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ARTICLE

100-fold but not 50-fold dystrophin overexpression aggravates electrocardiographic defects in the mdx model of Duchenne muscular dystrophy

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Dystrophin gene replacement holds the promise of treating Duchenne muscular dystrophy. Supraphysiological expression is a concern for all gene therapy studies. In the case of Duchenne muscular dystrophy, Chamberlain and colleagues found that 50-fold overexpression did not cause deleterious side effect in skeletal muscle. To determine whether excessive dystrophin expression in the heart is safe, we studied two lines of transgenic mdx mice that selectively expressed a therapeutic *minidystrophin* gene in the heart at 50-fold and 100-fold of the normal levels. In the line with 50-fold overexpression, minidystrophin showed sarcolemmal localization and electrocardiogram abnormalities were corrected. However, in the line with 100-fold overexpression, we not only detected sarcolemmal minidystrophin expression but also observed accumulation of minidystrophin vesicles in the sarcoplasm. Excessive minidystrophin expression did not correct tachycardia, a characteristic feature of Duchenne muscular dystrophy. Importantly, several electrocardiogram parameters (QT interval, QRS duration and the cardiomyopathy index) became worse than that of mdx mice. Our data suggests that the mouse heart can tolerate 50-fold minidystrophin overexpression, but 100-fold overexpression leads to cardiac toxicity.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common childhood lethal muscle disease caused by dystrophin deficiency. This X-linked disease mainly affects boys and young men. Introducing a functional dystrophin gene back to muscle by gene therapy holds a great promise to treat DMD. Ideally, for gene therapy one would like to express the full-length gene or the full-length cDNA from the endogenous promoter. This will allow for spatially and temporally regulated expression of a full-length protein to meet developmental and physiological needs of muscle. Unfortunately, the *dystrophin* gene is one of the largest genes in the mammalian genome. It greatly exceeds the packaging limit of most viral gene delivery vectors. This not only excludes the use of the full-length gene or cDNA as the therapeutic gene but also excludes the use of the endogenous dystrophin promoter in the expression cassette. To overcome these hurdles, investigators are forced to express a synthetic *mini/micro dystrophin* gene from a constitutive promoter (either ubiquitous or muscle-specific). A likely consequence of this approach is unchecked expression and the production of excessive amount of dystrophin. From the safety standpoint, it is essential to determine whether supraphysiological expression of a therapeutic mini/micro dystrophin gene can lead to deleterious side effects.

Despite intensive research and exciting progresses in the field of dystrophin gene replacement therapy, so far only one study has examined potential toxicity of dystrophin overexpression. Cox *et al.* generated a strain of full-length dystrophin overexpression transgenic mice on the background of dystrophin-null mdx mice.[1] The authors found that the dystrophin level in transgenic mice was 50-fold higher than that of normal mice. Despite excessive amount of dystrophin, surprisingly, skeletal muscle morphology and force were completely normal. This study suggests that skeletal muscle can tolerate supraphysiological levels of dystrophin.[1]

Heart disease is a leading cause of morbidity and mortality in DMD. To treat DMD heart disease, we need to deliver a functional dystrophin gene to the heart. It is thus important to determine whether supraphysiological dystrophin expression in the heart is safe. To address this critical question, we developed cardiac transgenic mdx mice that selectively overexpressed the *ΔH2-R19 minidystrophin* gene in the heart.[2] This minidystrophin gene has previously being shown to protect both skeletal muscle and the heart in mdx mice.[2–4] In a line of 50-fold overexpression, we observed the expected benefits of the *minidystrophin* gene.[2] However, cardiac toxicity was detected in a line that showed

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100-fold overexpression. Our results suggest that dystrophin overexpression in the heart is likely safe as long as it does not exceed 50-fold of the wild type level.

RESULTS

Generation of cardiac ΔH2-R19 minidystrophin overexpression transgenic mdx mice

To achieve heart specific overexpression of the therapeutic *ΔH2-R19 minidystrophin* gene, we used the 5.5 kb murine α-myosin heavy chain (αMHC) promoter.[5–7] This is the most commonly used promoter for cardiac transgenic studies and it drives transgene expression throughout the entire heart. Importantly, depending on the copy number, one can achieve a broad range of gene expression including supraphysiological expression with this promoter.[8–24] We obtained a total of 10 founder lines and nine lines were backcrossed to the congenic background of mdx mice.[2] In this study, we focused on lines 26 and 29.

Characterization of cardiac minidystrophin overexpressing transgenic mice

To determine the copy number of the minidystrophin gene in lines 26 and 29, we performed Southern blot. The 3.4 kb diagnostic band was detected in both lines (Figure 1a). On quantification, lines 26 and 29 contained 328.7 ± 11.5 and 5.3 ± 0.2 copies of the ΔH2-R19 minidystrophin gene, respectively (Figure 1b).

Next we examined the protein level by western blot. Both lines yielded the expected 220 kDa ΔH2-R19 minidystrophin band (Figure 1c). When compared with the level of full-length dystrophin in normal BL10 mice, the minidystrophin protein in lines 26 and 29 were 102.6 ± 4.3 and 50.8 ± 2.3 fold higher than that of normal mice, respectively (Figure 1d).

On immunofluorescence staining, ΔH2-R19 minidystrophin showed the expected sarcolemmal localization in the heart of line 29 mice (Figure 1e). However, minidystrophin was detected in both sarcolemma and sarcoplasm in cardiomyocytes of line 26 (Figure 1e, Supplementary Figure S1). On high magnification, sarcoplasmic minidystrophin staining displayed as small vesicles (Figure 1e).

Evaluation of heart histology and ECG in cardiac minidystrophin overexpression transgenic mice

On hematoxylin/eosin staining and Masson trichrome staining, heart histology of transgenic mice was indistinguishable from that of normal mice (see Supplementary Figure S2). To evaluate physiological consequences of minidystrophin overexpression, we performed 12 lead electrocardiogram (ECG) in 6-m-old mice. A characteristic change in DMD patients and mdx mice is tachycardia.[2,25–28] The heart

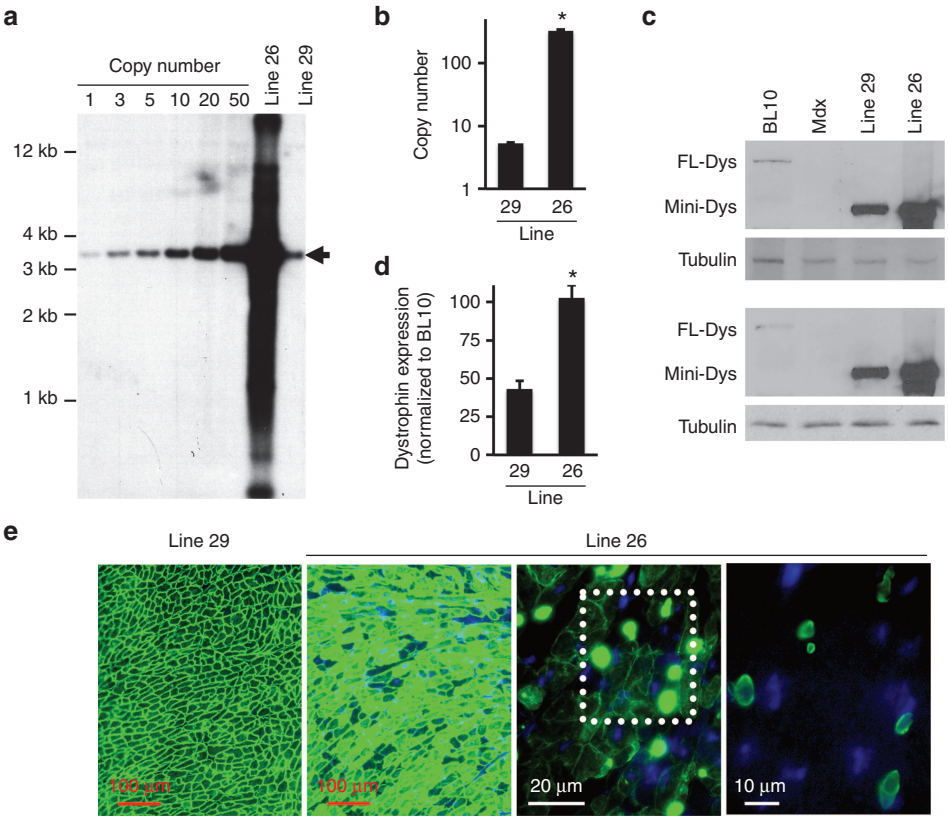

Figure 1 Transgenic overexpression of a therapeutic minidystrophin gene in the heart of mdx mice. (**a**) A representative Southern blot photomicrograph. Arrow, the 3.4 kb diagnostic band for transgenic mice. (**b**) Quantification of the minidystrophin gene copy number in transgenic mice. (**c**) Two representative dystrophin western blots of the heart of BL10, mdx and transgenic mice. (**d**) Quantification of minidystrophin expression. The level of expression was normalized to the loading control and BL10 control. (**e**) Representative dystrophin immunofluorescence staining from the heart of transgenic lines 26 and 29. The left panel (low-power images of line 29 heart) and the second to the left panel (low-power images of line 26 heart) had the identical exposure conditions. The middle panel of line 26 images shows a short-exposure, high-power photomicrograph. Excessively expressed dystrophin forms inclusion body inside cardiomyocytes. The right panel of line 26 images is an enlarged view of the boxed region of the middle panel and it was taken with a much reduced exposure time. The cytosolic dystrophin inclusion bodies appear as vesicles. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue color). Asterisk, significantly different from the other group.

rate was normalized in line 29 but not in line 26 (Figure 2). In fact, line 26 showed the same heart rate as that of mdx mice. The only ECG abnormality that was corrected in both lines was the PR interval (Figure 2b). When compared with BL10, mdx had a longer QT interval and QRS duration. These defects were completely corrected in line 29. Surprisingly, both parameters got worse in line 26. They were even significantly longer than those of mdx mice (Figure 2b). The Q-wave amplitude showed a peculiar trend. Line 26 was significantly shallower than all other strains. The cardiomyopathy index was used to evaluate overall electrophysiology in the heart. It was normalized in line 29 but was significantly higher than that of mdx in line 26.

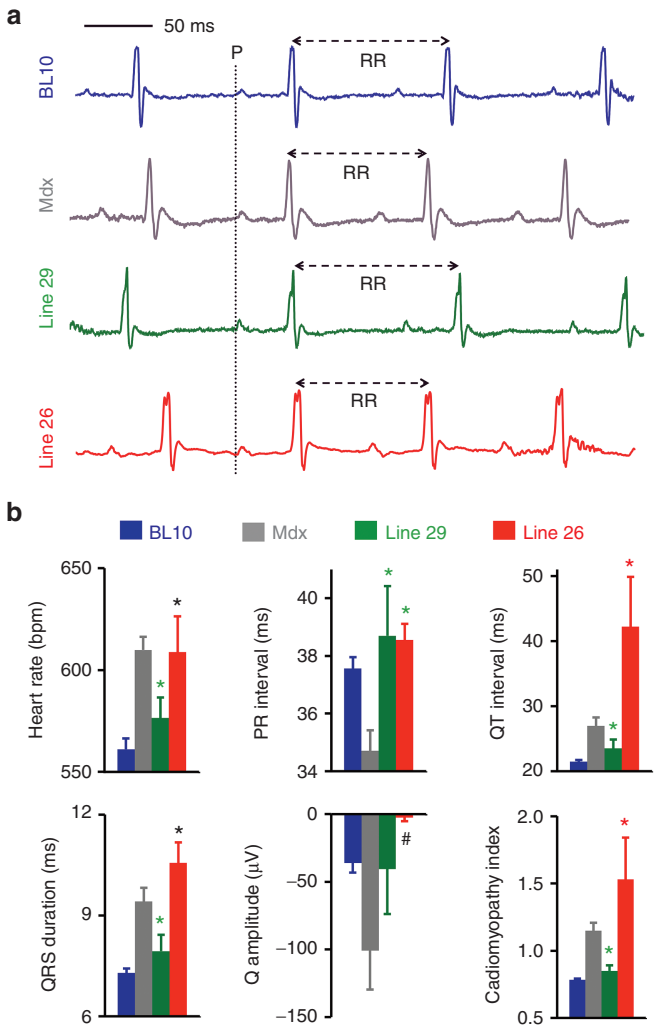

Figure 2 100-fold overexpression of the therapeutic ΔH2-R19 minidystrophin gene in the heart of transgenic line 26 worsened electrocardiogram (ECG) defects seen in mdx mice. (**a**) Representative ECG tracing from BL10, mdx, transgenic line 29 (50-fold overexpression) and line 26 (100-fold overexpression) mice. The dotted vertical line indicates the starting position of the P-wave. Respiratory rate (RR) duration (time between two neighboring heart beats) is clearly reduced in mdx mice and line 26, suggesting the presence of tachycardia in these two stains. In line 29, RR duration is similar to that of BL10 mice. (**b**) Quantitative comparison of the ECG profile from BL10, mdx, line 29, and line 26. Sample size: $n = 10$ for BL10 mice, $n = 9$ for mdx mice, $n = 17$ for line 29, and $n = 13$ for line 26. Green asterisk, results from transgenic mice are normalized to that of BL10 mice; black asterisk, results from transgenic mice are similar to that of mdx mice; red asterisk, results from transgenic mice are significantly worse than that of mdx mice; Pound sign, results from Line 26 mice are significantly different from all other lines (BL10, mdx and Line 29).

DISCUSSION

In this study, we examined the consequences of supraphysiological level minidystrophin expression in the heart of mdx mice. We found that 50-fold overexpression ameliorated ECG defects. In contrast, 100-fold overexpression not only failed to improve the outcome of the most of the ECG parameters but also aggravated abnormalities in several measures (such as the QT interval, QRS duration and cardiomyopathy index). Our results suggest that the murine heart has a quite impressive tolerance to dystrophin overexpression (up to 50-fold). However, when the level of expression becomes excessively high (*e.g.*, 100-fold), it will lead to cardiac toxicity. Specifically, overexpressed dystrophin formed aberrant cytosolic dystrophin vesicles and aggravated ECG abnormalities.

There has been significant progress in the development of DMD gene replacement therapy over the last few years. In particular, adeno-associated virus (AAV)-mediated *microdystrophin* gene delivery has yielded highly promising efficacy data in the murine and canine models.[29,30] Several clinical trials are currently in planning.[31] Despite these advances, one critical question remains incompletely answered. Specifically, how much dystrophin is too much? In other words, will dystrophin overexpression cause a problem? This is highly relevant because dystrophin is expressed from a constitutive promoter (either ubiquitous or muscle-specific) in all gene replacement therapy vectors and AAV is a long lasting virus.[32] To address this question, the Chamberlain laboratory, Seattle, WA studied transgenic mdx mice that had 50-fold dystrophin overexpression. Strikingly, no structural or functional abnormalities were found in skeletal muscle suggesting supraphysiological dystrophin expression is safe in skeletal muscle.[1]

Cardiac complications greatly compromise the life quality of DMD patients. A significant portion of patients dies from heart failure or sudden cardiac death. Hence, an effective DMD gene replacement therapy requires efficient delivery of a therapeutic dystrophin gene to the heart. Overexpression-induced cardiotoxicity is well documented in the literature.[11,14–16,20,33] For example, two fold overexpression of the green fluorescence protein, six fold overexpression of the adenosine receptor and 27-fold overexpression of myosin light chain 1 result in dilated cardiomyopathy.[14–16] Interestingly, depending on the protein being overexpressed, the heart seems to show different levels of tolerance. For example, 16-fold overexpression of myosin light chain 1 is not toxic.[14] Unfortunately, the tolerant range for dystrophin has never been determined. To address this unmet need, we generated cardiac dystrophin overexpressing mice. Since future clinical trials will likely use the abbreviated *dystrophin* gene, we overexpressed a therapeutic *minidystrophin* gene. Consistent with the finding of Cox *et al.*,[1] we did not detect any toxicity in the line with 50-fold minidystrophin overexpression. However, there was clear evidence of toxicity by the ECG assay when expression reached 100-fold of the normal. Based on these findings, we conclude that the heart has a relatively high safety margin for dystrophin overexpression. More specifically, 50-fold minidystrophin overexpression is not toxic to the mouse heart.

Several groups have tested AAV-mediated *dystrophin* gene replacement therapy for Duchenne cardiomyopathy in the mouse model.[25,34–40] Despite widespread expression throughout the entire heart, in none of these studies, AAV-mediated expression exceeded 10-fold of the normal dystrophin level. We have achieved cardiac AAV transduction in dogs and more recently demonstrated efficient

AAV micro-dystrophin expression in the heart of DMD dogs.[29,41–43] Two independent groups have also demonstrated AAV-mediated exon-skipping in dystrophic dog hearts.[44,45] Yet, it is still a great challenge to obtain saturated myocardial AAV transduction in the heart of a large mammal. We believe that with the current AAV technology, supraphysiologic dystrophin overexpression may not constitute a serious concern for Duchenne cardiomyopathy gene therapy. However, the development of novel AAV capsids, expression cassette and/or gene delivery methods may lead to significantly much higher transduction efficiency in the future.[46–48] The maximal tolerable dystrophin level described in our study will serve as an important reference to guide future studies.

The toxicity seen in the line of 100-fold overexpression suggests that a level higher than 50-fold may also cause harmful changes in skeletal muscle. Indeed, Harper *et al.* observed similar dystrophin aggregation vesicles in the quadriceps muscle of a microdystrophin transgenic line that specifically overexpressed the *ΔR2-R21+H3 microgene* in skeletal muscle.[3] On quantification of centrally located myonuclei of 6-m-old mice, the authors found ≤ 1%, 64% and 52% in BL10 (normal control), mdx, and ΔR2-R21+H3 microgene overexpression transgenic mice, respectively. Interestingly, in another line that expressed the *ΔR4-R23*, a structurally similar microgene (both microgenes have 4 repeats and 1 hinge), the percent of central nucleation was <1%. Although the authors did not quantify the level of overexpression, a rough evaluation based on the western blots in the paper suggests that the lines ΔR2-R21+H3 and ΔR4-R23 had a dystrophin level of ~90-fold and ~10-fold of BL10, respectively.

It is currently unclear how 100-fold dystrophin overexpression resulted in cardiac toxicity. We suspect that it may likely relate to the accumulation of excessive amount of dystrophin inclusion bodies in the cytosol. However, we believe the heart may tolerate limited levels of cytosolic dystrophin expression. In support of this notion, we did notice some dystrophin staining in the cytoplasm of cardiomyocytes in transgenic line 29 mice. Chamberlain also observed similar cytosolic dystrophin expression in the heart of their full-length dystrophin transgenic mice.[1] Besides the dystrophin level, it is also possible that the toxicity seen in line 26 may relate to the positional effect of transgene integration. Insertion may have either activated or shut down expression of other important cellular protein(s) and consequently result in toxicity. Nevertheless, the data of the current study as well as that of Cox *et al.* suggest that moderate dystrophin overexpression is relatively safe in muscle.[1] Tight control of dystrophin expression may not be necessary in DMD gene therapy.

MATERIALS AND METHODS

Experimental animals

All animal experiments were approved by the institutional animal care and use committee and were in accordance with NIH guidelines. All experimental mice were housed in a specific pathogen-free facility and kept under a 12 hours light (25 lux)/12 hours dark cycle with free access to food and water. C57Bl/10SnJ (BL10) and dystrophin-deficient C57Bl/10ScSn-*Dmd*^{mdx}/J (*mdx*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

The cardiac specific minidystrophin transgenic mice were generated at the University of Missouri transgenic core. The expression cassette consists of the αMHC promoter (a gift from Dr Jeffrey Robbins, Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Research Foundation, Cincinnati, OH), the *ΔH2-R19 minidystrophin* gene (a gift from Dr Jeffrey Chamberlain at the University of Washington, Seattle, WA) and the bovine growth hormone polyadenylation signal.[3,5,7] A total of 10 founders were identified and nine founders were backcrossed with mdx mice for 5–7 generations.[2] In this study, we evaluated incipient congenic mice from lines 26 and 29. Only male mice were used in the study. The ECG assay was performed in 6-m-old male mice.

Southern blot

Genomic DNA was extracted from the tail using a previously described high salt precipitation protocol.[49] A 414-bp BamH I (exon 4)/EcoR V (exon 7) double digested DNA fragment was used as the template for the Southern probe. Tail genomic DNA was digested with BamH I. After transfer to a nylon membrane, the blot was hybridized with a ^{32}P-labelled probe according to a previously published protocol.[49] The diagnostic band migrated at 3.4 kb. For the copy number control, the plasmid used for making transgenic mice was digested with BamH I and loaded on the same gel.

Immunostaining

Dystrophin immunofluorescence staining was performed essentially as we described before using a mouse monoclonal antibody against the C-terminal domain of dystrophin (Dys-2; 1:30; Vector Laboratories, Burlingame, CA).[36,50] Slides were viewed using a Nikon E800 fluorescence microscope. Photomicrographs were taken with a Qimage REtiga 1,300 camera (Burnaby, BC, Canada).[51]

Western blot

Membrane protein enriched microsomal preparation was extracted from the heart according to our published protocol.[52–54] 50 µg protein was separated on a 6% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, protein was transferred to a polyvinylidene fluoride membrane. The membrane was probed with the Dys-2 antibody (1:100). For the loading control, we used the α-tubulin antibody (1:3,000; clone B-5-1-2; Sigma, St Louis, MO). Western blot quantification was performed using ImageJ (http://rsbweb.nih.gov/ij/). The relative intensity of the respective protein band was normalized to the corresponding loading control in the same blot. To determine the relative expression level of minidystrophin, the relative band intensity of transgenic mice was further normalized to that of full-length dystrophin in BL10 mice in the same blot. We would like to point out that the full-length dystrophin protein might transfer to the membrane less efficiently than the minidystrophin protein during western analysis due to the large size of the full-length protein (427 kDa). To overcome this technical problem, we have conducted overnight transfer in western blot analysis. Furthermore, we confirmed efficient transfer by the lack of Coomassie blue staining of the polyacrylamide gel after transfer. Despite these efforts, we cannot completely exclude the possibility of incomplete transfer of trivial amount of full-length protein. There is still a very small likelihood that we may have underestimated the quantity of full-length dystrophin. Hence, the relative overexpression of minidystrophin could be slightly lower.

ECG

A 12-lead ECG assay was performed with an ECG recording system from AD Instruments (Model MLA0112S; Colorado Springs, CO) as described in the *Standard Operating Procedures (SOP's) for Duchenne Animal Models-Cardiac Protocols* (http://www.parentprojectmd.org/site/PageServer?pagename=Advance_researchers_sops).[54,55] Briefly, cardiac electric activity signals were processed with a single channel bioamplifier (Model ML132; AD Instruments). Averaged value from at least 1 minute continuous recording was used for ECG analysis by LabChart software (AD Instruments). The amplitude of the Q-wave was analyzed using the lead I tracing. The remaining ECG parameters were analyzed using lead II tracing results. The cardiomyopathy index is determined by dividing the QT interval by the PQ segment.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed with the Prism software (GraphPad, San Diego, CA). Statistical significance between two groups was determined by the Student's *t*-test. Statistical significance among different groups was determined by one-way analysis of variance followed by Tukey's *post hoc* analysis. Difference was considered statistically significant when $P < 0.05$.

CONFLICT OF INTEREST

D.D. is a member of the scientific advisory board for and an equity holder of Solid GT, a subsidiary of Solid Biosciences.

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