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Challenges and opportunities in dystrophin-deficient cardiomyopathy gene therapy

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Abstract

The last decade has evidenced unprecedented progress in gene therapy of Duchenne and Becker muscular dystrophy (DMD and BMD) skeletal muscle disease. Cardiomyopathy is a leading cause of morbidity and mortality in both patients and carriers of DMD, BMD and X-linked dilated cardiomyopathy. However, there is little advance in heart gene therapy. The gene, the vector, vector delivery, the target tissue and animal models are five fundamental components in developing an effective gene therapy. Intensive effort has been made in optimizing gene transfer vectors and methods. Systemic and/or local delivery of recombinant adeno-associated viral vector have resulted in widespread transduction in the rodent heart. The current challenge is to define other parameters that are essential for a successful gene therapy such as the best candidate gene(s), the optimal expression level and the target tissue. This review focuses on these long-ignored aspects and points out future research directions. In particular, we need to address whether all or only some of the recently developed mini- and microgenes are protective in the heart, whether partial correction can lead to whole heart function improvement, whether over-expression is hazardous and whether correcting skeletal muscle disease can slow down or stop the progression of cardiomyopathy. Discussion is also made on whether the current mouse models can meet these research needs.

INTRODUCTION

Dystrophin-deficient cardiomyopathy refers to the cardiac manifestation of three closely related diseases including Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and X-linked dilated cardiomyopathy (XLDC). The common genetic defect in these diseases is dystrophin gene mutation. The 427 kD muscle dystrophin protein has four functional domains including the N-terminal, central rod, cysteine-rich (CR) and C-terminal domains (Fig. 1). Together with dystroglycan and sarcoglycan, dystrophin and its partners form the dystrophin-associated glycoprotein complex (DGC) (1). The DGC discharges mechanical stress to the extra-cellular matrix and therefore stabilizes the sarcolemma during muscle contraction [reviewed in (2)]. The DGC also acts as a critical hub in several signal transduction pathways [reviewed in (3)].

DMD results from a complete loss of dystrophin. BMD is due to reduced protein expression and/or expression of a truncated but partially functional protein (4,5). XLDC is caused by selective loss of dystrophin in the heart (6,7). One-third of DMD patients show signs of cardiac dysfunction by mid-teenage and virtually all DMD patients develop cardiac damage by the end of their life. DMD patients (10–40%) eventually die from heart failure (8,9). It is estimated that cardiomyopathy can shorten the life expectancy of DMD patients by at least 2 years (10).

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Cardiac involvement is more prevalent in BMD patients. By age 40, more than 90% of BMD patients display some signs of heart disease (11-14). The high incidence in BMD is considered a natural consequence of a longer lifespan in these patients. It may have allowed more time for clinical signs to develop (12,13). In addition to patients, female carriers are also at high risk. Nearly half of them have electrocardiography (ECG) changes (15) and 7–18% suffer from heart function defect (11,14,16). Besides heart transplantation (17-20), symptom-relieving medicines (such as angiotensin-converting enzyme-inhibitors and β-blockers) are the only available treatment now (9). Currently, there is no cure.

HEART AND SKELETAL MUSCLE HAVE DIFFERENT NEEDS FOR DYSTROPHIN

To develop an effective gene therapy for dystrophin-null cardiomyopathy, one should always bear in mind that the heart is different from skeletal muscle. First, the anatomic structure of the contractile unit is different between cardiac and skeletal muscle. In contrast to the multinucleated syncytial myotube in skeletal muscle, cardiomyocytes are mainly single nucleated and are separated from each other by intercalated discs. Second, there is subtle difference in force development. In the absence of dystrophin, cardiomyocytes develop a higher than normal stretch-tension (21), but skeletal muscle develops an equal or lower than normal stretch-tension (22,23). Third, dystrophin shows distinctive expression patterns in two tissues. In skeletal muscle, dystrophin is concentrated at the costamere (24-27). Costameres are vinculin-rich subsarcolemmal rib-like structures. They anchor the Z-line to the sarcolemma and thus provide a mechanical linkage between contracting myofibrils and the extra-cellular matrix [reviewed in (27)]. Costameres are found in both skeletal and cardiac muscle (28,29). Interestingly, in the rodent heart, dystrophin is uniformly distributed along the sarcolemma and there is no preference for the costamere (30). [Although in the human heart dystrophin partially co-localizes with the costamere (31).] Another striking finding is the presence of dystrophin in the T-tubule membrane in the heart, but not in skeletal muscle (30-33). T-tubules are involved in excitation–contraction coupling but not in force transmission. The association of dystrophin in cardiac T-tubules suggests that dystrophin may play additional roles in the heart. Taken together, these subtle, but fundamental differences in dystrophin distribution emphasize the necessity of developing heart-specific gene therapy.

CHALLENGES IN HEART GENE THERAPY

The current success in skeletal muscle gene therapy is built on accumulative efforts from years' of transgenic studies (34-44). These experiments demonstrate that a level of 20% normal dystrophin is sufficient to maintain muscle function (36,37) and a 50-fold over-expression is not toxic (45). Unfortunately, similar studies have not been performed in the heart. A top priority in cardiac gene therapy is to address these fundamental questions.

Which gene(s) to use?

Theoretically, one would consider the endogenous full-length dystrophin cDNA as the ideal therapeutic gene. However, there are two potential problems. First, the full-length dystrophin cDNA is beyond the packaging capacity of most available viral vectors. Second, the wild-type dystrophin gene may not be the best gene in preventing/treating cardiomyopathy. It has been shown that the hinge 3 region is a cleavage site for coxsackievirus protease (46,47). Although an epidemic linkage remains to be established between coxsackievirus infection and heart failure in DMD/BMD/XLDC patients, Xiong *et al.* (48) have clearly shown that dystrophin deficiency is a predisposing factor to viral myocarditis and heart failure in mice. There is also anecdotal case report describing heart failure in BMD patient after a flu-like illness (49). It is possible that a modified gene may be therapeutically advantageous to the wild-type dystrophin

gene. In support of this notion, a recent study suggests that mutations in the hinge 3 region may protect DMD/BMD patients from dilated cardiomyopathy (50).

A 6 kb minigene and a 3.8 kb microgene are the two most promising candidate genes for skeletal muscle gene therapy. The minigene is identical to the full-length dystrophin cDNA except for a perfectly phased deletion in the rod domain (from hinge 2 to spectrin-like repeat 19). As this gene provides full protection in skeletal muscle, it may also yield a good protection in the heart. The only limitations are: (i) it requires two AAV virions for delivery (41,51); (ii) it carries the hinge 3 region and may be susceptible to coxsackieviral protease cut. The microgene does not carry the C-terminal domain and it also has a much larger deletion in the rod domain (from repeat 4 to 23). The microgene can be packaged in a single AAV virion but only provides basic protection in skeletal muscle (41,52,53). In a preliminary study, we found that AAV-mediated microgene expression can restore the DGC in the heart and protect the heart from stress-induced sarcolemmal injury (54) (Fig. 2). We also found a non-discriminative expression of microdystrophin along the entire sarcolemma in cardiomyocytes but a punctate expression in skeletal muscle myofibers (Fig. 2). This distribution profile is similar to the full-length protein (27,30,31). Despite the encouraging results, the inherent limitation in the microgene itself may prevent it from offering a full protection. In particular, comparing with other dystrophin isoforms, the microgene carries the least amount of genetic information. Furthermore, the consequence of the C-terminal domain deletion remains to be explored in the heart. Taking together, comprehensive studies are needed to determine whether the abbreviated mini- and microgenes can reduce heart pathology and maintain heart function.

How much dystrophin is enough?

Despite rapid progress in gene delivery technique, it is unrealistic to expect a 100% transduction of every single cell in the heart. New methods have been developed to deliver transgene to more than 50% heart cells in several rodent models with AAV vectors (55-58). The question is whether this is sufficient to protect the heart. To address this issue, we have produced genetically defined heterozygous female mice by crossing mdx and BL10 (59). Because of random X-chromosome inactivation, only half cardiomyocytes express dystrophin in heterozygous mice. In the hearts of heterozygous mice, some areas have more dystrophin positive cells, whereas other areas only have a few positive cells (Fig. 3). Heart function was examined in 3--month-old mice after β-isoproterenol challenge. Despite a partial dystrophin expression, stress-induced cardiomyopathy was significantly attenuated (Fig. 3). The most impressive correction was seen in the hemodynamic assay. In all parameters we examined, we observed a 100% protection (59). Although this is an encouraging finding, the lack of clinical heart disease in young mdx mice has limited the significance of the study. It remains to be determined whether dystrophin expression in half cardiomyocytes can protect the heart in symptomatic animals such as aged mdx mice.

A more relevant issue to clinical gene therapy is to determine the therapeutic threshold for the mini- and microgenes. These abbreviated synthetic genes may be less competent than the full-length gene. Furthermore, achieving ≥50% transduction efficiency in the human hearts is clearly a daunting task. Cardiac transgenic mice carrying mosaic expression of the mini- and microgenes and/or viral delivery will be needed to address this important question.

Will too much dystrophin be toxic?

An important finding in skeletal muscle transgenic study is that dystrophin over-expression is not toxic (45). However, in the reported study, dystrophin was only moderately over-expressed in the heart (45). Although the heart seems to be able to accommodate a limited range of transgene expression, numerous reports have revealed apparent cardiotoxicity when a transgene is over-expressed. This is true not only for transgenes that have biological activity

in the heart (60-63), it is also true for commonly used reporter proteins such as GFP (64) and proteins that are considered harmless such as the Gal4 yeast transcription factor (65).

Cardiac toxicity of dystrophin over-expression has not been studied. It is very likely that there exists an optimal range. The heart is known to display different levels of tolerance to different proteins. A six-fold higher expression of adenosine receptor causes dilated cardiomyopathy (63). However, a 16-fold over-expression of myosin light chain 1 is not toxic. Toxicity is observed only when the myosin light chain 1 level is 27-fold higher than normal (62). The toxic threshold is not known for dystrophin. Considering the high likelihood of cardiotoxicity from over-expression, one should be cautious in moving experimental heart gene therapy to patients before the tolerable range is determined and before the methods are developed to control gene expression. In the meantime, an urgent issue is to find out how much dystrophin is too much.

Where should the therapeutic gene(s) be delivered?

In general, the goal of gene therapy is to deliver the therapeutic gene to the place where it is missing. A logical assumption for heart gene therapy will be to restore dystrophin expression in cardiac muscle. However, some studies suggest that there are also skeletal muscle and vascular smooth muscle components in dystrophic heart diseases. The question is whether we need to restore dystrophin expression in all three muscles, including cardiac, skeletal and smooth muscle, in order to cure dystrophin-deficient heart diseases.

The pathogenesis of dystrophic heart disease is not completely understood. Several studies suggest that vasospasm from the disruption of the sarcoglycan complex is responsible for cardiomyopathy in certain types of limb girdle muscular dystrophy (such as in β- or δ-sarcoglycan-deficient mice) (66-68). However, recent studies by Wheeler *et al.* (69,70) suggest that vasospasm is not due to sarcoglycan deficiency. Rather it is caused by cytokines released from the injured cardiomyocytes and/or inflammatory cells in γ- or δ-sarcoglycan-deficient mice. Although it is tempting to hypothesize that vascular dysfunction may have contributed to the heart pathology in dystrophin-deficient disease, this hypothesis is not supported by experimental data. First, the sarcoglycan complex is not disturbed in mdx vascular smooth muscle (68). Second, a vasodilating drug (verapamil) that has effectively reduced cardiomyopathy in δ-sarcoglycan null mice does not protect the mdx mouse heart (68). Importantly, verapamil may trigger death in DMD patients (71). The most compelling evidence against the smooth muscle theory comes from a recent gene rescue experiment (70). In this study, wheeler *et al.* showed that rescuing the missing DGC component in vascular smooth muscle cannot stop heart disease in sarcoglycan-deficient mice. But restoring expression in cardiomyocytes can ameliorate heart disease (70). Taken together, rescuing smooth muscle dystrophin expression may not be necessary nor will it be protective in dystrophin-deficient heart disease.

Controversy exists as to whether skeletal muscle disease contributes to cardiomyopathy. Dystrophin-null mdx mouse is the most commonly used model for DMD. Adult mdx mice display mild skeletal muscle weakness and subtle heart pathology (72,73). However, when myoD (a skeletal muscle-specific transcription factor) is inactivated, mdx mice develop serious skeletal muscle disease. Interestingly, these dystrophin/myoD double-null (m-dko) mice also develop severe cardiomyopathy (74). As myoD is expressed only in skeletal muscle (not in the heart), it has been speculated that a secondary response to the severe skeletal muscle disease, rather than the loss of dystrophin itself, may be crucial for severe cardiomyopathy in these mice. This finding is reminiscent of an earlier patient study that showed a correlation between the severity of skeletal muscle disease and the development of heart disease (75). If, indeed, dystrophic cardiomyopathy is secondary to severe skeletal muscle damage, the therapy should then focus on treating skeletal muscle pathology.

This hypothesis is challenged by several studies. First, it has been demonstrated that the loss of dystrophin in the heart alone is sufficient to cause severe heart disease in XLDC patients. As dystrophin expression is not disturbed in skeletal muscle in these patients, the lack of cardiac dystrophin is apparently the primary cause of the heart pathology (7,76-78). Second, a study in γ-sarcoglycan-deficient mice suggests that transgenic rescue of skeletal muscle pathology has no beneficial effect on the heart (79). Heart disease is ameliorated only when the γ-sarcoglycan gene is expressed in cardiomyocytes (70).

In summary, the primary target tissue in gene therapy should be cardiac muscle. However, ameliorating skeletal muscle disease may further improve heart function.

MOUSE MODELS TO DEVELOP HEART GENE THERAPY

A number of mouse models have been developed to mimic human DMD/BMD. Among these, dystrophin-null mdx mice, m-dko and dystrophin/utrophin double knockout (u-dko) mice and m-dko mice are particularly attractive for heart gene therapy studies.

Mdx mice

Naturally occurring mdx mice result from a nonsense mutation in exon 23 (80,81). Skeletal muscle pathology in mdx mice has been extensively characterized. The heart in young mdx mice (<3-month-old) display infrequent and mild changes such as small degenerative foci and limited inflammation (72,73,82-85). By 6–8 months of age, the mdx heart starts to show moderate myocardial necrosis and fibrosis (68,72). Prominent heart pathology does not appear until mice are about 12-month-old (86) and it gets worse as mice age (86-88).

Several recent studies have begun to evaluate physiological changes in the mdx heart. Despite a lack of significant histology alteration in the young mdx heart, direct *ex vivo* measurement of myocardium contraction has revealed a substantial force reduction in 8–14-week-old mdx mice (89,90). However, this force decline is not sufficient to alter hemodynamic function unless mice are stressed (21,59,83,87,91,92). In contrast to young mdx mice, the features of dilated cardiomyopathy can be more easily detected in old mice. In one study, investigators reported echo-cardiography change in 10-month-old mdx mice (87). ECG study in old mdx mice also revealed similar findings as have been reported in DMD/BMD patients such as deep Q wave, increased R/S ratio, frequent premature ventricular contraction and reduced heart rate variability (86).

Despite a relatively mild cardiac phenotype at young age, mdx mice do develop heart disease when they get old. As mdx mice share exactly the same genetic defect as human patients and are readily available, they represent a valuable model for heart gene therapy studies.

U-dko mice

Utrophin is an ubiquitously expressed autosomal paralogue of dystrophin and it is up-regulated in mdx mice (45,92-96).

To test whether utrophin up-regulation compensates for the loss of dystrophin, u-dko mice were generated (73,97). In sharp contrast to the mild phenotype in mdx mice, u-dko mice are weaker and smaller. They show progressive muscle wasting, growth retardation, weight loss, scoliosis and contractures (Fig. 4). They die prematurely between the age of 8 and 10 weeks. As the catastrophic clinical progression in u-dko mice closely reproduces the severe phenotype of DMD patients, these mice are considered as excellent models for DMD gene therapy studies (53).

Two strains of u-dko mice were developed independently in two laboratories (73,97). Although the majority of mice in the strain reported by Grady *et al.* (73) show severe cardiomyopathy between 8 and 10 weeks of age (Fig. 4), the strain reported by Deconinck *et al.* (97) seems to display a relatively milder cardiac pathology. The heart weight/body weight ratio in 8–10-week-old Deconinck u-dko strain is not different from that of wild-type littermates (90). In a semi-quantitative assay, the histopathology of 10-week-old Deconinck strain u-dko heart also scores similarly to the mdx heart (85). The exact mechanism for the different heart phenotype in these two strains is not clear. It may relate to the difference in the genetic background on which these mice are generated. Alternatively, it may reflect the difference in gene knockout strategy. All utrophin isoforms are inactivated in the Grady strain, whereas only the largest utrophin is inactivated in the Deconinck strain.

M-dko mice

The mild phenotype in mdx mice has been partially accredited to extremely efficient muscle regeneration as demonstrated by muscle hypertrophy and the prevalence of centrally located nuclei (82,98-100). The activated satellite cells are responsible for skeletal muscle regeneration. The absolute number of satellite cells is not altered in mdx mice, but their regenerative capacity is enhanced (101,102).

MyoD is a basic helix-loop-helix myogenic transcription factor that is specifically involved in satellite cell activation (103). To test whether an increased muscle regenerative capacity has contributed to the relatively healthy appearance of mdx mice, m-dko mice were generated by crossing myoD knockout mice and mdx mice (101). As expected, m-dko mice develop severe dystrophic signs as seen in patients, such as substantially reduced muscle mass, pronounced scoliosis, abnormal waddling gait and premature death at ~ 12 months of age (74) (Fig. 4). Surprisingly, m-dko mice also develop severe dilated cardiomyopathy (74) (Fig. 4). As myoD is only required in skeletal muscle development (104), it is thought that cardiomyopathy in m-dko mice is secondary to severe skeletal muscle disease (74). M-dko mice provide a unique variant to mdx and u-dko mice. Genetically, m-dko heart is identical to human patients. Phenotypically, m-dko heart displays the exactly same progressive dilated cardiomyopathy as seen in patients.

In summary, mdx mice (especially old mice), u-dko and m-dko mice constitute a great resource to study dystrophin-null cardiomyopathy and to develop gene therapy. Because of the difference in gene mutation and the degree of the pathology, a comprehensive study including all three models will likely overcome the limitations of the single model and yield useful information to guide human gene therapy.

SUMMARY AND FUTURE DIRECTION

The enthusiasm for gene therapy of dystrophin-deficient cardiomyopathy has reached a record high level. Tremendous progress has been made in developing heart gene delivery vehicles, in optimizing heart gene transfer methods and in generating novel synthetic mini- and microgenes. Now the challenges are to understand the basic parameters. In particular, we need to know whether we should correct every single cardiomyocyte in order to achieve organ level improvement. We need to know whether treating the heart is sufficient to alleviate the cardiac phenotype. Most importantly, we need to know which candidate dystrophin gene(s) will work in the heart and how much protein will be required. It is now the golden time to systemically address these important questions in experimental animal models. Knowledge in these areas will pave the way to eventually curing dystrophin-null cardiomyopathy in human patients.

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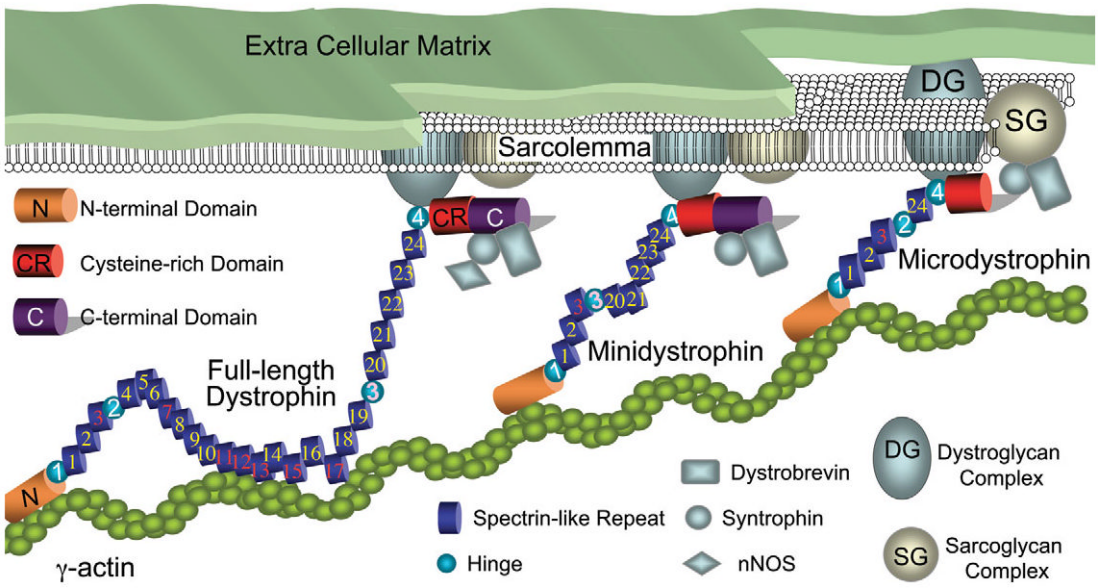

Figure 1.

Schematic outline of full-length dystrophin, minidystrophin and microdystrophin and their interaction with other cellular proteins. Spectrin-like repeats are numbered from 1 to 24 (positively charged repeats are in red color, other repeats are in yellow color). Proline-rich hinges are numbered from 1 to 4. Hinge 3 is in pink color to indicate that it can be cleaved by viral protease. Hinge 2 to repeat 19 are deleted in minidystrophin. Repeat 4–23 and the C-terminal domain are deleted in microdystrophin. Not drawn to scale.

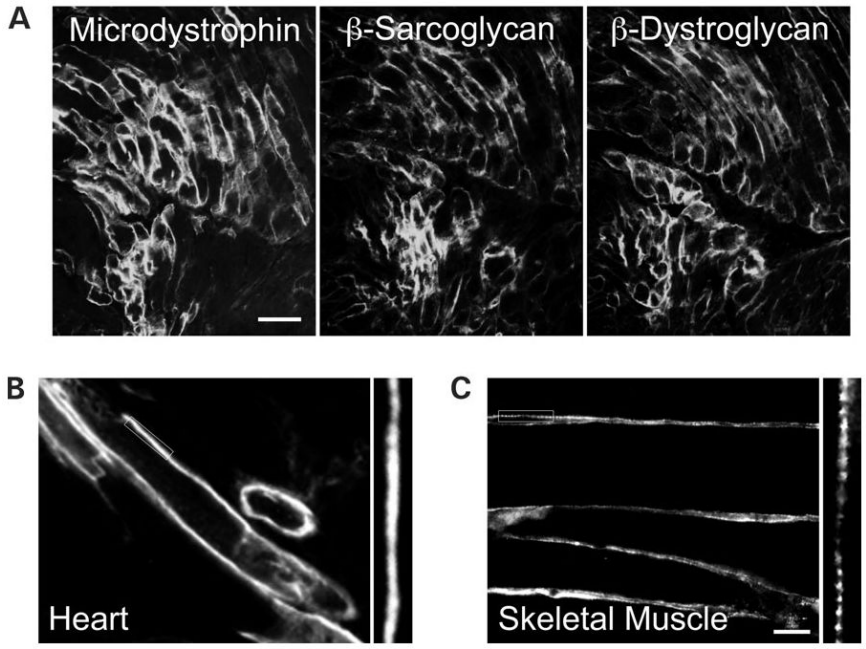

Figure 2.

Microdystrophin restores the DGC and displays a no-interrupted expression pattern along the sarcolemma in the mdx heart. Microdystrophin was delivered to the neonatal mdx heart by AAV vector. Transgene expression was examined when mice were 10-month-old. (**A**) Serial sections showing co-localization of microdystrophin with two other major components of the DGC, β-sarcoglycan and β-dystroglycan. Scale bar: 50 μm. Adapted from Yue *et al*. (2003) *Circulation*, **108**, 1626. (**B**) Continuous labeling of microdystrophin along the sarcolemma in the heart. (**C**) To study microdystrophin expression in skeletal muscle, an AAV virus carrying the microgene was injected into the limb muscle in 2-month-old mdx mice. Immunofluorescence staining was performed 3 months later. Microdystrophin displays a punctate staining pattern in skeletal muscle. Scale bar: 20 μm (this bar also applies to B). In B and C, high magnification photomicrographs of the boxed areas are highlighted in the sides, respectively.

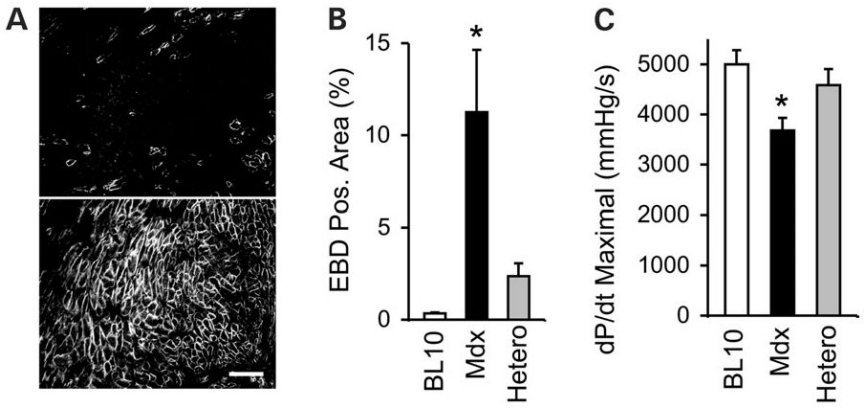

Figure 3.

Mosaic dystrophin expression in heterozygous mdx mice protects the heart from stress-induced cardiomyopathy. (**A**) Dystrophin expression in the heart of heterozygous female mice. Top and bottom panels depict regions of low and high dystrophin expression, respectively. Scale bar: 100 μm. (**B**) Quantification of sarcolemma damage (EBD positive area) in the hearts of different mouse strains following β-isoproterenol challenge. (**C**) Correction of hemodynamic defect in heterozygous mice. Bar graph shows the d*P*/d*t* maximal. Asterisk denotes results in mdx were significantly different from these in BL10 or heterozygous mice. EBD, Evans blue dye; Hetero, heterozygous mice. Adapted from Yue *et al.* (2004) *Hum. Mol. Genet.*, **13**, 1669.

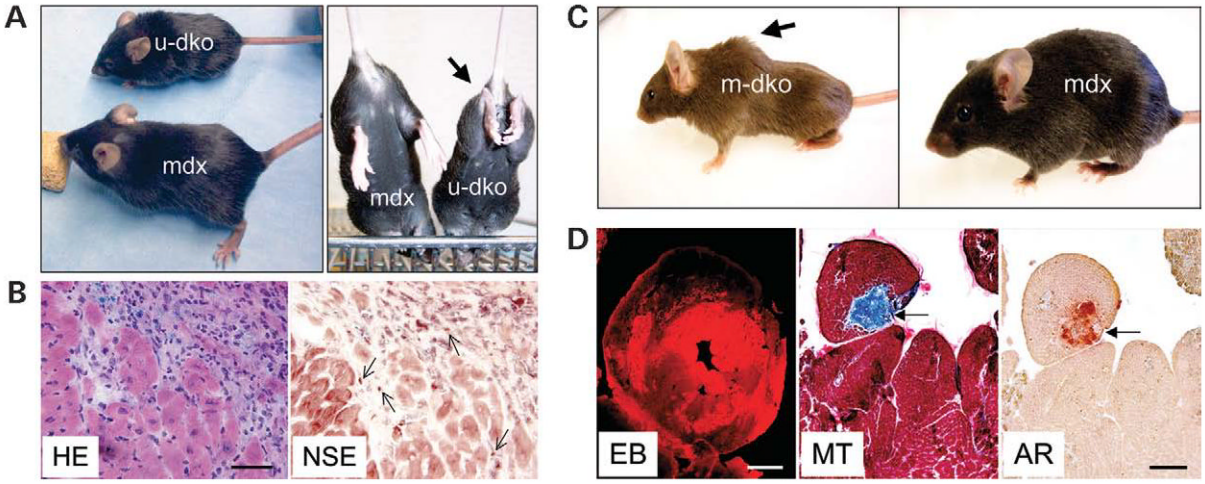

Figure 4.

Utrophin/dystrophin (u-dko) and myoD/dystrophin (m-dko) double knock-out mice recapitulate the clinical phenotype in DMD patients. (**A**) U-dko mice are significantly smaller than age and sex-matched mdx mice (left panel). U-dko mice also display abnormal hind limb contracture (arrow, right panel). (**B**) Inflammation in the heart of a 2-month-old Grady strain u-dko mouse. HE, hematoxylin–eosin stain; NSE, non-specific esterase stain for macrophage. Arrows, macrophage. Scale bar: 50 μm. (**C**) Scoliosis (arrow) is a common feature in 5-month-old m-dko mice, but it is absent in age- and sex-matched mdx mice. (**D**) Representative heart pathology in m-dko mice. EBD uptake in 4-month-old m-dko heart; scale bar: 1 mm. MT and AR, Masson trichrome (for fibrosis, arrow) and Alizarin red (for calcification, arrow) stain, respectively; scale bar: 100 μm.