Chapter 6

Low dystrophin levels in heart are sufficient to delay heart failure in mdx mice

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Abstract

Aim: Duchenne muscular dystrophy is a muscular dystrophy 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 heart is limited. Improved quality and function of only skeletal muscle potentially accelerates the development of cardiomyopathy. Our study aimed to elucidate which dystrophin levels in heart are required to prevent or delay cardiomyopathy in mice.

Methods and Results: Heart function and pathology assessed with magnetic resonance imaging, histology and gene expression analysis were compared between 2, 6 and 10-month-old *mdx-Xist*$^{\Delta hs}$ mice, expressing low dystrophin levels (3-15%) based on skewed X-inactivation, dystrophin-negative *mdx* mice, and wild type mice of corresponding genetic backgrounds.

Mdx mice developed severe dilated cardiomyopathy and hypertrophy, whereas the onset of heart pathology was delayed and function greatly improved in *mdx-Xist*$^{\Delta hs}$ mice. The ejection fraction, the most severely affected parameter for both ventricles, correlated to dystrophin expression and the percentage of fibrosis. Fibrosis was reduced from 9.8% in *mdx* to 5.4% in *mdx-Xist*$^{\Delta hs}$ mice. Additionally, expression of genes involved in heart pathology normalized towards wild type levels in older *mdx-Xist*$^{\Delta hs}$ mice.

Conclusions: These data suggest that mosaic expression of 3-15% dystrophin in heart is sufficient to delay the onset and ameliorate cardiomyopathy in mice.

Introduction

Duchenne muscular dystrophy (DMD) is the most common inherited neuromuscular disorder. It is characterized by severe and progressive muscle wasting and affects 1 in 3500 newborn boys. In DMD patients, the synthesis of functional dystrophin proteins is prevented by mutations in the *DMD* gene, located on the X-chromosome (Muntoni et al. 2003). Dystrophin is part of the dystrophin-associated protein complex, which links the actin cytoskeleton to the extracellular matrix and stabilizes the sarcolemma during muscle contractions (Blake et al. 2002). In DMD patients, this link is absent, making fibers vulnerable to exercise-induced damage, leading to repeated cycles of de- and regeneration. Upon exhaustion of the regenerative capacity, fibers are replaced by fibrotic and fat tissue, resulting in loss of ambulation in the second decade of life. Pre-clinical cardiac involvement is sometimes already observed in young patients (aged <6 years) and its frequency increases with age. Later in life, it progresses into clinically relevant cardiomyopathy affecting 40% of 10-year-old patients (Nigro et al. 1990). Old patients are dependent on assisted ventilation and at that stage the vast majority suffers from dilated and hypertrophic cardiomyopathy of the left ventricle, while the right ventricle and atrium remain unaffected (Connuck et al. 2008;van Bockel et al. 2009). Eventually, patients die of heart or respiratory failure in their third or fourth decade (Gulati et al. 2005).

Becker muscular dystrophy (BMD) is a milder form of muscular dystrophy, caused by in-frame mutations in the *DMD* gene, which result in reduced expression of dystrophin and/or internally truncated, but partly functional dystrophin. The skeletal muscle pathology is less severe than in DMD patients, and patients usually remain ambulant until later in life. However, in 70% of the BMD patients a more severe cardiomyopathy is observed at time of clinical presentation accounting for 50% of deaths (Connuck et al. 2008;Yilmaz et al. 2008;Kaspar et al. 2009). Especially BMD patients with very mildly affected skeletal muscles can develop severe cardiomyopathy (Melacini et al. 1996). Dilated cardiomyopathy is also found in DMD and BMD carriers, who express 50% of dystrophin in heart. Only 17% of the carriers exhibit some skeletal

muscle weakness, whereas 23% have dilated cardiomyopathy or left-ventricular dilation (Hoogerwaard et al. 1999).

There is no cure for DMD, but several potential therapies aiming at dystrophin restoration are under investigation in clinical trials (Bowles et al. 2012;Malik et al. 2010;Skuk et al. 2007), with antisense-mediated exon skipping being closest to clinical application (Goemans et al. 2011;Cirak et al. 2011). Unfortunately, the efficiency of targeting the heart seems less than for skeletal muscle for the exon skipping approaches currently tested in clinical trials (Alter et al. 2006;Bostick et al. 2009;Townsend et al. 2008;Heemskerk et al. 2010;Heemskerk et al. 2009;Malerba et al. 2011;Wu et al. 2011). Eventually, this might result in partial dystrophin restoration in skeletal muscle accompanied by improved function, but without restoration in the heart. Given that both BMD patients and 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.

The most commonly used mouse model for DMD, the *mdx* mouse, also suffers from dilated cardiomyopathy. In non-treated *mdx* mice, increased activity (e.g. voluntary wheel running), accelerates the progression of dilated cardiomyopathy (Jearawiriyapaisarn et al. 2010;Quinlan et al. 2004;Spurney et al. 2008;Costas et al. 2010). In line with this, skeletal muscle restricted dystrophin restoration improves voluntary activities thereby increasing the workload of the heart and exacerbating heart pathology (Malerba et al. 2011;Townsend et al. 2008). By contrast, it has been shown that high dystrophin levels in skeletal muscles and diaphragm only, improve heart function in *mdx* mice (Crisp et al. 2011).

To study which dystrophin levels are needed to prevent or delay the onset of heart failure, we assessed heart function and pathology over time in *mdx-Xist*$^{\Delta hs}$ mice. These mice originate from crossing *Xist*$^{\Delta hs}$ females (expressing intact dystrophin but carrying a mutation in the promoter of the *Xist* gene which coordinates X-inactivation (Newall et al. 2001)), with dystrophic *mdx* males. In the female offspring (*mdx-Xist*$^{\Delta hs}$), 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 (van Putten et al. 2012a). We show that dystrophin levels of 3-15% in heart are sufficient to delay the onset of dilated cardiomyopathy and reduce the severity of pathology in *mdx* mice.

Material and methods

Animal care

Breeding pairs of *mdx* (C57BL/10ScSn-mdx/J) males and *Xist*$^{\Delta hs}$ females gave birth to *mdx-Xist*$^{\Delta hs}$ females (van Putten et al. 2012a). The *Xist*$^{\Delta hs}$ model (Newall et al. 2001) 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 tail tips 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 conforms with the Directive 2010/63/EU of the European Parliament.

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 anaesthetized by inhalation of 2% isoflurane in a 1:1 mixture of pure oxygen and air. Mice were scanned in a vertical 9.4T magnet with 89 mm bore size, equipped with a 1 T/m gradient system (Bruker

BioSpin, Germany), using a quadrature birdcage coil (inner diameter 3 cm). Image acquisition was done with Bruker Paravision 5.0 software and took ~30 min including animal setup. A retrospectively-gated Intragate sequence was used with flip angle 10°; repetition time 8.5 ms; echo time 1.86 ms; field-of-view 2.56x2.56 cm^2; matrix 192x192; 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. Respiration rate was monitored with a respiratory pad and kept constant at 50-80 respirations per minute. 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 (van der Geest and Reiber 1999). For each picture, endocardial and epicardial contours of both ventricles were drawn manually. The end diastolic and systolic phase was computed by the software and a maximum mass difference of 10% was accepted. When this criteria was met, End Diastolic and Systolic Volume (EDV and ESV, respectively) 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.

Serum biomarker level analysis

For analysis of cardiac Troponin I and N-terminal Pro Brain Natriuretic Peptide levels, blood (~1mL) was drawn from the anaesthetised mouse via the eye after which it was 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.

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 and RNA isolation. Sections were stained with goat-anti-Collagen type 1 (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 DC350FX 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).

Dystrophin determination by western blot

Heart and diaphragm muscles were homogenized and dystrophin levels quantified by western blotting using the Odyssey system as described previously (van Putten et al. 2012a). The lowest concentration in the standard curve was 3%.

Gene expression analysis with qPCR

Total RNA was isolated with TRIzole (Invitrogen, the Netherlands) in MagNA Lyser Green Beads tubes (Roche diagnostics Ltd, UK) and purified with the NucleoSpin RNA II kit (Bioke, the Netherlands). RNA integrity and concentration was determined with a total RNA nano-chip assay on a labchip bioanalyzer (Agilent, the Netherlands). Random hexamer primers were used for cDNA synthesis and gene expression was determined by Sybr Green based Real Time qPCR (95°C 10 sec, 60°C 30 sec, 72°C 20 sec for 45 cycli followed by melting curve analysis) on the Roche Lightcycler 480 (Roche diagnostics Ltd, UK). *Gapdh* was used as a reference gene. The C_p values were obtained with the second derivative maximum method and primer efficiencies and gene expression was determined with LinREgPCR version 11.1 (Ramakers et al. 2003). The expression of C57BL/10ScSnJ mice was set to one.

Statistics

Statistical analyses were performed using SPSS 17.0.2. (SPSS, Inc., Chicago, IL, USA). Heart function was compared over time with the two-way analysis of variance (ANOVA). In case of significance (P<0.05), a LSD correction for multiple testing was applied. Correlations for the 10-month-old *mdx-Xist*$^{\Delta hs}$ mice were tested with the Spearman correlation test. Values of P<0.05 were considered significant. The one-way ANOVA was conducted for comparison of the histological and gene expression data. In case of significance (P<0.05), the Bonferroni post hoc test was performed.

Results

Low dystrophin levels in 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 groups of female *mdx* (no dystrophin), *mdx-Xist*$^{\Delta hs}$ (low dystrophin), C57BL/10ScSnJ (wild type control) and *Xist*$^{\Delta hs}$ (wild type control) mice aged 2, 6 and 10 months. We chose a cross-sectional design to allow post-mortem histological validation. To assess the correlation between dystrophin levels and heart function in individual mice, 27 *mdx-Xist*$^{\Delta hs}$ mice were scanned per time point, while 6 mice were scanned for the control groups. Dystrophin levels in the heart of *mdx-Xist*$^{\Delta hs}$ mice varied between 3-15% 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 (Figure 1A-B). MRI scans were positioned on the left ventricle and the ejection fraction (EF), stroke volume (SV), end diastolic volume (EDV), cardiac output (CO) and end systolic mass of both ventricles were assessed.

Overall, the heart function of the two wild type strains was largely comparable, but differed slightly, probably due to differences in the genetic background. In *mdx* mice, heart function of both ventricles deteriorated with age, most evidently for the EF and SV, while it remained constant in both wild type models (Figure 1C-F, Table 1), as reported previously (Verhaart et al. 2011). Expression of 3-15% dystrophin in heart prevented EF and SV to drop in *mdx-Xist*$^{\Delta hs}$ 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*$^{\Delta hs}$ 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*$^{\Delta hs}$ mice (P<0.05). The EDV and CO of both ventricles 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. In contrast, the expression of <15% dystrophin in *mdx-Xist*$^{\Delta hs}$ mice hearts completely prevented hypertrophy.

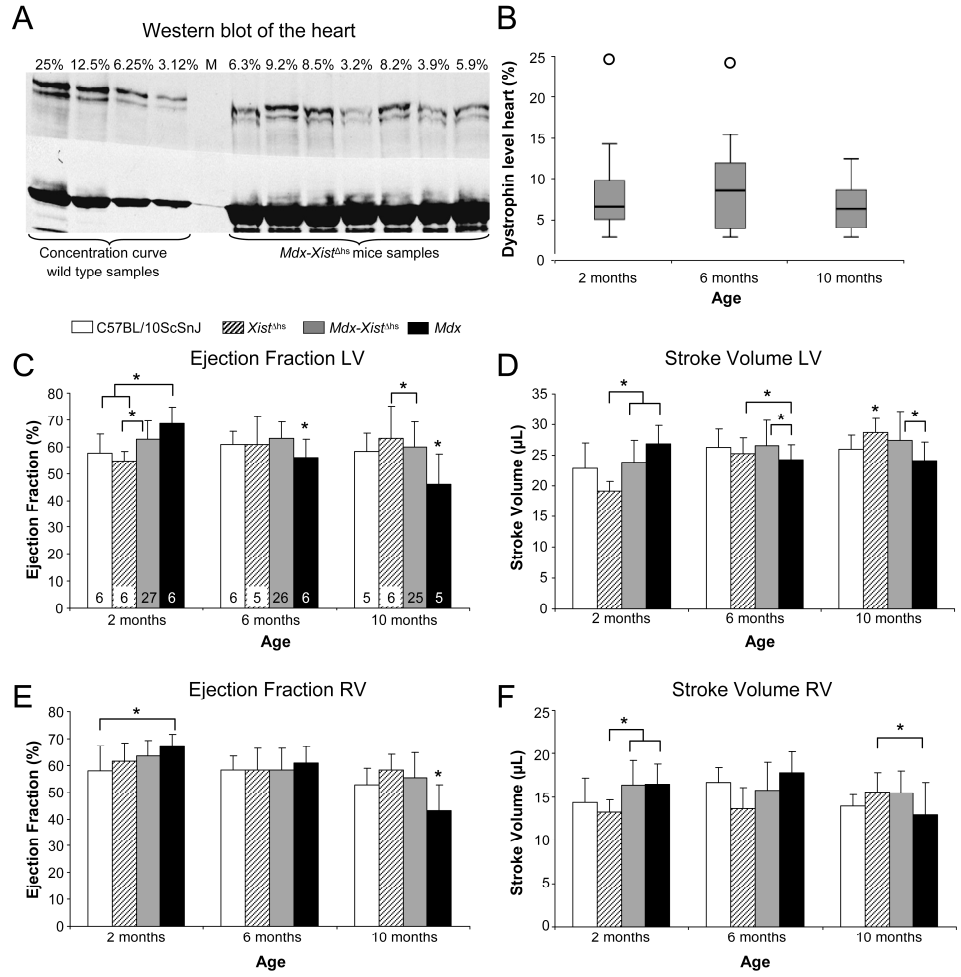

Figure 1. Dystrophin levels and heart function of *mdx-Xist*$^{\Delta hs}$ mice. (A) Dystrophin levels were assessed by western blot for all *mdx-Xist*$^{\Delta hs}$ mice, as shown in the representative blot. Myosin was used as a loading control. M=marker. **(B)** Dystrophin levels are plotted in the box plot. The line dissecting the box resembles the median, outer bars of the box resemble the lower and upper quartile. The bars show maximum and minimum values considered excepting outliers (indicated by ○). Dystrophin levels varied between 3-15%, n = 81 and no increment was observed in older animals. **(C-F)** The EF and SV of both ventricles remained stable in wild type mice, but declined in *mdx* mice, which was prevented in the *mdx-Xist*$^{\Delta hs}$ mice. LV=left ventricle, RV=right ventricle. Bars represent mean values and the standard deviation. Asterisks indicate a difference of P<0.05.

The right ventricular mass did not differ between *mdx* and C57BL/10ScSnJ mice, but was higher than that of *mdx-Xist*$^{\Delta hs}$ and *Xist*$^{\Delta hs}$ mice (P<0.01), which were comparable. This indicates that hypertrophy was restricted to the left ventricle. Heart rate increased in *mdx* mice with age (P<0.001), but remained stable over time for the other models. Heart rate of 2 months old *Xist*$^{\Delta hs}$ mice was higher than that of both *mdx* and *mdx-Xist*$^{\Delta hs}$ mice (P<0.01). In old *mdx* mice, heart rate was higher than that of both *mdx-Xist*$^{\Delta hs}$ and *Xist*$^{\Delta hs}$ mice (P<0.01).

Table 1. Heart function.

Age in months	2	2	2	2		6	6	6	6		10	10	10	10		
Mouse strain	Wt	*Xist*$^{\Delta hs}$	*Mdx-Xist*$^{\Delta hs}$	*Mdx*	Changes at 2 months	Wt	*Xist*$^{\Delta hs}$	*Mdx-Xist*$^{\Delta hs}$	*Mdx*	Changes at 6 months	Wt	*Xist*$^{\Delta hs}$	*Mdx-Xist*$^{\Delta hs}$	*Mdx*	Changes at 10 months	Change over time within strain
Left ventricle																
EF (%)	57.6±7.3	54.6±3.8	62.8±6.9	68.7±6.2	*mdx-Xist*$^{\Delta hs}$ vs *Xist*$^{\Delta hs}$ P<0.05 *mdx* vs wt P<0.05, *Xist*$^{\Delta hs}$ P<0.01	61.0±4.6	60.9±10.4	63.3±6.0	55.9±6.9	*mdx* vs wt P<0.05, *mdx-Xist*$^{\Delta hs}$ and *Xist*$^{\Delta hs}$ P<0.01	58.4±6.9	63.3±11.7	59.8±9.6	46.1±11.4	*mdx-Xist*$^{\Delta hs}$ vs *Xist*$^{\Delta hs}$ P<0.05 *mdx* vs all P<0.01	*mdx* decrease P<0.01 from 6 months onwards
SV (µl)	22.9±4.1	19.1±1.6	23.8±3.7	26.8±3.1	*Xist*$^{\Delta hs}$ vs *mdx-Xist*$^{\Delta hs}$ and *mdx* P<0.01	26.3±3.0	25.2±2.7	26.6±4.1	24.3±2.4	*mdx* vs *Xist*$^{\Delta hs}$ P<0.01, *mdx-Xist*$^{\Delta hs}$ P<0.05	26.0±2.3	28.8±2.3	27.4±4.7	24.2±3.0	*Xist*$^{\Delta hs}$ vs wt and *mdx-Xist*$^{\Delta hs}$ P<0.05, *mdx* P<0.01 *mdx* vs *mdx-Xist*$^{\Delta hs}$ P<0.05	*Xist*$^{\Delta hs}$ and *mdx-Xist*$^{\Delta hs}$ increase P<0.01 from 6 months onwards
EDV (µl)	39.8±5.3	35±2.4	38.1±4.5	39.4±6.6	NS	43.1±3.9	42.3±7.9	42.5±8.2	43.9±4.9	NS	45.1±6.9	47.8±15.7	46.5±8.7	54.4±11.7	NS	All strains increase P<0.01 from 6 months onwards
CO (µl/min)	8229±1801	7877±343	8506±1079	9403±2105	NS	9418±1758	10365±1754	10022±1421	8908±1587	NS	10995±2196	11762±1577	10178±2026	10637±1207	NS	All strains increase P<0.01 from 6 months onwards
ES mass (mg)	63.5±7.6	55.5±4.4	59.5±7.1	69.5±10.0	*mdx* vs all P<0.01	70.3±10.1	80.4±11.5	83.0±10.1	84.2±8.3	*mdx* vs all P<0.01	86.8±12.6	91.1±16.8	93.6±10.3	108±20.9	*mdx* vs all P<0.01	All strains increase P<0.01 from 6 months onwards
Right ventricle																
EF (%)	58.1±9.2	61.5±6.8	63.5±5.9	67.4±4.2	*mdx* vs wt P<0.05	58.2±5.3	58.2±8.1	58.3±7.9	60.9±6.3	NS	52.9±5.9	58.2±5.9	55.4±9.5	43.1±9.8	*mdx* vs all P<0.01	*mdx-Xist*$^{\Delta hs}$ decrease P<0.05 at 6 months and P<0.01 at 10 months *mdx* decrease P<0.01 at 10 months
SV (µl)	14.3±2.9	13.3±1.4	16.5±2.8	16.5±2.3	*Xist*$^{\Delta hs}$ vs *mdx-Xist*$^{\Delta hs}$ and *mdx* P<0.05	16.7±1.7	13.6±2.5	15.8±3.3	17.8±2.4	NS	14.0±1.3	15.6±2.3	15.5±2.5	12.9±3.8	*mdx* vs *Xist*$^{\Delta hs}$ P<0.05	*mdx* decrease P<0.05 at 10 months
EDV (µl)	25.1±6.0	21.9±3.4	26.0±4.7	24.6±4.2	NS	28.8±1.6	23.9±5.4	27.6±7.1	29.7±5.9	NS	26.5±1.7	27.1±5.7	28.9±7.1	30.8±9.4	NS	All strains increase P<0.01 at 6 months
CO (µl/min)	5166±1260	5492±426	5892±1050	5800±1373	NS	6002±1177	5640±1433	5938±1205	6448±919	NS	5859±665	6372±1173	5733±1056	5621±1365	NS	NS
ES mass (mg)	17.9±5.5	13.9±2.4	15.5±2.2	18.9±3.8	wt vs *Xist*$^{\Delta hs}$ P<0.05 *mdx* vs *Xist*$^{\Delta hs}$ and *mdx-Xist*$^{\Delta hs}$ P<0.01	20.3±1.7	19.2±5.4	19.4±4.7	22.8±2.2	wt vs *Xist*$^{\Delta hs}$ P<0.05 *mdx* vs *Xist*$^{\Delta hs}$ and *mdx-Xist*$^{\Delta hs}$ P<0.01	21.7±2.3	19.1±2.6	20.6±2.7	24.1±5.6	wt vs *Xist*$^{\Delta hs}$ P<0.05 *mdx* vs *Xist*$^{\Delta hs}$ and *mdx-Xist*$^{\Delta hs}$ P<0.01	All strains increase P<0.01 from 6 months onwards
Heart rate	362±58	414±18	363±34	352±49	*Xist*$^{\Delta hs}$ vs *mdx* and *mdx-Xist*$^{\Delta hs}$ P<0.01	388±24	410±38	383±39	364±33	NS	424±80	409±39	374±35	442±34	*mdx* vs *Xist*$^{\Delta hs}$ and *mdx-Xist*$^{\Delta hs}$ P<0.01	*mdx* increase with P<0.01 from 10 months onwards

Values are mean ± standard deviation. Wt indicates C57BL/10ScSnJ mice; EDV, end diastolic volume; CO, cardiac output; EF, ejection fraction; SV, stroke volume; ES mass, end systolic mass; NS, non significant; vs, versus.

Dystrophin levels of 10-month-old *mdx-Xist*$^{\Delta hs}$ mice correlated with the EF of both ventricles (left R=0.464 *P*=0.019, right R=0.471 *P*=0.017), which was also the most severely affected parameter in *mdx* mice (Figure 2A-B). As heart function has been described to normalize by dystrophin expression in the diaphragm (Crisp et al. 2011), we also assessed this for 10-month-old *mdx-Xist*$^{\Delta hs}$ mice. Dystrophin levels of the diaphragm varied between 1.5-38% (*mean* = 11.5% *stdev* = 8.8) and did not correlate to dystrophin levels in 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 heart and diaphragm might have a cumulative beneficial effect on heart function (Figure 2C-D). To elucidate this, we grouped the EF of 10-month-old *mdx-Xist*$^{\Delta hs}$ mice based on their dystrophin levels in heart and diaphragm into the following groups; mice with <4% dystrophin in heart and >4% in diaphragm (heart; median 3.0% *stdev* = 0.29, diaphragm; median 8.67% *stdev* = 3.07, *n* = 7), >4% dystrophin in heart and <4% in diaphragm (heart; median 7.64% *stdev* = 1.13, diaphragm; median 2.63% *stdev* = 0.88, *n* = 5) and >4% dystrophin in heart and diaphragm (heart; median 6.31% *stdev* = 3.04, diaphragm; median 15.23% *stdev* = 9.31, *n* = 13) (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 expression of >4% dystrophin in heart only improves EF of both ventricles, while expression of >4% (but less than 14%) dystrophin in diaphragm only does not.

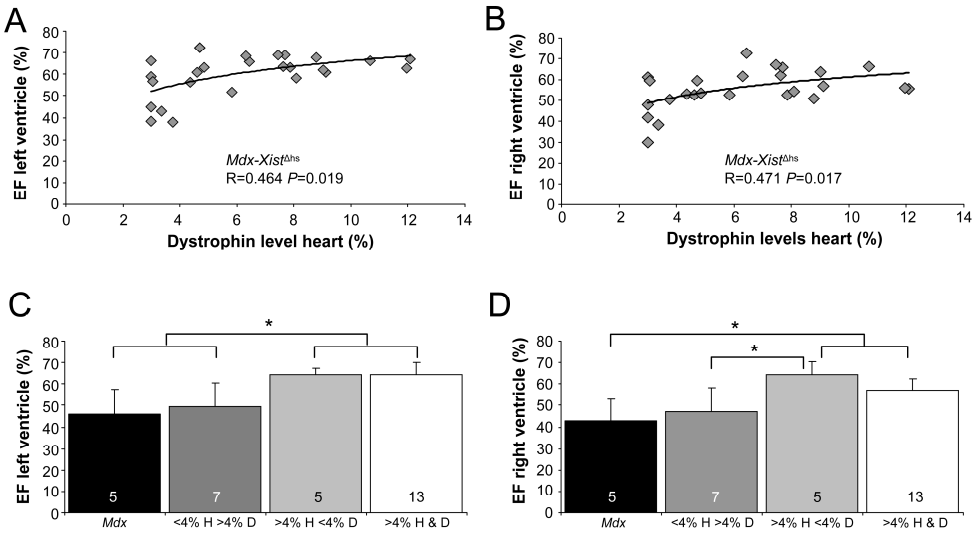

Figure 2. Correlation between dystrophin expression and heart function. (A-B) Dystrophin levels of the heart correlated to EF of both ventricles in 10-month-old *mdx-Xist*$^{\Delta hs}$ mice (left R=0.464 *P*=0.019 and right R=0.471 *P*=0.017). **(C-D)** <4% dystrophin in heart did not restore EF despite dystrophin expression in the diaphragm. EF levels were improved in mice expressing >4% dystrophin in heart regardless of expression levels in the diaphragm. H=heart, D=diaphragm. Bars represent mean values and the standard deviation. Asterisks indicate a difference of *P*<0.05.

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 (Figure 3A). Cardiac Troponin I levels did not differ between *mdx* and *mdx-Xist*$^{\Delta hs}$ mice, nor did they correlate to heart dystrophin levels (R=0.004 *P*=0.986). Levels did correlate to CO and SV 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.

Low dystrophin levels reduce fibrosis

Transverse cross-sections of the heart were stained with Collagen type I, to determine the percentage of fibrotic tissue (see Supplementary Figure 1 for representative examples). No fibrosis was detected in 2-month-old mice, whereas levels were elevated in 6-month-old *mdx* and *mdx-Xist*$^{\Delta hs}$ compared to wild type mice (2.4% and 3.4% vs 2%, Table 2). Due to high individual variation only the increment of the *mdx-Xist*$^{\Delta hs}$ mice reached significance (*P*<0.05). Fibrosis was most prominent in 10-month-old *mdx* mice, while its formation was partly prevented in *mdx-Xist*$^{\Delta hs}$ 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-Xist*$^{\Delta hs}$ hearts did not correlate with fibrosis (R= -0.234 *P*=0.240, Figure 3B). Fibrosis negatively correlated to the EF of both ventricles (left R= -0.512 *P*=0.009, right R= -0.594 *P*=0.002) (Figure 3C-D). This concurs with the above described correlation between the EF of both ventricles and dystrophin levels Heart mass did not correlate with fibrosis (left R= 0.253 *P*= 0.222, right R= 0.285 *P*=0.167).

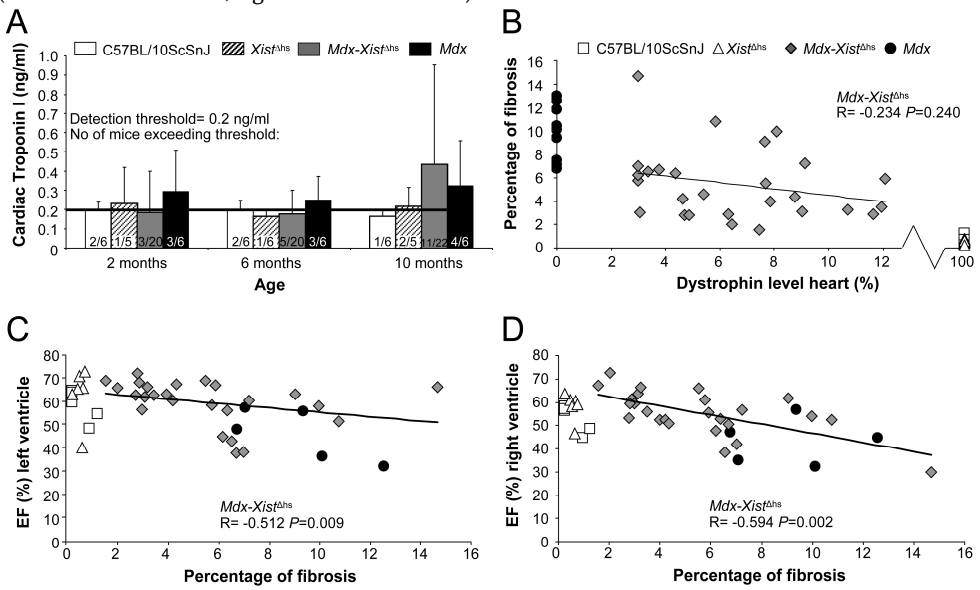

Figure 3. Cardiac Troponin I levels and correlation between fibrosis, dystrophin expression and heart function. Bars represent mean values and the standard deviation. **(A)** Serum cardiac Troponin I levels exceeded the detection threshold of 0.2 ng/µl in some samples from all models. Levels did not differ between models or correlate to dystrophin expression in the heart (R=0.004 *P*=0.986). **(B)** Scatter plot of the percentage of fibrosis versus dystrophin levels of the heart of 10-month-old mice. Although *mdx-Xist*$^{\Delta hs}$ mice had less fibrosis than *mdx* mice, dystrophin levels did not correlated to fibrosis (R= -0.234 *P*=0.240). **(C-D)** Fibrosis levels of 10-month-old *mdx-Xist*$^{\Delta hs}$ mice negatively correlated to EF of both ventricles (left R= -0.512 *P*=0.009, right R= -0.594 *P*=0.002).

Table 2. Fibrosis and gene expression of the heart.

Age in months	2	2	2	2		6	6	6	6		10	10	10	10	
Mouse strain	Wt	*Xist*$^{\Delta hs}$	*Mdx-Xist*$^{\Delta hs}$	*Mdx*	Changes at 2 months	Wt	*Xist*$^{\Delta hs}$	*Mdx-Xist*$^{\Delta hs}$	*Mdx*	Changes at 6 months	Wt	*Xist*$^{\Delta hs}$	*Mdx-Xist*$^{\Delta hs}$	*Mdx*	Changes at 10 months
Histopathology															
Fibrosis	0.57±0.15	0.47±0.25	1.06±1.07	1.34±1.12	NS	0.66±0.31	0.40±0.15	3.45±2.84	2.43±1.04	*mdx-Xist*$^{\Delta hs}$ vs wt $P<0.01$ and *Xist*$^{\Delta hs}$ $P<0.05$	0.54±0.37	0.55±0.18	5.43±3.01	9.84±2.35	*mdx-Xist*$^{\Delta hs}$ vs all $P<0.01$ *mdx* vs all $P<0.01$
Gene expression															
Lgals3	1±0.75	0.17±0.27	0.72±1.35	1.13±1.04	NS	1±0.77	1.02±0.25	4.06±3.93	3.07±3.16	NS	1±0.51	0.87±0.77	2.65±1.85	4.09±2.75	*mdx* vs wt and *Xist*$^{\Delta hs}$ $P<0.01$
Cd68	1±0.22	1.12±0.21	1.38±1.05	1.63±0.97	NS	1±0.36	1.08±0.47	2.33±1.51	1.17±0.29	*mdx-Xist*$^{\Delta hs}$ vs wt $P<0.05$	1±0.29	0.88±0.11	2.00±0.86	1.76±0.71	*mdx-Xist*$^{\Delta hs}$ vs wt and *Xist*$^{\Delta hs}$ $P<0.01$
Ctgf	1±0.46	1.63±0.50	1.89±0.83	1.48±0.65	*mdx-Xist*$^{\Delta hs}$ vs wt $P<0.05$	1±0.48	1.83±0.65	2.13±0.74	1.12±0.43	*mdx-Xist*$^{\Delta hs}$ vs wt and *mdx* $P<0.01$	1±0.36	0.88±0.26	1.20±0.41	1.79±0.72	*mdx* vs wt and *Xist*$^{\Delta hs}$ $P<0.01$, vs *mdx-Xist*$^{\Delta hs}$ $P<0.05$
Nox4	1±0.32	0.62±0.31	0.84±0.30	0.82±0.22	NS	1±0.51	0.85±0.20	1.63±0.88	1.85±0.50	NS	1±0.50	0.58±0.20	2.06±0.86	3.39±1.29	*mdx-Xist*$^{\Delta hs}$ vs wt $P<0.05$ and *Xist*$^{\Delta hs}$ $P<0.01$ *mdx* vs all $P<0.01$
Serca2a	1±0.14	1.21±0.17	1.17±0.37	1.10±0.26	NS	1±0.20	1.01±0.14	0.80±0.18	0.75±0.24	NS	1±0.30	1.47±0.22	0.94±0.33	0.28±0.19	*Xist*$^{\Delta hs}$ vs wt $P<0.05$ and *mdx-Xist*$^{\Delta hs}$ $P<0.01$ *mdx* vs all $P<0.01$
Nppa	1±0.51	0.47±0.31	0.79±0.56	0.93±0.29	NS	1±0.74	0.67±0.25	0.95±0.55	1.70±0.67	*mdx* vs *Xist*$^{\Delta hs}$ and *mdx-Xist*$^{\Delta hs}$ $P<0.05$	1±0.73	0.77±0.24	1.37±0.78	3.99±2.11	*mdx* vs all $P<0.01$

Values are mean ± standard deviation. Wt indicates C57BL/10ScSnJ mice; NS, non significant; vs, versus.

Concurring with histopathological and functional data, expression levels of 2-month-old dystrophic mice were not elevated except for *Ctgf*, which was increased in *mdx-Xist*$^{\Delta hs}$ compared to C57BL/10ScSnJ mice. With age, expression of several genes increased in *mdx* mice, but to a lesser extent in *mdx-Xist*$^{\Delta hs}$ mice. In 6-month-old *mdx* mice, expression of *Nppa* was elevated. Gene expression of 10-month-old *mdx* mice was greatly increased, while levels of *mdx-Xist*$^{\Delta hs}$ mice fell between those of *mdx* and wild type mice for most genes. By contrast, levels of *Serca2a* dropped in *mdx* mice, but were similar for *mdx-Xist*$^{\Delta hs}$ and C57BL/10ScSnJ mice.

Discussion

Potential therapeutic strategies for DMD are currently tested in clinical trials and encouraging results have been published for dystrophin restoration in skeletal muscles (Cirak et al. 2011;Goemans et al. 2011). Unfortunately, the heart seems to be a less efficient target as several therapeutic approaches fail to restore dystrophin expression in this organ or are less efficient in heart than skeletal muscle (Bowles et al. 2012;Cirak et al. 2011;Goemans et al. 2011;Malik et al. 2010;Skuk et al. 2007). Studies in *mdx* mice suggest that functional improvement in skeletal muscles may worsen cardiomyopathy (Malerba et al. 2011;Townsend et al. 2008). It is, however, unknown whether low dystrophin levels can preserve function and prevent or delay heart pathology.

We used skewed X-inactivation as a tool to generate mice with low dystrophin levels in heart and skeletal muscles. Heart function of *mdx* mice progressively declines with age, resulting in severe dilation of both ventricles in 10-month-old mice (Quinlan et al. 2004;Spurney et al. 2008;Verhaart et al. 2011;Zhang et al. 2008). With age, they develop severe fibrosis and upregulate genes involved in pathology and function, whereas this remains stable in wild type mice (Au et al. 2011;Li et al. 2009). Interestingly, expression of 3-15% dystrophin in 10-month-old *mdx-Xist*$^{\Delta hs}$ mice ameliorates the age-dependent loss of heart function and pathology, as heart function parameters, fibrosis and gene expression partly normalize towards wild type levels. Whereas EF and SV are affected already in *mdx* mice aged 6 months, this is observed later (10 months) and to a lesser extent in *mdx-Xist*$^{\Delta hs}$ mice.

It was hypothesized that the combined expression of dystrophin in heart and diaphragm might cumulatively improve heart function, as others observed beneficial effects of dystrophin expression in diaphragm on heart function (Crisp et al. 2011). Expression of >4% dystrophin in diaphragm does not improve heart function (EF, the most affected parameter) in *mdx-Xist*$^{\Delta hs}$ mice, whereas similar levels in heart do. Expression of >4% dystrophin in both heart and diaphragm does not further improve heart function indicating that the cumulative beneficial effect of dystrophin expression in diaphragm on heart function is limited. The discrepancy between our findings and those reported by Crisp et al. might be caused by the low dystrophin levels in diaphragm in this study compared to the high dystrophin levels in their study. This suggests that for a protective effect on heart function probably high dystrophin levels in diaphragm are needed.

Dystrophin levels of the *mdx-Xist*$^{\Delta hs}$ mice do not correlate with fibrosis, whereas, fibrosis correlates to the EF of both ventricles, which is in concordance with literature (Li et al. 2009). It should be stressed that *mdx-Xist*$^{\Delta hs}$ mice also express low dystrophin levels in skeletal muscle leading to improved muscle quality and function, which has been reported to increase the workload for the heart, thereby potentially worsening cardiomyopathy. Whether 3-15% dystrophin in heart is still protective when forced strenuous exercise is applied remains to be elucidated. Hearts of *mdx* mice are hypertrophic from the age of 2 months onwards, a pathological feature which is not observed in any of the *mdx-Xist*$^{\Delta hs}$ mice. Our finding (assessing

the mass of the entire ventricle) concurs with the report of Costas et al. (assessing ventricular wall thickness (Costas et al. 2010)), but is in contrast with work from others who observe hypertrophy from 6 months onwards (measuring heart to body weight ratios) (Bia et al. 1999;Quinlan et al. 2004). The difference in age of onset might be caused by differences in sensitivity of the quantification methods used.

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 (Matsumura et al. 2007). Additionally, cardiac Troponin I levels of idebenone treated *mdx* mice have been reported to normalize towards wild type levels (Buyse et al. 2009). Unexpectedly, serum cardiac Troponin I values observed in our study did not correspond with those obtained by Buyse et al. However, personal communication revealed that their values were in the in pg/ml range instead of the published ng/ml, making their observations fall in the same range as ours. Although heart function of *mdx* mice is severely impaired, serum cardiac Troponin I levels barely exceeds the detection threshold of the kit. Thus, we feel 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 (van Bockel et al. 2009;Schade van Westrum et al. 2006). In our hands, NT-proBNP levels are undetectable even in 10-month-old *mdx* mice. Therefore we propose this marker not suitable 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 analysed MRI data available for the right ventricle. All scans were analysed in the same manner and up to exactly the same height in the right ventricle to ensure comparability. However, the top part of the right ventricle was not scanned and analysed, which 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 (Crisp et al. 2011;Verhaart et al. 2011).

In summary, we have shown that expression of 3-15% dystrophin not only delays the onset of cardiomyopathy, but also largely 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.

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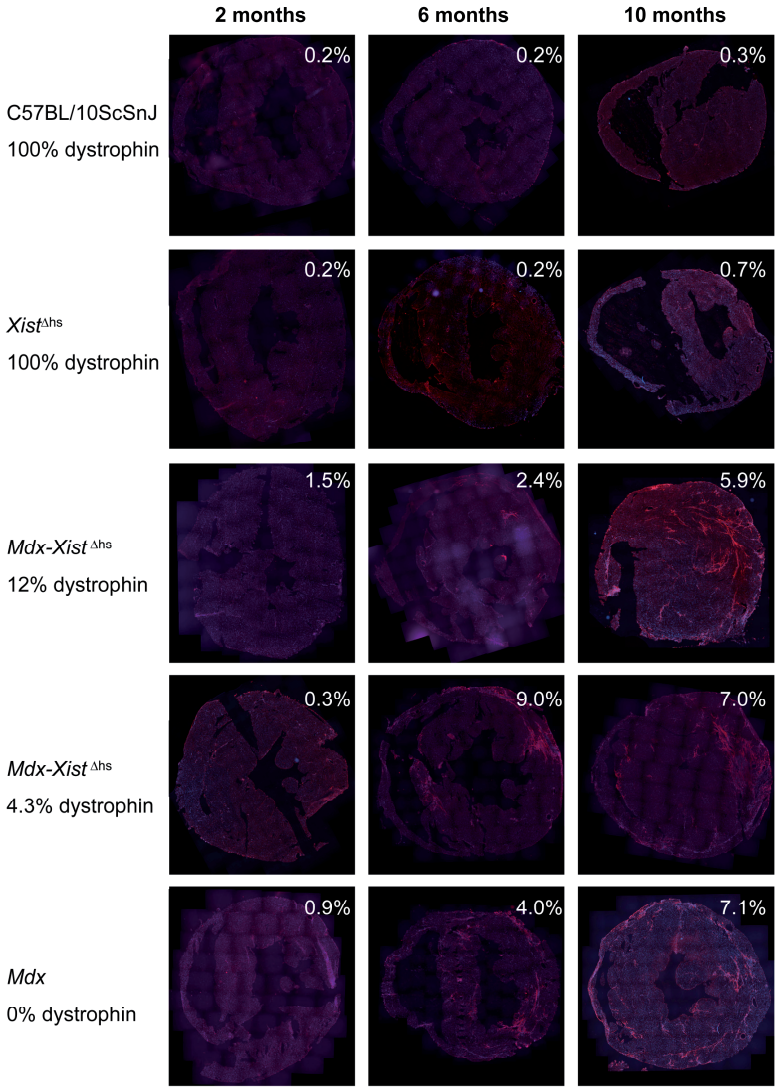
Conflict of Interest

None declared



Supplementary Figure 1. Representative pictures of fibrosis in the heart. Mean dystrophin levels are indicated for each model and the percentage of fibrosis is given for each picture. No fibrosis was evident in any of the models aged 2 months, nor in wild type mice regardless of their age. From 6 months onwards, increased levels of fibrosis were observed in *mdx* and *mdx-Xist*$^{\Delta hs}$ mice. Ten months old *mdx* mice had extensive areas of fibrosis, covering ~10% of the heart, whereas this was partly prevented in *mdx-Xist*$^{\Delta hs}$ mice. The blue spots are stitching artifacts.