AAV-2 viruses (10^{10} particles) were delivered either to the heart or to the left hind limb of the newborn *mdx* pups, and β-galactosidase activity was quantified 1 month later. Consistent with our previous study in the adult *mdx* skeletal muscle,[14] the CMV and the CK6 promoter led to similarly high levels of gene expression in the neonatal *mdx* limb muscle (Figure 2). However, the CMV promoter led to a substantially higher expression in the heart [$(2.96\pm0.26)\times10^6$ relative units/mg] than the CK6 promoter [$(7.23\pm0.98)\times10^4$ relative units/mg]. The CK6 promoter is derived from mouse muscle creatine kinase gene regulatory elements.[18] Transgenic studies have shown that the muscle creatine kinase promoter drives ≈50-fold higher expression in skeletal muscle than in cardiac muscle.[19] Our results agreed with the previous publication and highlighted the importance of selecting appropriate gene expression cassettes for DMD heart gene therapy.

Long-term gene transfer is required for DMD therapy. To evaluate whether neonatal gene transfer can mediate persistent transgene expression in the heart, we compared AAV-mediated expression at 1 and 6 months after infection. To expand our findings to other transgenes, AV.RSV.Alkaphos was used in this study. The RSV promoter was as effective as the CMV promoter in the *mdx* muscle.[14] Because AAV-5 mediates better transduction in the *mdx* muscle than AAV-2,[13] we decided to use AAV-5 in this and other experiments described later. As shown in Figure 3, quantitative analysis revealed nearly a 3-fold increase in alkaphos-positive heart cells at 6 months after infection. It is possible that multiple copies of virus genomes may have been delivered to some progenitor cells. Subsequent cell division may split the episomal AAV genomes into more cells. Interestingly, in addition to the efficient transduction of the inner myocardium layer, we also observed a fairly efficient transduction of the outer layer (Figure 3). This mirrored the findings of Christensen et al.[12] Transduced cells were also observed through the middle myocardium layer (Figure 3).
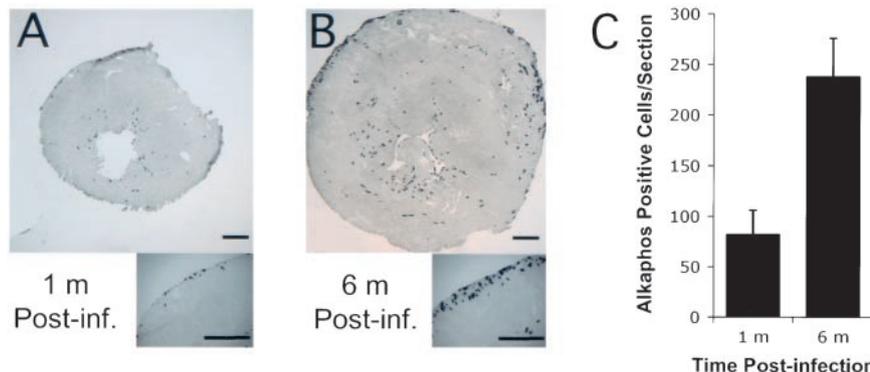

Figure 3. Long-term AAV transduction in *mdx* heart. Alkaphos-positive cells were quantified at 1 month (A) and 6 months (B) after AV.RSV.Alkaphos infection. Preferential transduction of outer myocardium layer is shown in enlarged photomicrographs at bottom right of A and B. Bar=500 μm. Data in C represent mean±SEM (n=6 for each group).
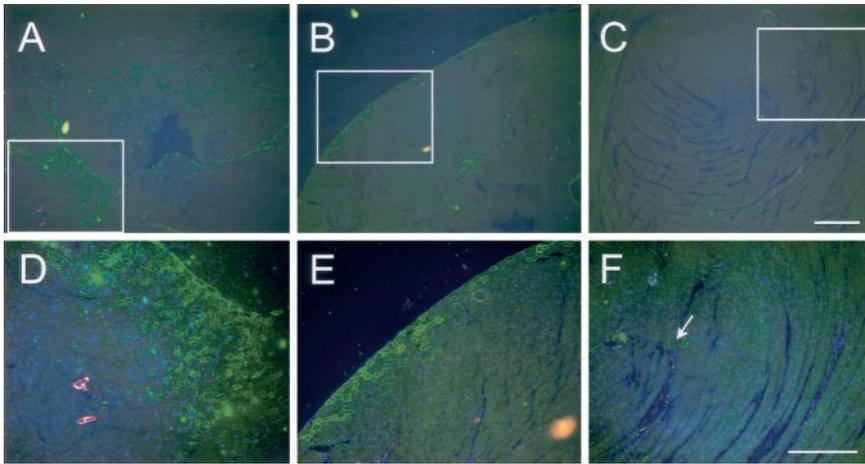
Figure 4. Persistent high-level microdystrophin expression in the *mdx* heart. At 10 months after AV.CMV.ΔR4 infection, ΔR4 microdystrophin expression was detected by immunostaining with polyclonal anti–dystrophin N-terminal antibody (DAPI counterstain). A, B, D, and E are representative photomicrographs from 10 independently infected *mdx* hearts. C and F are from mock-infected *mdx* heart. D, E, and F are enlarged photomicrographs of boxed regions in A, B, and C, respectively. Bar=500 μm. A few revertant cells are seen in C and F (arrow).

Microdystrophin Expression in the *Mdx* Heart Restored DGC

Microdystrophin genes are highly truncated versions of the dystrophin gene. We sought to determine whether microdystrophins could be used to treat the *mdx* cardiomyopathy. As shown in Figure 4, efficient microdystrophin expression was detected at both the inner (Figure 4, A and D) and the outer (Figure 4, B and E) layers of the myocardium at 10 months after infection. In uninfected control *mdx* heart, we observed only rare revertant cardiomyocytes (Figure 4, C and F). The cardiomyocyte-specific microdystrophin expression was further demonstrated by double immunostaining with antibodies against dystrophin N-terminal and α-actinin (Figure 5). Additional Western blot also confirmed microdystrophin expression in AAV-infected *mdx* heart (data not shown).

The biological function of dystrophin is performed in conjunction with the DGC (reviewed by Blake et al[20] and Rando[21]). In the absence of dystrophin, the DGC is destabilized, and this loss of DGC contributes greatly to the muscle pathology. Consequently, DGC restoration is a critical therapeutic end point (reviewed by Chamberlain[4] and Straub and Campbell[22]). The DGC in cardiac muscle is similar to that in skeletal muscle.[23] An immunoelectron microscopic study in cardiac muscle revealed a close interaction between the dystrophin cysteine-rich and C-terminal domains and β-dystroglycan.[24] These 2 domains are important for recruiting the other DGC components (reviewed in Reference 20). Because the C-terminal domain is completely deleted in our construct, we investigated whether AAV-mediated microdystrophin expression could restore the DGC in the *mdx* heart. As shown in Figure 6A, ΔR4 microdystrophin was detected only in AAV-infected *mdx* heart by a human-specific anti–dystrophin N-terminal antibody. Importantly, other components of DGC (such as β-sarcoglycan and β-dystroglycan) were also detected in AAV-infected but not mock-infected *mdx* heart. Additional immunostainings were performed on serial sections of AAV-infected *mdx* heart. Consistent with the data shown in Figure 6A, ΔR4 microdystrophin expression successfully restored β-sarcoglycan and β-dystroglycan in virus-transduced cardiomyocytes (Figure 6B). These data demonstrated that AAV-mediated microdystrophin expression was sufficient to restore the DGC in the *mdx* heart.

Microdystrophin Expression in the *Mdx* Heart Improved Sarcolemma Integrity

EBD uptake is a hallmark for dystrophin-deficient myofibers and reflects the susceptibility of these fibers to mechanical stress. β-Isoproterenol was administered to the virus-infected *mdx* mouse to create a mechanical challenge to the heart.[16] As shown in Figure 7, microdystrophin expression indeed improved the sarcolemma integrity and prevented EBD uptake. Quantitative examination of 4 independent AAV-infected *mdx* heart (≈300 cells/heart were evaluated) demonstrated
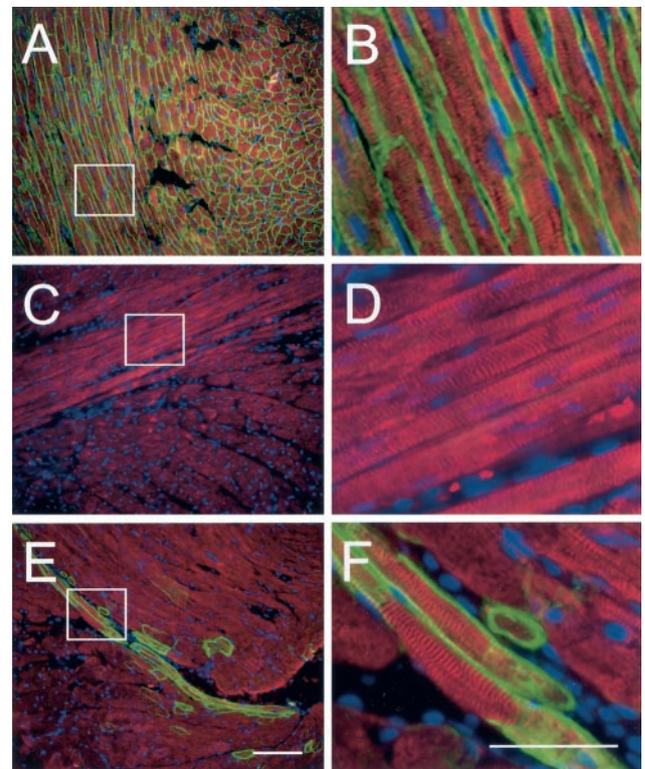

Figure 5. AAV-mediated microdystrophin expression was localized to *mdx* cardiomyocytes. A and B, BL10 heart; C and D, *mdx* heart; E and F, AAV-infected *mdx* heart. B, D, and F are enlarged photomicrographs of boxed regions in A, C, and E, respectively. Dystrophin was detected with a polyclonal anti–dystrophin N-terminal antibody. Cardiomyocytes were revealed by a monoclonal antibody against sarcomeric marker α-actinin. E, Bar=100 μm. F, Bar=50 μm.
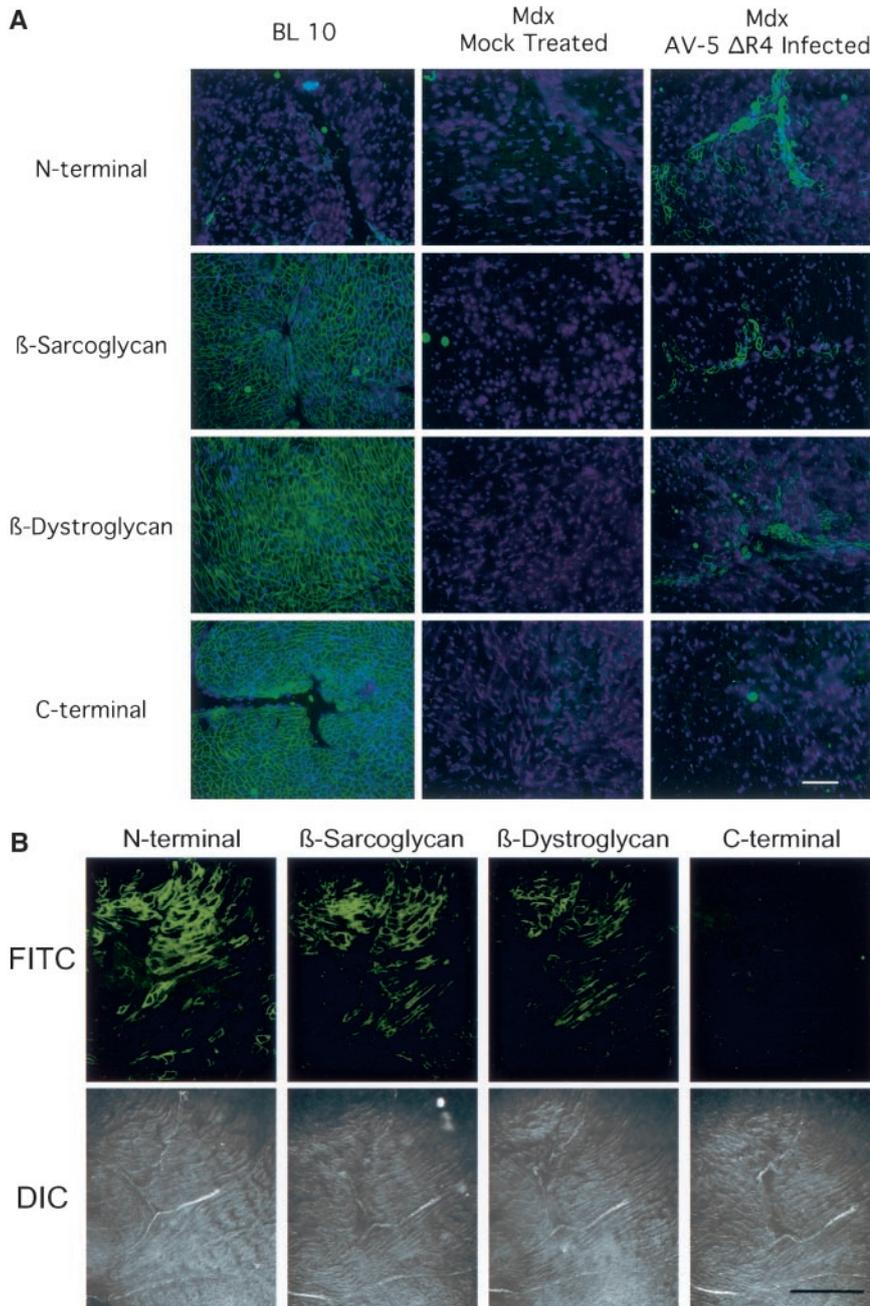
A

B

Figure 6. AAV-mediated microdystrophin expression restored DGC. A, Mock-treated and AAV-infected *mdx* hearts and age-matched BL10 heart were evaluated with monoclonal antibodies specific to N-terminal (human-specific) and C-terminal of dystrophin, β-sarcoglycan, and β-dystroglycan. Nuclei were counterstained with DAPI. Bar=100 μm. B, Serial sections of virus-specific microdystrophin expression and recruitment of β-sarcoglycan and β-dystroglycan. DIC indicates differential interference contrast. Bar=200 μm.

that all the EBD-positive cells were negative for microdystrophin and none of the AAV-infected cells were leaky to EBD.

Discussion

Cardiomyopathy is one of the two most important causes leading to the death of DMD patients.[1] It is also a major health threat to an even larger population of BMD patients and female DMD carriers. Unlike the cardiomyopathy in some other forms of muscular dystrophy (eg, sarcoglycan-sarcospan–deficient muscular dystrophy) that responds to pharmacological intervention,[25] gene therapy is perhaps the most promising treatment for cardiomyopathy in DMD and BMD. In this study, we applied a novel heart gene transfer technique to evaluate the therapeutic potential of AAV-

microdystrophin vector in the *mdx* heart. Our results, for the first time, demonstrated that the C-terminal deleted microdystrophin was sufficient to restore the DGC and improve sarcolemma integrity in the *mdx* heart.

The posterior left ventricle wall is the most commonly affected area in DMD.[26] It is currently unclear whether gene therapy of DMD cardiomyopathy requires efficient gene transfer to every cardiomyocyte in the heart or whether transduction of a subset of cells at the posterior left ventricular wall is sufficient. Consistent with the findings by Christensen et al,[12] we also observed a preferential transduction at the inner and the outer layers of the myocardium (Figures 3 and 4). The inner myocardium transduction may have derived from a passive diffusion of AAV across endocardium. It is currently unclear how intracavity delivery leads
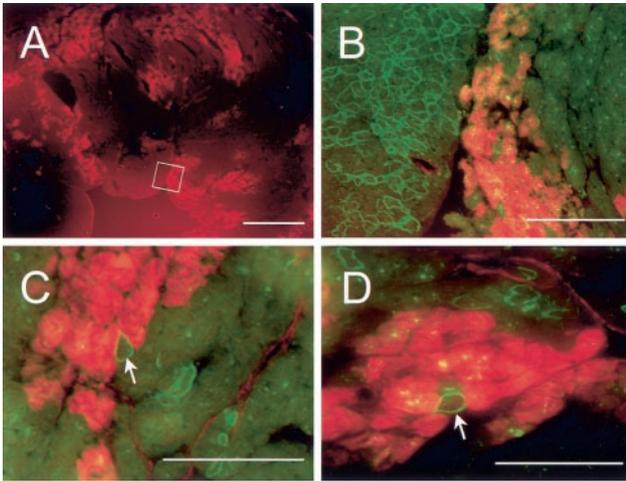
Figure 7. AAV-mediated microdystrophin expression improved sarcolemma integrity. Sarcolemma integrity was evaluated by EBD uptake after β-isoproterenol challenge. Microdystrophin was revealed by a human dystrophin N-terminal–specific antibody (Dys-3). A, EBD uptake in *mdx* heart; B, enlarged photomicrograph of boxed region in A. C and D, exclusion of EBD in individual AAV-transduced cardiomyocytes (arrows). A, B, C, and D, bars=500, 200, 100, and 100 μm, respectively.

to expression at the outer layer. Occasionally, transduced cells were observed in the middle myocardium layer. Because these cells cannot be reached by simple diffusion, we suspect that the transduction may have occurred through coronary artery distribution. It is likely that further optimization of gene transfer techniques will be necessary to target a greater proportion of the heart cells to achieve therapeutic effects in patients.

In this study, we also evaluated the effect of the promoter and the persistence of gene transfer. The muscle creatine kinase promoter and its derivatives (such as CK6) have been used extensively to drive muscle-specific expression (reviewed in Reference 4). Our data suggest that the CK6 promoter is less optimal in cardiac muscle than it is in skeletal muscle (Figure 2). Novel promoters need to be developed for high-level expression in both skeletal and cardiac muscle. Kinetic study at 1 and 6 months after injection revealed a persistent transduction. Interestingly, we also observed a 3-fold increase in the absolute number of transgene-positive cells over this period (Figure 3C). We suspect that the increase in the number of AAV-transduced cells may have resulted from the limited proliferation of AAV-infected progenitor cells.

Whether the lack of dystrophin in the heart is directly responsible for the DMD cardiomyopathy has been debated. Although cardiomyopathy has been described in old *mdx* mice (>20 months old),[27] studies in young *mdx* mice (<30 weeks old) revealed infrequent pathological changes in the heart.[28] These observations have been partially attributed to the compensatory upregulation of the dystrophin homologue utrophin in the *mdx* mouse (reviewed in Reference 20). However, no apparent histological abnormalities were observed in hearts of young mice (<7 weeks old) lacking both utrophin and dystrophin.[29] Even in relatively old dystrophin/utrophin double-knockout mice (7 to 11 weeks old), only

50% to 60% of the mice showed cardiac pathology.[29] Megeney et al[30] recently reported an extensive skeletal muscle dystrophy and severe cardiomyopathy in the MyoD-knockout *mdx* mouse. Because MyoD is expressed only in skeletal muscle, Megeney et al proposed that the secondary response to the severe skeletal muscle damage, rather than the loss of dystrophin itself, might be crucial for the cardiomyopathy in DMD. This hypothesis is challenged by several recent studies showing a direct link between the loss and/or remodeling of dystrophin and the nondystrophic cardiomyopathy.[31,32] To understand the pathogenesis of DMD cardiomyopathy, it will be important to determine whether dystrophin expression in skeletal or heart muscle alone can rescue cardiomyopathy. A suggestive answer to this question is provided by a recent transgenic study in the γ-sarcoglycan–deficient mouse.[33] The lack of γ-sarcoglycan leads to the limb girdle muscular dystrophy 2C in humans. Zhu et al[33] found that the genetic rescue of skeletal muscle pathological conditions by skeletal muscle–specific expression of γ-sarcoglycan had no beneficial effect on the cardiomyopathy. Taken together, these recent results indicate that the loss of dystrophin is probably the most important pathogenic factor for the cardiomyopathy in DMD. Restoration of the missing dystrophin with a functional homologue such as a microdystrophin not only in skeletal muscle but also in the heart will be needed to eventually cure DMD. Our study provides an important first step toward this goal.

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