Low dystrophin levels in heart can delay heart failure in mdx mice

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ABSTRACT

Duchenne muscular dystrophy is caused by mutations that prevent synthesis of functional dystrophin. All patients develop dilated cardiomyopathy. Promising therapeutic approaches are underway that successfully restore dystrophin expression in skeletal muscle. However, their efficiency in the heart is limited. Improved quality and function of only skeletal muscle potentially accelerate the development of cardiomyopathy. Our study aimed to elucidate which dystrophin levels in the heart are required to prevent or delay cardiomyopathy in mice.

Heart function and pathology assessed with magnetic resonance imaging and histopathological analysis were compared between 2, 6 and 10-month-old female mdx-Xist Δhs mice, expressing low dystrophin levels (3–15%) in a mosaic manner based on skewed X-inactivation, dystrophin-negative mdx mice, and wild type mice of corresponding genetic backgrounds and gender.

With age mdx mice developed dilated cardiomyopathy and hypertrophy, whereas the onset of heart pathology was delayed and function improved in mdx-Xist Δhs mice. The ejection fraction, the most severely affected parameter, for both ventricles, correlated to dystrophin expression and the percentage of dystrophin-negative cells in the heart.

These data suggest that mosaic expression of 4–15% dystrophin in the heart is sufficient to delay the onset and ameliorate cardiomyopathy in mice.

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1. Introduction

Duchenne muscular dystrophy (DMD) is the most common inherited neuromuscular disorder. In DMD patients, the synthesis of functional dystrophin proteins is prevented by mutations in the DMD gene [1]. Dystrophin negative fibers are vulnerable to exercise-induced damage, leading to loss of ambulation in the second decade of life. Cardiomyopathy is increasingly observed from 10 years of age onwards [2]. Old patients depend on assisted ventilation and at that stage the vast majority suffers from dilated and hypertrophic cardiomyopathy, of which the progression is accelerated by voluntary activity, increasing the heart workload and exacerbating heart failure [3,4]. Eventually, patients die of heart or respiratory failure in their third or fourth decade [5].

Becker muscular dystrophy (BMD) patients have in-frame mutations in the DMD gene, resulting in expression of internally deleted, but partly functional dystrophins. The skeletal muscle pathology is less severe than in DMD patients, but a more severe cardiomyopathy is observed, especially in BMD patients with a mild muscle phenotype, accounting for 50% of deaths [3,6,7]. Dilated cardiomyopathy is also occasionally found in DMD and BMD carriers, who express 50% of dystrophin in the heart [8].

There is no cure for DMD, but several potential therapies aiming at dystrophin restoration are under investigation in clinical trials [9–13]. Unfortunately, the efficiency of targeting the heart is limited. Eventually, this might result in partial dystrophin restoration in skeletal muscle, accompanied by improved muscle function, in the absence of restoration in the heart [14–16]. Given that BMD patients and some DMD/BMD carriers exhibit more pronounced cardiomyopathy than DMD patients, increased physical activity is likely to put a larger workload on the heart, thereby potentially exacerbating the development of cardiomyopathy, although other factors, like competition between utrophin and dystrophin, might also play a role.

The dystrophin-negative mdx mouse also suffers from dilated cardiomyopathy, of which the progression is accelerated by activity [17–21]. In line with this, partial dystrophin restoration in skeletal muscle improves voluntary activity, increasing the heart workload and exacerbating heart pathology [15]. By contrast, high dystrophin levels in skeletal muscles and diaphragm only, either improve heart function in 6 month old mice [22], or prevent worsening of heart failure in exercise challenged 23 month old mdx mice [23]. In addition, an internally deleted dystrophin (mini-dystrophin) transgene has been delivered to the heart using adeno

Abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy.

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associated viruses (AAV9). This improved heart function and partially prevented myocardial fibrosis, especially when mice were treated before they were 17 months [24,25].

From carrier mdx mice, it is known that 50% of dystrophin in the heart is sufficient to prevent development of heart pathology [26]. To study whether low dystrophin levels are enough to prevent or delay the onset of heart failure, we assessed heart function and pathology over time in mdx-XistΔhs mice. These mice originate from crossing XistΔhs females (expressing wild type dystrophin but carrying a mutation in the promoter of the Xist gene, which coordinates X-inactivation [27]), with mdx males (which do not express dystrophin and have a normal Xist gene). In the female offspring (mdx-XistΔhs), the X-chromosome expressing the mutated Xist gene, but intact dystrophin, is preferentially inactivated, resulting in expression of varying, low dystrophin levels in skeletal muscle and heart [28]. We show that mosaic expression of dystrophin in 4–15% of the cardiomyocytes (at wild type intensities) is sufficient to delay the onset of diluted cardiomyopathy and reduce the severity of pathology in mdx mice.

2. Methods

2.1. Animal care

Breeding pairs of mdx (C57BL/10ScSn-mdx/J) males and XistΔhs females (BL/6 background) gave birth to mdx-XistΔhs females [28]. The XistΔhs model [27] was a kind gift from prof. Dr. Brockdorff (MRC Clinical Sciences Centre London, UK, current affiliation Department of Biochemistry, University of Oxford, UK). Genotyping was performed on DNA obtained from ear biopsies by PCR analysis. Mice were housed in individually ventilated cages with 12-h light-dark cycles and had ad libitum access to standard chow and water. All experiments were approved by the Animal Experimental Commission (DEC) of the LUMC and conform with the Directive 2010/63/EU of the European Parliament.

2.2. MRI heart function analysis

MRI analysis was performed at the Faculty of Science of the University of Leiden to which the mice were transported a week before analysis to acclimatize. Mice were anesthetized by inhalation of 2% isoflurane in a 1:1 mixture of pure oxygen and air. Respiration rate was monitored with a respiratory pad and kept constant at 50–80 respirations per minute. Mice were scanned in a vertical 9.4 T magnet with 89 mm bore size, equipped with a 1 T/m gradient system (Bruker BioSpin, Germany), field-of-view 2.56 × 2.56 cm²; matrix 192 × 192; and in-plane resolution 133 μm. We made 8–9 short-axis slices of the heart of 1 mm thick and 200 repetitions per slice. The navigator echo was placed in-slice. Images were reconstructed to 18 frames per heart cycle. Per time point 200 repetitions per slice. The navigator echo was placed in-slice. Six of the 135 mice scanned were not used for analysis as the angle of the scan was incorrect.

MASS for MICE software (developed at the LUMC, Division of Image Processing (LKEB)) was used for image analysis [29]. For each picture, endocardial and epicardial contours of both ventricles were drawn manually. The end diastolic and systolic phase were computed by the software and a maximum mass difference of 10% was accepted. When this criteria was met, end diastolic volume (EDV) and end systolic volume (ESV) were determined and based on these values stroke volume (SV = EDV-ESV), ejection fraction (EF = (SV/EDV)*100%) and cardiac output (CO = SV*heart rate) were calculated automatically. Left ventricular end diastolic thickness and end systolic wall thickening were measured for the mid-section of the heart.

2.3. Serum biomarker level analysis

For analysis of cardiac Troponin I and N-terminal Pro Brain Natriuretic Peptide (NT-proBNP) levels, blood (~1 ml) was drawn from the anesthetized mouse via the eye after which animals were sacrificed by cervical dislocation. Blood was collected in a non-coated eppendorf tube, allowed to clot at room temperature for 10 min and stored on ice. Samples were centrifuged at 1700 g for 10 min at 4 °C and stored at −80 °C. Levels of cardiac Troponin I and NT-proBNP were measured using the High Sensitive Mouse Cardiac Troponin-I ELISA Kit (Life Diagnostics, USA) and the ELISA Kit for NT-proBNP (Uscn Life Science Inc., China), respectively.

2.4. Histological examination

Tissues were snap frozen in 2-methylbutane (Sigma Aldrich, the Netherlands) cooled in liquid nitrogen. Sections of 8 μm were made on Superfrost Plus slides (Thermo Fishes Scientific, Menzel-Gläser, Germany) with a Shandon cryotome (Thermo Fisher Scientific Co., USA) along the entire length of the heart with an interval of 240 μm between the sections. Excess tissue was used for protein isolation. Sections were stained with goat-anti-Collagen type I (dilution 1:100, 1310-01 Southern Biotech, USA), donkey-anti-goat Alexa594 (dilution 1:1000, Invitrogen, the Netherlands) and DAPI. A fluorescent microscope (Leica DM RA2) was used to examine the sections at a 16 times magnification and images covering the entire heart were captured with a Leica DC500FX snapshot camera. The percentage of fibrotic tissue was assessed with Imagej software (Rasband W.S., ImageJ, U.S. National Institutes of Health, USA, http://rsb.info.nih.gov/ij/, 1997–2008). The number of arterioles, cardiomyocyte size and distribution of fibrosis were determined on sections stained with Harris Haematoxylin and Eosin (Sigma Aldrich, the Netherlands), which were examined with a light microscope (Leica SP5, Leica Microsystems, United Kingdom). The minimal Feret’s diameter of the cardiomyocytes was measured with Imagej software on four pictures taken at a 40 times magnification. The severity of fibrosis, ranked into six categories, was assessed for ten regions to assess its distribution.

2.5. Dystrophin determination by western blot

The heart, diaphragm and quadriceps muscles were homogenized and dystrophin levels quantified by western blotting using the Odyssey system as described previously [28]. The lowest concentration in the standard curve was 3%. Blots were stained overnight with NCL-DYS1 (dilution 1:125, Novacasta, UK) and MF20 (MF20 dilution 1:20000, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) for dystrophin and myosin respectively. The fluorescent IRDye 800CW goat-anti-mouse IgG (dilution 1:5000, Li-Cor, USA) was used as secondary antibody.

2.6. Statistics

Statistical analyses were performed using SPSS 17.0.2. (SPSS, Inc., USA). Heart function was compared over time with the two-way analysis of variance (ANOVA). In case of significance (P < 0.05), a Fisher’s Least Significant Difference-procedure (LSD) was applied to distinguish between heart function of different strains and to analyze within a mouse strain whether there are differences in heart function over time (so between 2, 6 and 10 month old mice). For ESV data, a log transformation was performed to correct for inequality of the data. Correlations for the 10-month-old mdx-XistΔhs mice were tested with the Spearman correlation test. P < 0.05 was considered significant. The one-way ANOVA was conducted for comparison of the histological and wall thickness data. In case of significance (P < 0.05), the Bonferroni post hoc test was performed. For Fig. 1B, data are presented as medians.
3. Results

3.1. Low dystrophin levels in the heart prevent the age-dependent decline of heart function

To determine whether low dystrophin levels can prevent or delay the onset of dilated cardiomyopathy, heart function was determined by MRI in female mdx (no dystrophin), mdx-Xist<sup>Δhs</sup> (low dystrophin), C57BL/10ScSnJ (wild type) and Xist<sup>Δhs</sup> (wild type) mice aged 2, 6 and 10 months. We chose a cross-sectional design to allow post-mortem histological validation. Dystrophin was expressed in a mosaic manner in the hearts of mdx-Xist<sup>Δhs</sup> mice, where cardiomyocytes were either dystrophin positive at wild type intensities or negative as previously reported [28] (Supplementary Figure A). Dystrophin levels in the heart of mdx-Xist<sup>Δhs</sup> mice varied between 3 and 15% and were similar for the different age groups (2 months; 3–13.8%, 6 months; 3–14.9%, 10 months; 3–12.1%) as assessed by western blot (Figs. 1A–B).

The heart function of the two wild type models was largely comparable, but differed slightly, probably due to differences in the genetic background. In 2 month old mdx mice ejection fraction (EF) is stronger than that of wild type mice. With age, function of both ventricles deteriorated, most evidently for the EF and stroke volume (SV), while it remained constant in both wild type models (Figs. 1C–D, Supplementary Table A), as reported previously [30]. Expression of 3–15% dystrophin in the heart prevented EF and SV to drop in mdx-Xist<sup>Δhs</sup> mice as levels remained stable over time and were higher than mdx mice from the age of 6 months onwards (P < 0.05). The EF and SV of mdx-Xist<sup>Δhs</sup> mice were similar to levels of the wild type stains at 6 months, and for the left ventricle only lower than 10-month-old Xist<sup>Δhs</sup> mice (P < 0.05). The end systolic volume (ESV) of the left ventricle was increased in 10-month-old mdx mice (P < 0.01), while levels of mdx-Xist<sup>Δhs</sup> mice remained similar to wild types. The end diastolic volume (EDV) and cardiac output (CO) of both ventricles and ESV of the right ventricle did not differ between the mouse models.

End systolic mass of both ventricles increased with age in all mice. However, the left ventricular mass of mdx mice was greater than that of all other mice regardless of their age (P < 0.01), indicative of hypertrophy of the heart. This was not observed for mdx-Xist<sup>Δhs</sup> mice. The minimal Feret’s diameter of cardiomyocytes showed a similar pattern, and tended to be larger in 10-month-old mdx than C57BL/10ScSnJ mice (P = 0.056, Supplementary Table B). The left ventricular lateral free wall thickness in diastole was larger in Xist<sup>Δhs</sup> than the other models aged 10 months (P < 0.05 Supplementary Table B), while thickening in systole was larger in Xist<sup>Δhs</sup> compared to both dystrophic models (P < 0.01). For both parameters no difference was observed in the interventricular septum.

Heart rate increased in mdx mice with age (P < 0.001), but remained stable over time for the other models. Heart rate of 2-month-old Xist<sup>Δhs</sup> mice was higher than that of both mdx and mdx-Xist<sup>Δhs</sup> mice (P < 0.01). In old mdx mice, heart rate exceeded both mdx-Xist<sup>Δhs</sup> and Xist<sup>Δhs</sup> mice (P < 0.01).

Dystrophin levels of 10-month-old mdx-Xist<sup>Δhs</sup> mice correlated with the EF of the left ventricle (R = 0.464 P = 0.019), which was also the most severely affected parameter in mdx mice (Fig. 2A). As heart function has been described to normalize by dystrophin expression in the diaphragm [22], we also assessed this for 10-month-old mdx-Xist<sup>Δhs</sup> mice. Dystrophin levels of the diaphragm varied between 1.5 and 38% (mean = 11.5% sdev = 8.8) and did not correlate to dystrophin levels in the heart (R = 0.04 P = 0.849). Dystrophin levels in the diaphragm only correlated with the EDV of the right ventricle (R = 0.492 P =
0.017), however, the EDV did not differ between any of the models. We hypothesized that the combined presence of dystrophin in the heart and diaphragm might have a cumulative beneficial effect on heart function (Fig. 2B). To study this, we grouped the EF of 10-month-old mdx−/XistΔ mice based on their dystrophin levels in the heart and diaphragm into the following groups; mice with <4% dystrophin in the heart and >4% in diaphragm (heart; median 3.0% stdev = 0.29, diaphragm; median 8.67% stdev = 3.07), >4% dystrophin in the heart and <4% in diaphragm (heart; median 7.64% stdev = 1.13, diaphragm; median 2.63% stdev = 0.88) and >4% dystrophin in the heart and diaphragm (heart; median 6.31% stdev = 3.04, diaphragm; median 15.23% stdev = 9.31). There were no mice with <4% dystrophin in both heart and diaphragm. The EF of both ventricles of mice expressing <4% dystrophin in heart and >4% in diaphragm was as low as age-matched mdx mice. The EF of mice expressing >4% dystrophin in heart and <4% or >4% in diaphragm was higher than in mdx mice (P < 0.05). This indicates that the expression of >4% dystrophin in the heart only improves EF of both ventricles, while expression of >4% (but less than 14%) dystrophin in diaphragm only does not (data for the right ventricle are not shown). We performed the same analysis for the quadriceps, but as for the diaphragm, no effect of dystrophin levels in quadriceps on heart function was found (data not shown).

We also assessed levels of potential serum biomarkers for heart failure; cardiac Troponin I and N-Terminal Pro Brain Natriuretic Peptide (NT-proBNP). Detectable concentrations of cardiac Troponin I were observed for some mice for all models and at all ages, but were slightly more often found in serum of dystrophic mice (Fig. 3A). Cardiac Troponin I levels did not differ between mdx and mdx−/XistΔ mice, nor did they correlate to heart dystrophin levels (R = 0.004 P = 0.986). Levels did correlate to SV and CO of the right ventricle (R = −0.535 P = 0.015 and R = −0.483 P = 0.031 respectively). NT-proBNP levels were undetectable in any of the mouse models at any age.

3.2. Low dystrophin levels partly reduce fibrosis

Transverse cross-sections of the heart were stained with Collagen (Fig. 3B). From Supplementary Figure B for representative examples). No fibrosis was detected in 2-month-old mice, whereas levels were slightly elevated in 6-month-old mdx and mdx−/XistΔ compared to wild type mice (2.4% and 3.4% vs 2%, Fig. 3B). Due to high individual variation only the increment of the mdx−/XistΔ mice reached significance (P < 0.05). Fibrosis was most prominent in 10-month-old mdx mice and was predominantly found in the lateral wall of the left ventricle (Fig. 3C). Its formation was partly prevented in mdx−/XistΔ mice (9.8% vs 5.4%, P < 0.01), although levels were still higher than wild type (P < 0.01). Dystrophin levels in the mdx−/XistΔ hearts did not correlate with fibrosis (R = −0.234 P = 0.240, Fig. 3D). Fibrosis negatively correlated to the EF of the left ventricle (R = −0.512 P = 0.009) (Fig. 3E). This concurs with the above described correlation between the EF and dystrophin levels. Heart mass of the left ventricle did not correlate with fibrosis (R = 0.253 P = 0.222). The number of arterioles did not differ between 10-month-old mdx and wild type mice (mean number of arterioles ±stdev mdx 4.75 ± 3.46 vs C57BL/10ScSnJ 4.25 ± 2.25, XistΔ 4.71 ± 2.63, all groups n = 6).

4. Discussion

Potential therapeutic strategies for DMD are currently tested in clinical trials and encouraging results have been published for dystrophin restoration in skeletal muscles [12,13]. Unfortunately, several therapeutic approaches fail or are less efficient in restoring dystrophin expression in the heart [9–13]. It is unknown whether low dystrophin levels in the heart can preserve function and prevent or delay heart pathology.

We used skewed X-inactivation as a tool to generate mice expressing low dystrophin levels in a mosaic manner in the heart and skeletal muscles. Both C57BL/10ScSnJ and XistΔ (BL/6 background) wild type mice were investigated to eliminate potential strain effects. Heart function of mdx mice progressively declines with age, resulting in dilatation of both ventricles in 10-month-old mice [18,19,30,31]. With age, the mdx heart becomes fibrotic, and genes involved in pathology are upregulated, whereas this remains stable in wild type hearts [32,33]. Interestingly, expression of 3–15% dystrophin in 10-month-old mdx−/XistΔ mice ameliorates the age-dependent loss of heart function and pathology, as heart function parameters, fibrosis and hypertrophy partly normalize towards wild type levels. Although EF was higher in 2 month old mdx compared to wild type mice levels, they fell within the normal range. From the age of 6 months onwards, EF, SV and ESV are already affected in mdx mice, while this is observed later (10 months) and to a lesser extent in mdx−/XistΔ mice. We did not observe a decrease in CO, probably due to an increased heart rate of the mdx mice, which was higher than all models except C57BL/10ScSnJ mice. This is likely due to the higher standard deviation in that group caused by two mice (st.dev. 80 bpm for C57BL/10ScSnJ while 40 bpm for the other models).

These data concur with a study we published recently involving a more severe mouse model also lacking utrophin (mdx/utrn−/−/XistΔ) [34]. Expression of 4% dystrophin in skeletal muscle greatly improved survival to over a year, whereas dystrophin and utrophin double knockout mice normally die before 12 weeks of age. Ejection fraction in 10 month old mdx/utrn−/−/XistΔ mice with median heart dystrophin levels of 4.3% was severely reduced, but partly normalized in mice with median heart dystrophin levels of 29.7%. Here, we observe that EF partly normalizes to wild type levels in 10 month old mdx−/XistΔ mice expressing lower dystrophin levels (<15%), indicating that utrophin-negative mice need higher dystrophin levels to improve cardiomyopathy to a similar extent. This results most likely from the
It was hypothesized that the combined expression of dystrophin in the heart and diaphragm might cumulatively improve heart function 

(M. van Putten et al. / Journal of Molecular and Cellular Cardiology 69 (2014) 17–23)
potentially giving the mdx-XistΔhs mice an early start advantage. It is possible that the development of fibrosis is linked to ischemia, e.g. due to reduced capillary supply in the dystrophic heart. This was not assessed in the current study. However, it is known that dystrophin negative fibers are prone to damage and that the disposition and loss of nNOS are playing big roles in the development of heart failure, so ischemia may contribute to the pathology but is probably not the main driver.

The hearts of mdx mice are hypertrophic from the age of 2 months onwards, a pathological feature which is not observed in mdx-XistΔhs mice. Our finding [assessing the mass of the entire ventricle] concurs with the report of Costas et al. [assessing ventricular wall thickness for a single point [20]], but is in contrast with work from others who observe hypertrophy from 6 months onwards [measuring heart to body weight ratios] [18,35]. The difference in age of onset might be caused by differences in sensitivity of the quantification methods used. Unfortunately, we did not collect data on heart and body weight, so it was not feasible to confirm this.

The applicability of cardiac Troponin I and NT-proBNP as biomarkers for heart failure was also assessed. Cardiac Troponin I has previously been used for detection of cardiac infarcts and was reported to be a useful marker for heart function in DMD and BMD patients [36]. Additionally, cardiac Troponin I levels of idebenone treated mdx mice have been reported to normalize towards wild type levels [37]. Unexpectedly, our serum cardiac Troponin I values did not correspond with those obtained by Buyse et al. However, personal communication revealed that these values were in the pg/ml range instead of the published ng/ml, making their observations fall in the same range. Although heart function of mdx mice is impaired, serum cardiac Troponin I levels barely exceed the detection threshold of the kit. Thus, we feel that the usefulness of cardiac Troponin I as a biomarker for cardiomyopathy in dystrophic mouse models is limited. Based on literature, the applicability of NT-proBNP is questionable as well, as positive and negative correlations with heart function in DMD and BMD patients have been published [4,38]. In our hands, NT-proBNP levels in serum are undetectable even in 10-month-old mdx mice. Therefore we propose that serum NT-proBNP levels are not suitable markers for the assessment of heart failure in mdx mice younger than 10 months.

Our study is partly limited by the fact that scans only entirely cover the left ventricle, while the top part of the right ventricle is not scanned. At initiation of this study, our primary focus was the left ventricle, but with interesting findings published on the right ventricle meanwhile, we also analyzed MRI data available for the right ventricle. All scans were analyzed in the same manner and up to the same height to ensure comparability. Lacking the upper ~25% of the right ventricle resulted in a lower CO for the right compared to the left ventricle. It may also be the reason that we observe a more severe phenotype for the left than the right ventricle, while others have reported the opposite [22,30].

Inherent to the mdx-XistΔhs model, only females of a mixed background were assessed. Gender is known to influence cardiac function in animals and human. Female mdx mice aged 22 months develop more pronounced cardiomyopathy than males [39]. We can only speculate whether similar dystrophin levels will be protective in male mice as well. Since the mdx mouse has a BL/10 background and the XistΔhs a BL/6 background, the resulting females have a mixed background. We therefore used two wild type models as controls, although we cannot rule out that heart function is impacted differently by loss of dystrophin in the two different backgrounds. An alternative strategy would have rule out that heart function is impacted differently by loss of dystrophin as well. Since the mdx mice younger than 10 months.

In summary, we have shown that expression of 4–15% dystrophin not only delays the onset of cardiomyopathy, but also ameliorates its severity in 10-month-old mdx mice. These observations suggest that treatments restoring only low dystrophin levels in the heart of DMD patients may have benefits on heart function.


