

AWARD NUMBER: W81XWH-18-1-0624

TITLE: “Improving Cardiorespiratory Performance in DMD via Combinatorial Dystrophin and Ribonucleotide Reductase Gene Therapy”

PRINCIPAL INVESTIGATOR: Dr. Guy L. Odom

**CONTRACTING ORGANIZATION: University of Washington
Seattle WA 98195**

REPORT DATE: Oct 2019

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Fort Detrick, Maryland 21702-5012**

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14. ABSTRACT Duchenne muscular dystrophy (DMD) is caused by mutations in the gene encoding dystrophin, a structural protein that links the cytoplasmic contractile components of muscle cells to the extracellular matrix. Our dual target research strategy mediated by recombinant adeno-associated viral vectors (rAAV) utilizes dystrophin replacement (structural-based therapy) coupled with contractile performance enhancement via ribonucleotide reductase (RNR) expression (contractile augmentation therapy)). Therefore, we will address the underlying DMD structural deficit (lack of dystrophin), protecting muscle cells from further injury, while simultaneously improving cardiac muscle contractility & relaxation via a promising new nucleotide therapy. We recently published data (see appendix) demonstrating rAAV/RNR treatment improves cardiac performance of deteriorating or aged (22-24 month-old) hearts of <i>dmd</i> mice, and that improved performance was sustained long-term. In fulfillment of Aim-1 for this grant, following vector production/ purification (Oct-Nov, 2018), <i>dmd</i> mice were administered rAAV's (Dec-Jan 2018/19). Echocardiography has been performed on the majority of treated mice with end points forthcoming on the final 2-cohorts of animals over the coming weeks (Nov. 2019). End points for the 4-cohorts of treated mice for Aim-1/Phase-1 are currently in process (Oct-Nov 2019) where we have performed cardiac perfusion assays (baseline performance, pressure-volume relationships, & high-workload performance) in ~60% of the mice at the time of writing this report.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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1. INTRODUCTION:

Subject: Dystrophin provides a structural link between the inside and outside of muscle cells to protect against damage during exercise. Duchenne muscular dystrophy (DMD) patients carrying mutations to the dystrophin gene gradually lose muscle function and ultimately die due to weakened heart and breathing muscles.

Purpose: Two current limitations of DMD cardiac gene therapy include: (1) overcoming the preexisting damage to muscles caused by disease progression that ultimately leads to cardiomyopathy-or worse (heart failure), and (2) the inability to restore lost function of those muscle cells that express a highly truncated dystrophin protein (i.e. micro-dystrophin, currently advancing in clinical trials). These challenges limit the effective response of strategies for sustained protective therapy.

SCOPE: To address these concerns, we propose a two-pronged approach: dystrophin replacement (structural-based therapy) coupled with contractile performance enhancement via ribonucleotide reductase (RNR) expression (contractile augmentation therapy). Cardiac muscle lacks the ability to regenerate, so endowing existing cardiomyocytes that actively contribute to rhythmic contractions with the ability for sustained increases in force production (via elevated RNR and dATP) represents a mechanistic based therapy at the level of the muscle contractile unit (sarcomere) that increases whole heart function. We have demonstrated that the contractile protein myosin can utilize dATP as a preferred energy that results in stronger, faster heart muscle cell contraction as well as faster relaxation to improve whole heart function for both systole and diastole. This RNR strategy may be even more effective when combined with dystrophin replacement strategies to improve the stability of muscle cell structure. Our DOD proposal is clearly focused on bringing successful cardiac gene therapies for DMD into clinical application in the next 3-5 years with the goal of making a significant clinical impact.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Adenosine triphosphate
Deoxyadenosine triphosphate
Becker muscular dystrophy
Dilated cardiomyopathy
Dystrophin Glycoprotein Complex
Duchenne muscular dystrophy
Dystrophin
Echocardiography
Heart failure
Muscle creatine kinase
recombinant Adeno-associated viral vector
Ribonucleotide reductase
Rate Pressure Product
Tissue Doppler Imaging
Micro-dystrophin

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Task 1: We will test the hypothesis that the treatment of dystrophic mice with AAV-mediated RNR can halt adverse cardiac remodeling progression, and increase cardiac contractile performance when combined with AAV-mediated μ Dys. These will be tested in mdx^{4cv} dystrophic mice. (Mos 1-12)

Task 2: Investigate the hypothesis that AAV6-mediated μ Dys and RNR treatments will *rescue* function in a more progressive and severely affected model of DMD (Mos. 13-24). These *in vivo* studies will be tested in $mdx/utrn^{fl/fl}$ dystrophic mice. (Mos 11-24) The *in vitro* studies that are part of RNAi knockdown experiments will commence in year-1 to potentially substantiate *in vivo* efforts of Aim-2 in year-2.

Task 3: These studies will test the hypothesis that AAV-mediated RNR expression can enhance the functional capacity of dystrophin gene editing in an advanced model of DMD. These *in vivo* studies will be tested in *mdx/utrn*^{+/-} dystrophic mice. (Mos 22-36)

What was accomplished under these goals?

Task 1: We will test the hypothesis that the treatment of dystrophic mice with AAV-mediated RNR can halt adverse cardiac remodeling progression, and increase cardiac contractile performance when combined with AAV-mediated μ Dys. These will be tested in *mdx*^{4cv} dystrophic mice (N=6). (Mos 1-12)

- 1g.** For production of rAAV6 vectors, 293T cells in roller bottles are co-transfected with the rAAV transgene vector and the helper plasmid pDG6. Viral vector is purified by cellular lysis, followed by affinity chromatography over a heparin column using an AKTApurifier 10 high performance liquid chromatography machine, and dialyzed against Hanks balanced saline solution. The vector titer is determined by quantitative Southern blot analysis, using plasmid standards (Odom & Chamberlain labs). (Mos. 1-2; 13-14; & 25-26)

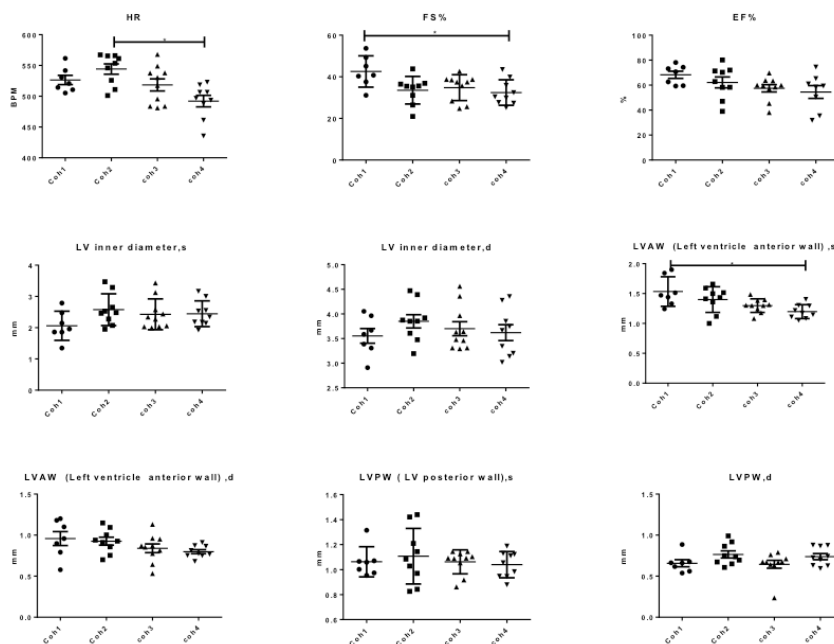
Plasmid production, vector production, vector purification, vector quantification (southern blot & qPCR) has been successfully carried out with resulting titers for rAAV6-delta-CMV-Luc, rAAV6-CK8e-RNR, and rAAV6-CK8e-uDys yielding titers of 8.5×10^{13} , 1×10^{14} , and 2×10^{13} vg/ml respectively.

- 1h.** Systemic injections to mice are administered in a 200 μ l bolus injection via the retro orbital sinus route comprising HBSS containing up to 2×10^{14} vg/kg of rAAV6 vector (Odom lab).

Following baseline functional assessment by echocardiography, wild-type and *mdx*^{4cv} mice were enrolled into respective cohorts 1-4. For aim-1/phase-1 (Langendorff endpoint), mice (N=8) were administered vector on December 11th 2018 (cohort-1), December 18th 2018 (cohort-2), December 27th 2018 (cohort-3), and January 3rd 2019 (cohort-4). For aim-1/phase-2 (Metabolomics endpoint), mice (N=8) were administered vector on January 29th 2018 (cohort-1 & -2), and February 6th 2018 (cohort-3 & -4).

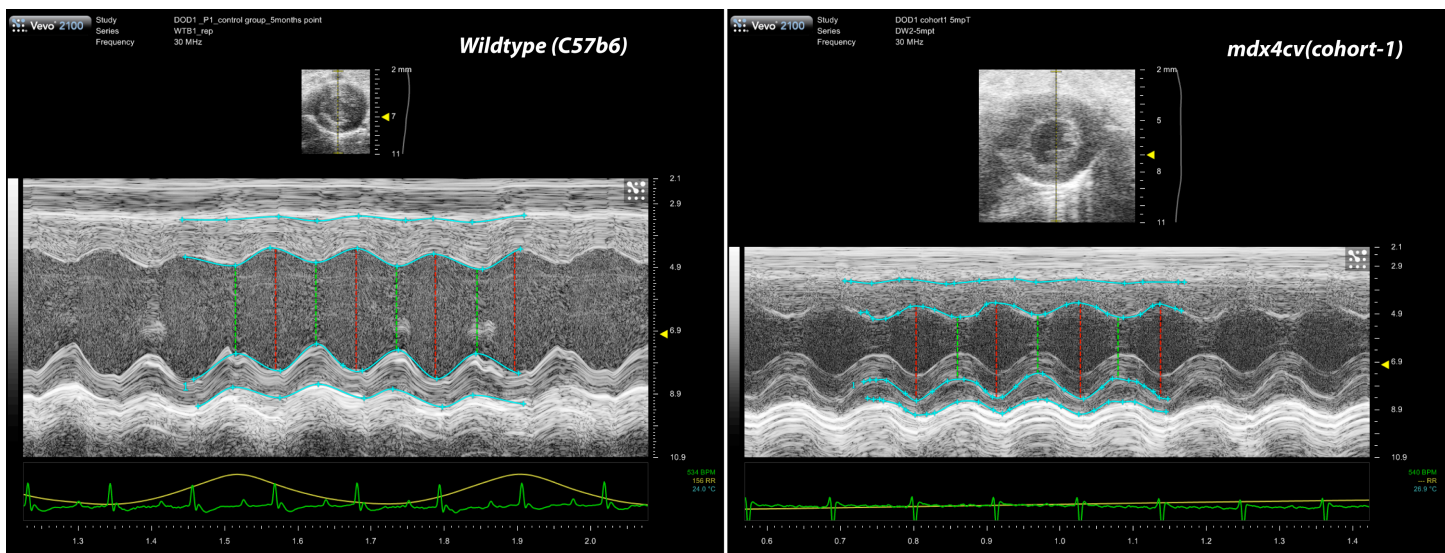
- 1a.** Echocardiography will be performed using a Visualsonics Vivo2100 and standard imaging planes: M-mode, conventional and Tissue Doppler imaging (TDI). Assayed parameters include stroke volume, cardiac output, LV end-systolic (LVESD) and end-diastolic (LVEDD), fractional shortening, LV wall mass MPI (Tei index, the proportion of systole that is isovolumic), and Ea/Aa (early/late diastolic wall-motion velocity ratio). These assays will continue throughout the project as part of the goals of each specific aim (Regnier & Odom Labs). (Mos. 1-36)

DOD 5-mth echo (systolic function)

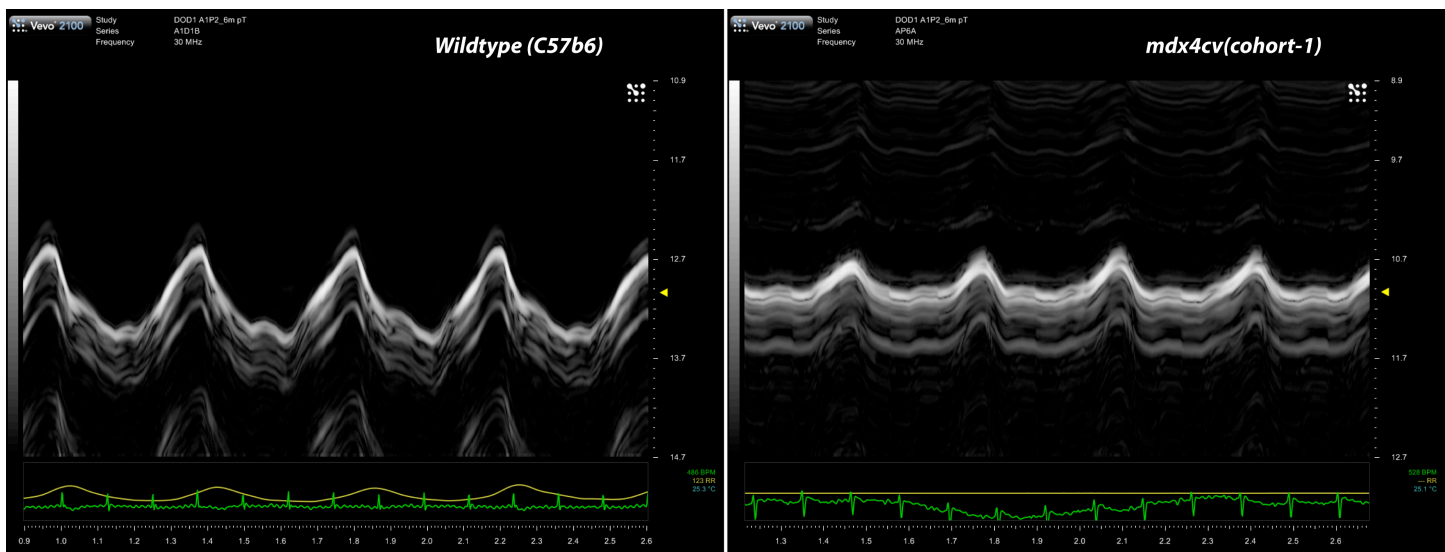


We have performed echocardiography imaging and analysis on mice enrolled in Aim-1/Phase-1 of this project. Compiled at left is the most recent time point completed for the 4-cohorts of mice (coh1 = *mdx*^{4cv}+control vector, coh2 = *mdx*^{4cv}+RNR vector, coh3=*mdx*^{4cv}+uDys vector, coh4=*mdx*^{4cv}+RR vector & uDys vector). Wild-type mice have also been completed (not shown). The research scientist performing the imaging analysis remains blinded with respect to animal enrollment within the individual cohorts until the conclusion of this study. There was no significant difference between cohorts 1-4 contractile properties such as fractional shortening (FS%) and ejection fraction (EF%). Nor was there any significant difference with regard to anatomical geometrys such as left ventricular (LV) inner diameter

during systole (s) or diastole (d), as well as LV anterior wall (aw) or LV posterior wall (pw). Below (next page) are representative M-mode images for wildtype and *mdx*^{4cv}.



We have also performed diaphragm sonography imaging and analysis to a similar extent on mice enrolled in Aim-1/Phase-2 of this project- end-point imaging remains to be collected which will be performed in the month to follow the submission of this report. Below shows a representative image for wildtype and mdx4cv mice, where each “peak” represents a contractile phase of a given respiratory cycle.



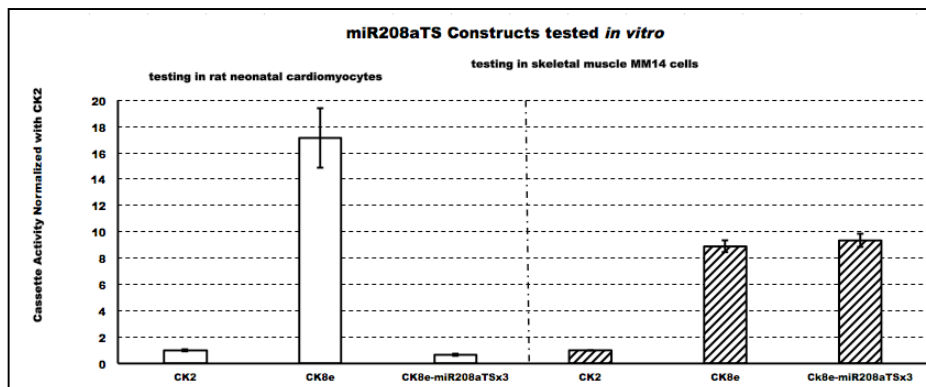
- 1b.** Langendorff perfusion assays will be performed to determine basal function & high workload challenge capacities in treated and control hearts. Hearts are perfused at a constant pressure of 80mmHg with a modified Krebs-Henseleit (KH) buffer supplemented with glucose and pyruvate. Left ventricular (LV) function is monitored during the experiment via a water-filled balloon inserted to the LV and connected to a pressure transducer. LV systolic pressure (LVSP), end diastolic pressure (EDP), heart rate (HR), and minimum and maximum rate of pressure change in the ventricle ($\pm$ dP/dt) are obtained from the attached data acquisition system (PowerLab, ADInstruments, Colorado Springs, CO). Pressure-volume relationships (i.e., Frank-Starling curves) are assessed by gradually increasing the volume of the LV balloon. Following baseline measures, hearts are allowed to equilibrate for 5 minutes and then the perfusate will be changed to the same buffer as above except for the addition of 4.0 mmol/L CaCl_2 to simulate a high workload (HWL) challenge for 20 minutes (Regnier & Odom Labs). (Mos. 9-11; 21-23; 33-35)

We have performed cardiac perfusion assays (Langendorff) on 24 mice enrolled in Aim-1/Phase-1 of this project. The remaining perfusions are currently scheduled to be completed on October 30-31 2019 (7 mice), and November 12-13 (8 mice).

Task 2: Investigate the hypothesis that AAV6-mediated μ Dys and RNR treatments will *rescue* function in a more progressive and severely affected model of DMD (Mos. 13-24). These *in vivo* studies will be tested in *mdx/utrn*^{-/-} dystrophic mice (N=6). (Mos 11-24) The *in vitro* studies that are part of RNAi knockdown experiments will commence in year 1 to fulfill cohort-4 *in vivo* efforts of Aim-2.

In addition to the tasks/responsibilities described for Aim-1, the following will additionally be conducted pertaining to Aim-2:

- **2a.** Current “best” RCs will be modified by addition of candidate enhancer & control element additions, and assayed for increased transcriptional activity via sequential test systems that begin with muscle cell cultures. Quantitative assays utilize Alkaline phosphatase, Luciferase, and Renilla reporter genes. For studies utilizing RNAi knock-down the 3’UTR will be modified with miR target sites as described in the application. (Hauschka and Odom Labs). (Mos. 1-24).



We have performed *in vitro* co-transfection studies utilizing rat neonatal cardiomyocytes demonstrating an ~90-95% “knock-down” of CK8e-driven expression (by reporter gene assays). The reference plasmid (CK2-luciferase) was included as an additional control for exceedingly low levels of activity. The cardiomyocyte knock-down was accomplished by including 3-copies of the miR-208A target or seed sequence into the 3’ UTR of the reporter

cassette. We also demonstrated similar expression levels (based on luciferase reporter gene expression) in mouse differentiated skeletal muscle cultures (MM14 cells). Presently we are moving this design into the AAV system in an effort to evaluate its effectiveness *in vivo*, with vector production scheduled to commence within 4-6 weeks following the submission of this report.

What opportunities for training and professional development has the project provided?

As it relates to the funding of this project, 2 conferences have been attended over the past fiscal year, with abstracts listed below.

1. Kolwicz SC, Hall JK, Murry J, Moussavi-Harami F, Flint G, Chen X, Hauschka SD, Chamberlain JS, Regnier M, and Odom GL. A cardiac function-enhancing gene therapy approach via deoxy-ATP elevation rescues cardiac dysfunction in a DMD cardiomyopathy mouse model. Parent Project for Muscular Dystrophy Annual Conference, Orlando FL, June 27-30, 2019.

2. Robeson-KZ, Moussavi-Harami F, Davis J, Odom GL, Regnier M. New Promoters to Improve the Efficiency of Cardiac Gene Therapies Using 2 Deoxy-ATP. BCVS, July 29-Aug 1, 2019.

Additionally, with the official opening event on October 16th 2019 for The Center for Translational Muscle Research at UW Medicine (Award Number P30AR074990, Dr. Michael Regnier-Director); where research work was presented by Dr. Guy Odom (entitled below). Briefly, the center provides a unifying organization for muscle research in the heart of Seattle’s biomedical research community. The Center will facilitate new collaborations, recruit new investigators, and offer access to state of the art approaches to accelerate translational muscle research efforts happening at UW Medicine.

Gene Therapy Rescues Cardiac Dysfunction in Duchenne Muscular Dystrophy Mice by Elevating Cardiomyocyte Deoxy-Adenosine Triphosphate.

How were the results disseminated to communities of interest?

Work currently *in press* is now available on the Journal of the American College of Cardiology: Basic to Translational Science website, scheduled to be published in the November issue.

<http://basictranslational.onlinejacc.org/content/early/2019/09/25/j.jacbts.2019.06.006>

Additionally, there are ongoing plans to disseminate these research efforts within the upcoming University of Washington, Institute of Stem Cell & Regenerative Medicine (ISCRM) newsletter (November-December, 2019).

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period we will be concluding the efforts of Aim-2 within this DMDRP-IIRA. Vector production will be initiated within a month to follow the submission of this report. Currently, our goal is to administer vector to mice in December, 2019. The animals will be followed to endpoint, which is anticipated to occur in the summer of 2020.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change.

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

The laboratory of the PI (as well as Co-Investigators Hauschka & Bengtsson) was physically relocated to the University of Washington, South Lake Union (SLU) campus during the month of February 2019. This provided for a closer proximity to our collaborator, Dr. Michael Regnier, whose lab resides in the same building one floor below. This move also had the advantages of being more integrated with members of the UW ISCRM and the Center for Cardiovascular Biology many of which are centered on the SLU campus.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

Kolwicz SC, Flint GV, Hall JK, Moussavi-Harami F, Chen X, Hauschka SD, Regnier M, and **Odom GL**. Gene Therapy Rescues Cardiac Dysfunction in Duchenne Muscular Dystrophy Mice by Elevating Cardiomyocyte Deoxy-Adenosine Triphosphate. JACC Basic to Translation Research, August 2019 (*in press*)

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers and presentations.

Nothing to Report.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

US Provisional Patent filed-June 25th 2019.

Title: Use of ribonucleotide reductase alone or in combination with micro-dystrophin to treat Duchenne muscular dystrophy striated muscle disease.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Guy L. Odom
Project Role: PI
No Change

Name: Michael Regnier
Project Role: Collaborator
No Change

Name: Stephen D. Hauschka
Project Role: Co-Investigator
No Change

Name: Niclas Bengtsson
Project Role: Co-Investigator
No Change

Name: Galina Flint
Project Role: Research Scientist
No Change

Name: Quynh Nyugen
Project Role: Research Scientist
No Change

Name: Xiolan Chen
Project Role: Research Scientist
No Change

Name: James Moore
Project Role: Research Scientist
No Change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Guy L. Odom (PI)- Nothing to Report.

Niclas Bengtsson (Co-I)- Nothing to Report.

Stephen D. Hauschka (Co-I)- Nothing to Report.

Michael Regnier (Collaborator)- see below.

COMPLETED

1 R56 AG 055594-01 (M. Regnier, PI)

Title: Myocardial Infarct in Aging Animals and dATP Therapy

Time commitment: 1.8 calendar months (15% effort)

Supporting agency: NIH

Funding agency contact:

Program Official: Dr. Candace L. Kerr

National Institute on Aging

301-496-6402

candace.kerr@nih.gov

Performance period: 9/30/17-8/31/18

Level of funding: \$387,863 (total direct costs)

Goal: The goal of this project is two-fold 1) to determine how age compounds the effect of myocardial infarct (MI) on heart function and how this affects skeletal muscle function and exercise tolerance; and 2) to determine the ability of 2 deoxy-ATP (dATP) to affect heart and skeletal muscle performance, metabolism and exercise tolerance in age and MI induced heart failure.

Specific aims:

Aim 1. To determine how aging influences the effect of MI on the contractile function of cardiac and skeletal muscle and exercise capacity.

Aim 2. To determine how RNR over-expression and elevated dATP can restore contractile function of cardiac and skeletal muscle, and exercise capacity of aging rodents both before and after MI. We have previously studied the effect of cardiac-specific elevation of dATP on young post-MI heart and cardiac muscle performance.

Aim 3. To determine how RNR over-expression and elevated dATP affect the metabolism of cardiac and skeletal muscle from aging and infarcted rodents.

Overlap: There is no scientific or budgetary overlap.

4 R01 HD 048895-11 (M. Bamshad, PI)

Title: Genetic and Molecular Basis of Congenital Contractures

Role: Co-Investigator

Time commitment: 0.96 calendar months (8% effort)

Supporting agency: NIH

Funding agency contact:

Program Official: Dr. Reiko Toyama

National Institute of Child Health & Human Development

301-435-2723

toyamar@mail.nih.gov

Performance period: 7/1/12-6/30/18

Level of funding: \$341,901 (NCE)

Goal: The goal of this project is to study myosin and thin filament regulatory proteins associated with human distal arthrogryposis, and the dysfunction of skeletal muscle contraction. Feasibility of novel gene therapies will also be studied.

Specific aims:

Aim 1: Discover genes for both well-known and novel forms of Distal Arthrogryposis.

Aim 2: Determine the mechanisms of contractile apparatus dysfunction caused by DA-associated mutations of myofilament proteins.

Overlap: There is no scientific or budgetary overlap.

NSF CBET-1509106 (N. Sniadecki, PI)

Title: Development of a Micropost Approach for the Contractile Maturation of iPS-Derived Cardiomyocytes

Role: Co-Investigator

Time commitment: 0.18 calendar months (1.5% effort)

Supporting agency: NSF

Funding agency contact:

Program Manager: Michele Grimm

CBET Div Of Chem, Bioeng, Env, & Transp Sys

ENG Directorate for Engineering

703-292-4641

mgrimm@nsf.gov

Performance period: 6/1/15-5/31/19

Level of funding: \$454,800

Goal: The goal of this project is to develop technologies that enhance the maturation of patient derived iPS muscle cells *in vitro* with the capability to longitudinally assess mechanical performance.

NEW

NIH P30 AR074990-01 (Michael Regnier, Daniel Raftery, Thomas Daniel, Multi-PI)

Title: UW Center for Translational Muscle Research

Time commitment: 1.56 calendar months (13% effort)

Supporting agency: NIH/NIAMS

Grants Officer: Teresa Do, Grants Management Specialist, NIH/NIAMS, 6701 Democracy Blvd., Democracy I, Suite 800, Bethesda, MD 20892-4872, 301-594-3512, Teresa.Do@nih.gov.

Performance period: 04/05/19-02/29/24

Level of funding: \$500,000 (Year 1 direct costs)

Goals: The goal of this center is to provide a unifying resource and state of the art approaches to enhance skeletal muscle research at the UW and to recruit new investigators into the field. The proposed Center will offer tools, facilities and expertise in a combination available only at the UW to facilitate novel insights to muscle pathologies and move new therapeutics towards the clinic and the marketplace.

Specific aims:

The UW CTMR will:

- (1) Provide leadership and training that promotes novel interdisciplinary research and fosters new collaborations in translational muscle research locally and at other US and international institutions.
- (2) Provide services and scientific resources to accelerate progress on existing projects and facilitate development of novel high risk, high reward projects that can lead to significant advances and follow-on funding.
- (3) Become a centralizing agent that coordinates research efforts, provides multi-project data analysis and mining, and multi-scale mechanistic interpretations with predictive power.
- (4) Develop state of the art and novel approaches that drive muscle disease and treatment research forward.
- (5) Provide outreach activities to A) recruit new scientists and clinician investigators into muscle research and B) disseminate important research findings to the scientific community and the public.

Overlap: There is no scientific or budgetary overlap.

NIH RM1 (James Spudich, Daniel Bernstein, Multi-PI)

Role: University of Washington sub-award PI

Title: A Multiscale hiPSC Platform to Solve the Genotype-Phenotype Conundrum in Inherited Disease

Time commitment: 2.88 calendar months (24% effort)

Supporting agency: NIH/NIGMS

Grants Officer: Jessica Dean, Grants Management Specialist, NIH/NIGMS, Room 2AN32F, 45 Center Drive MSC 6200, Bethesda, MD, 20892-6200, 301-451-2447, deanje@mail.nih.gov.

Performance period: 04/01/19-03/31/24

Level of funding: \$371,066 (Year 1 direct costs to Regnier)

Goals: The overarching goal of our interdisciplinary team is to understand how changes in intrinsic force generation and the mechanical environment affect the structure and function of striated muscle.

Specific aims:

- (1) Determine how structural changes in myosin affect the chemo-mechanical properties of the myosin-actin interaction for individual and small assemblies of motor proteins.
- (2) Determine how changes in myosin kinetics and force production influence the growth, maturation, and function of single hiPSC-CMs and multicellular tissue constructs.
- (3) Determine how changes in myosin kinetics and force production influence the growth, maturation, and function of engineered hiPSC skeletal myotubes/myocytes.

UW will perform mechanical assessments of hiPSC-CMs (Aim 2) and hiPSC-SMs (Aim 3) containing myosin mutations that will be provided by the Stanford group. We will also perform molecular dynamics simulations

of myosin and model sarcomere function for these mutations. Measurements will be made for single cells and individual myofibrils isolated from cells. We will identify magnitude and kinetic parameters that correlate with measures of myosin motors mechanics and actin motility (Aim 1).

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not Applicable

QUAD CHARTS:

Not Applicable

- 9. APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

See below.

USE OF RIBONUCLEOTIDE REDUCTASE ALONE OR IN COMBINATION WITH MICRO-DYSTROPHIN TO TREAT DUCHENNE MUSCULAR DYSTROPHY STRIATED MUSCLE DISEASE

Inventors:

Guy L. Odom
Michael Regnier
Jeffrey S. Chamberlain
Stephen D. Hauschka

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant No. W81XWH-18-1-0624, awarded by the Department of Defense, and Grant Nos. R01 HL128368, R56 AG055594, and RO1 HL122332 awarded by the National Institutes of Health. The government has certain rights in the invention.

USE OF RIBONUCLEOTIDE REDUCTASE ALONE OR IN COMBINATION WITH MICRO-DYSTROPHIN TO TREAT DUCHENNE MUSCULAR DYSTROPHY STRIATED MUSCLE DISEASE

BACKGROUND OF THE INVENTION

[1] Duchenne muscular dystrophy (DMD) is caused by mutations in the gene encoding dystrophin, a protein that links the cytoplasmic contractile components of muscle cells to the extracellular matrix. The clinical manifestations of disease progression include severe peripheral muscle weakness, respiratory insufficiency, and cardiomyopathy that advances to heart failure in many patients.

[2] A need exists to rescue muscle function in subjects with muscular dystrophy, such as DMD.

SUMMARY OF THE INVENTION

[3] The present disclosure relates to a cardiac function-enhancing gene therapy approach that targets myosin in contractile filaments by overexpressing the enzyme ribonucleotide reductase (RNR). RNR converts ADP to deoxy-ADP (dADP), which is rapidly converted to dATP in cells. In humans, RNRs may be encoded by the RRM1 and RRM2 genes. Duchenne muscular dystrophy (DMD) is caused by mutations in the gene encoding dystrophin, a protein that links the cytoplasmic contractile components of muscle cells to the extracellular matrix. When dystrophin is absent or aberrant, the compromised linkage function may cause membrane damage during muscle contraction, which may lead to progressive structural and functional deterioration in cardiomyocytes and skeletal muscle cells. The clinical manifestations of disease progression may include severe peripheral muscle weakness, respiratory insufficiency, and cardiomyopathy that may advance to heart failure in patients. Gene replacement approaches for DMD in animal models and patients can partially ameliorate muscle functional deficits, though given the progressive nature of the disease, it is unclear whether these approaches can adequately address the associated cardiomyopathy.

[4] In the present study, the relative cardiac responses in an advanced-age DMD cardiomyopathy mouse model following intravenously administered recombinant adeno-associated viral (rAAV) vectors carrying muscle-specific micro-dystrophin (μ Dys) or ribonucleotide reductase (RNR) were compared. The results from this study demonstrate that both μ Dys and RNR treatments of DMD hearts may rescue baseline cardiac dysfunction and high workload contractile performance in isolated heart preparations. Although systolic function may be significantly improved by expression of μ Dys, both systolic and diastolic function may be greatly improved with cardiac-specific RNR therapy. This suggests that RNR may be a beneficial contractile augmentation therapy for DMD.

[5] The present disclosure relates generally to methods of treating a subject having muscular dystrophy or DMD. The present disclosure also relates generally to methods of prophylactically treating a subject at risk of developing muscular dystrophy or DMD.

[6] In certain embodiments, the methods may include administering a pharmaceutical composition including an RRM1 gene, an RRM2 gene, and a delivery vehicle to a subject. In specific embodiments, the methods may include administering a pharmaceutical composition including an RRM1 gene and an RRM2 gene coupled to a regulatory cassette to a subject. In further embodiments, the

methods may include administering a pharmaceutical composition including an RRM 1 gene, an RRM2 gene, a regulatory cassette, and a delivery vehicle to a subject.

[7] In specific embodiments, the methods may include administering a first pharmaceutical composition including an RRM1 gene in a first delivery vehicle and a second pharmaceutical composition including an RRM2 gene a second delivery vehicle, such that the first delivery vehicle and the second delivery vehicle are not the same vehicle. In additional embodiments, the methods may include administering a first pharmaceutical composition including an RRM1 gene coupled to a first regulatory cassette in a first delivery vehicle and a second pharmaceutical composition including an RRM2 gene coupled to a second regulatory cassette in a second delivery vehicle, such that the first delivery vehicle and the second delivery vehicle are not the same vehicle.

[8] In yet another embodiment, the methods may include administering a pharmaceutical composition including (i) an RRM1 gene and/or an RRM2 gene, operably coupled to a first regulatory cassette; (ii) a micro-dystrophin gene encoding a protein, operably coupled to a second regulatory cassette; and (iii) one or more delivery vehicles. In alternative embodiments, the methods may include administering a pharmaceutical composition including (i) an RRM 1 gene, operably coupled to a first regulatory cassette; (ii) an RRM2 gene, operably coupled to a second regulatory cassette; (iii) a micro-dystrophin gene encoding a protein, operably coupled to a third regulatory cassette; and (iv) one or more delivery vehicles.

[9] In certain embodiments, the methods may include administering (i) a first pharmaceutical composition including an RRM1 gene, an RRM2 gene, a first regulatory cassette, and a first delivery vehicle, and (ii) a second pharmaceutical composition including a micro-dystrophin gene, a second regulatory cassette, and a second delivery vehicle, such that the first delivery vehicle and the second delivery vehicle are separate delivery vehicles. In another embodiment, the methods may include administering (i) a first pharmaceutical composition including an RRM1 gene, a first regulatory cassette, and a first delivery vehicle, (ii) a second pharmaceutical composition including an RRM2 gene, a second regulatory cassette, and a second delivery vehicle; and (iii) a third pharmaceutical composition including a micro-dystrophin gene, a third regulatory cassette, and a third delivery vehicle, such that the first delivery vehicle, the second delivery vehicle, and the third delivery vehicles are separate delivery vehicles.

[10] The regulatory cassettes may be selected from at least one of a CK8 promoter, a cardiac troponin T (cTnT) promoter, and/or another suitable regulatory cassette.

[11] In some embodiments, the methods may include administering (i) a first pharmaceutical composition including an RRM1 gene, an RRM2 gene, a cTnT promoter, and a first delivery vehicle, and (ii) a second pharmaceutical composition including a micro-dystrophin gene, a CK8 promoter, and a second delivery vehicle.

[12] In certain embodiment, the methods may include administering (i) a first pharmaceutical composition including an RRM1 gene, a cTnT promoter, and a first delivery vehicle, (ii) a second pharmaceutical composition including an RRM2 gene, a cTnT promoter, and a second delivery vehicle; and (iii) a third pharmaceutical composition including a micro-dystrophin gene, a CK8 promoter, and a third delivery vehicle.

[13] DNA may be introduced into a subject's cells in several ways. There are transfection methods, including chemical methods such as calcium phosphate precipitation and liposome-mediated transfection, and physical methods such as electroporation. There are also methods that use recombinant viruses. Current viral-mediated gene delivery methods include, but are not limited to, retrovirus, adenovirus, herpes virus, pox virus, and adeno-associated virus (AAV) vectors.

[14] In certain embodiments, the delivery vehicle includes an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus vector (rAAV).

[15] In further embodiments, the pharmaceutical compositions may be configured to reduce a pathological effect or symptom of a muscular dystrophy. The muscular dystrophy may be selected from at least one of myotonic muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, congenital

muscular dystrophy, oculopharyngeal muscular dystrophy, distal muscular dystrophy, Emery-Dreifuss muscular dystrophy, and/or another suitable muscular dystrophy.

BRIEF DESCRIPTION OF THE DRAWINGS

[16] Other features and advantages of the disclosure will become more fully apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

[17] Figure 1: **(A)** Graphic representation of rAAV6 vectors utilized in the present disclosure. The ribonucleotide reductase vector contains the human cDNA for the RRM1 and RRM2 subunits whose expression is driven by the cardiac specific (cTnT455) regulatory cassette (RC). The human micro-dystrophin (Δ R2-R15/ Δ R18-R22/ Δ ACT) vector has expression driven by the CK8 muscle specific RC. The control vector utilized in the present study carries the firefly luciferase transgene whose CMV early promoter/enhancer RC has been deleted. **(B)** Outline of animal enrollment, vector administration, and experimental protocols implemented following a treatment period of 5 months.

[18] Figure 2: Kaplan Meier analysis was performed on mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). All mice were followed for 20 weeks post injection.

[19] Figure 3: Hearts were isolated from mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). Age-matched, non-diseased, non-treated wild-type mice were used as controls (WT, n=8). All hearts were perfused with a glucose-pyruvate buffer. Functional assessment was performed at spontaneous heart rates. **(A)** Left ventricular developed pressure (LVDevP, the difference between systolic and diastolic pressures). **(B)** Rate pressure product (RPP, the product of LVDevP and HR). **(C)** Negative rate of pressure change calculated by the first derivative of the descending LV pressure wave (-dP/dt), used as an index of ventricular relaxation. **(D)** Positive rate of pressure change calculated by the first derivative of the ascending LV pressure wave (+dP/dt), used as an index of ventricular contractility. * P<0.05 mdx^{4cv} vs. WT; # P<0.05 mdx^{4cv} +RNR vs. mdx^{4cv} .

[20] Figure 4: Hearts were isolated from mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). Age-matched, non-diseased, non-treated wild-type mice were used as controls (WT, n=8). The pressure-volume relationship (*i.e.*, Frank-Starling mechanism) was evaluated by gradually increasing the volume of the LV balloon. Hearts were paced at 450 bpm throughout the protocol. **(A)** Left ventricular systolic pressure (LVSP). **(B)** LV end-diastolic pressure (LVEDP). **(C)** Left ventricular developed pressure (LVDevP, the difference between systolic and diastolic pressures). **(D)** Negative rate of pressure change calculated by the first derivative of the descending LV pressure wave (- dP/dt), and is used as an index of ventricular relaxation. **(E)** Positive rate of pressure change was calculated by the first derivative of the ascending LV pressure wave (+dP/dt), and is used as an index of ventricular contractility. * P<0.05 mdx^{4cv} vs. WT; # P<0.05 mdx^{4cv} +RNR vs. mdx^{4cv} ; \$ P<0.05 mdx^{4cv} + μ Dys vs. mdx^{4cv} .

[21] Figure 5: Hearts were isolated from mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). Age-matched, non-diseased, non-treated wild-type mice were used as controls (WT, n=4). All hearts were perfused with a glucose-pyruvate buffer containing high calcium (4.0 mmol/L) to simulate a high workload challenge for 20 min. Hearts were paced at 450 pm throughout the protocol. **(A)** Left ventricular developed pressure

(LVDevP, the difference between systolic and diastolic pressures). **(B)** Rate pressure product (RPP, the product of LVDevP and HR). **(C)** Positive rate of pressure change calculated by the first derivative of the ascending LV pressure wave (+dP/dt), is used as an index of ventricular contractility. **(D)** Negative rate of pressure change calculated by the first derivative of the descending LV pressure wave (-dP/dt), is used as an index of ventricular relaxation. * $P < 0.05$ mdx^{4cv} vs. WT; # $P < 0.05$ $mdx^{4cv} + RNR$ vs. mdx^{4cv} ; \$ $P < 0.05$ $mdx^{4cv} + \mu Dys$ vs. mdx^{4cv} .

[22] Figure 6: At 5-months following vector administration, cryosections were prepared and immunostained with antisera against dystrophin or ribonucleotide reductase. A considerable level of protein is detected for each ribonucleotide reductase subunit-1 (human specific) as indicated by immunofluorescent staining (Red) localized primarily within the cytoplasm of cardiomyocytes with occasional perinuclear accumulation (upper panel). Noteworthy on the lower panel, is the robust level of expression for dystrophin in WT and in aged mdx^{4cv} mice treated with AAV6-CK8-micro-dystrophin (laminin staining, inset image).

[23] Figure 7: Representative full-view photomicrographs of Masson trichrome staining of the hearts from mdx^{4cv} mice displaying control vector (ΔCMV), and rAAV6-treated with either RNR or μDys from mdx^{4cv} mice. Similarly, 20X enlarged view of the corresponding images (*) is shown.

[24] Figure 8: **(A)** Body weights and **(B)** heart weights were obtained from mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase ($mdx^{4cv} + RNR$, n=6), or micro-dystrophin ($mdx^{4cv} + \mu Dys$, n=5). **(C)** Heart weights (HW) were normalized to body weights (BW) to obtain HW/BW ratio. No statistical differences were noted among the variables.

[25] Figure 9: **(A)** RNR and μDys protein expression detection as revealed by immunoblotting of cardiac whole tissue lysates using either RRM1, RRM2 or anti-dystrophin antibody. **(B)** HPLC-MS/MS intracellular [dNTP] quantification from methanol extracted cardiac tissue. **(C)** qPCR analysis of vector genomes from cardiac tissue revealed similar vector genomes being represented for all treated cohorts.

[26] Figure 10: Comparison of specific force generation per body weight by wild type control mice (WT), mdx^{4cv} mice treated with control vector (MDX), mdx^{4cv} mice treated with micro-dystrophin (MDX+ μDys), and mdx^{4cv} mice treated with micro-dystrophin and RNR (MDX+ μDys +RNR).

DETAILED DESCRIPTION

INTRODUCTION

[27] Duchenne Muscular Dystrophy (DMD) and its milder and allelic form, Becker muscular dystrophy (BMD), are the most frequent muscular dystrophies, occurring once in ~5000 male births, and are due to mutations in the dystrophin gene (1). DMD patients typically die due to cardiac and respiratory muscle failure; thus, maintenance of adequate function in both cardiac and skeletal muscle is important for optimal DMD therapy. The primary function of dystrophin is to provide a structural role by mechanically linking the subsarcolemmal cytoskeleton to the extracellular matrix (ECM) through the dystrophin-glycoprotein complex (DGC) (2). This linkage transmits the forces of contraction to the extracellular matrix (ECM) and protects muscles from contraction-induced injury (3-7). In addition to a structural or mechanical role, the DGC also serves as a scaffold for cytoplasmic and membrane-associated

signaling proteins and ion channels (8-11). The complete absence of dystrophin results in drastic reductions of all DGC components (12-14). Together, an absence of dystrophin and reduction in the DGC components causes membrane destabilization and permeability defects that lead to myofiber degeneration, repeated cycles of degeneration/regeneration, and the gradual replacement of muscle fibers with fibrotic, connective, and adipose tissue.

[28] In contrast, some in-frame deletions, truncations, and missense mutations lead to reduced dystrophin expression associated with milder phenotypes. These pathologies are largely curtailed in mouse (*mdx*) and canine (*cxmd*) models of DMD following the vector mediated delivery of muscle-specific expression of highly functional miniaturized versions of dystrophin, micro-dystrophin (μ Dys) (15-24). In *mdx* mice, muscle pathology may be milder than in humans; however, the dystrophic phenotype may worsen with increasing age including the development of cardiac dysfunction (25-32). Administration of rAAV-mediated μ Dys therapy in *mdx* mice preceding the onset of cardiomyopathy may be highly cardioprotective (33-35). However, when *mdx* mice are treated with μ Dys at a late stage of cardiomyopathy, such as would be the case for a number of DMD patients, a full rescue of the dysfunctional cardiac phenotype is not achieved (30,35-37).

[29] The present disclosure relates to a cardiac function-enhancing gene therapy approach that may target myosin in contractile filaments and overexpress the enzyme ribonucleotide reductase (RNR). RNR converts ADP to deoxy-ADP (dADP), which may be rapidly converted to dATP in cells. In numerous *in vitro* studies, it has been shown that dATP may increase cross bridge binding and cycling, which may result in stronger, faster contraction and faster relaxation (38-46). It has also been reported that dATP may improve contractile properties of myocardium from end-stage human heart failure (HF) *in vitro* (43) and dogs with end-stage idiopathic dilated cardiomyopathy (47). In normal rodent muscle, it has been reported that increases in cardiomyocyte and cardiac function may occur with as little as ~1% of the ATP pool in the dATP form (40,48). Similarly, it has been shown that rAAV-mediated delivery of RNR under cardiac specific regulatory control may result in enzyme overexpression exclusively in cardiomyocytes and significantly improved left ventricular function without adverse cardiac remodeling in normal and infarcted rodent hearts (49). Studies also indicate that dATP could rescue the pre-load responsiveness of failing hearts, suggesting restoration of the abnormal Frank-Starling Law of the Heart that may occur in HF.

[30] In the current study, the relative therapeutic capacity of CK8-driven microdystrophin (μ Dys) or cTnT-driven ribonucleotide reductase (RNR), via intravenously administered recombinant adeno-associated viral (rAAV) vectors in an advanced age, DMD cardiomyopathy mouse model, were compared. A restoration of myocardial workload was demonstrated as indicated by rate pressure product (RPP), for baseline function in *mdx*^{4cv} mice treated with RNR. This outcome was primarily attributed to the normalization of left ventricular developed pressure (LVDevP). Although *mdx*^{4cv} mice treated with μ Dys appeared to normalize LVDevP, this did not result in a significant increase in RPP. Upon further evaluation of cardiac function, the pressure-volume relationship revealed that systolic pressure response with increased preload was significantly improved with the treatment of either RNR or μ Dys. However, only RNR treatment resulted in significant improvements in diastolic functional parameters, returning them to values that were similar to wild-type control hearts. As a further assessment of cardiac function, hearts were tested using a high workload challenge protocol. Both RNR and μ Dys treatments improved systolic function in *mdx*^{4cv} hearts without compromising cardiac reserve. These positive results suggest that targeted expression of RNR within the myocardium may significantly improve contractile performance in an advanced age model of DMD cardiomyopathy and may have therapeutic implications for muscular dystrophy and DMD patients.

METHODS

[31] The following examples are illustrative of disclosed methods and compositions. In light of this disclosure, those of ordinary skill in the art will recognize that variations of these examples and other examples of the disclosed methods and compositions would be possible without undue experimentation.

1. Animal experiments

[32] Male wild-type C57Bl/6J (The Jackson Laboratory, Bar Harbor, ME) and *mdx*^{4cv} (generated in house) mice were utilized for these studies (17). All animals were experimentally manipulated in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Washington. Experimental mice were administered vector at 22-24 months of age via the retro-orbital sinus with a 200- μ l bolus injection in Hanks Balanced Saline Solution (HBSS) at a dose of 2×10^{14} vg/kg. All mice were housed in a specific-pathogen free animal care facility using a 12-hr light/12-hr dark cycle with access to food and water ad libitum.

2. Vector production

[33] Recombinant AAV genomes containing the CK8 regulatory cassette (expressed exclusively in skeletal and cardiac muscle) and the human codon optimized (GenScript) μ Dys (Δ R2-15/ Δ R18-22/ Δ CT) (24), followed by the rabbit beta-globin poly-adenylation (pA) signal, were generated using standard cloning techniques. The rAAV genomes containing the cardiac-muscle specific cTnT455 regulatory cassette, the codon optimized human RNR transgene flanked by 100-bp UTR's, and the rabbit beta-globin pA were generated as previously described (49). The 'dead' rAAV genomes or promoter-less firefly luciferase followed by the human growth hormone (hGH) pA (kindly provided by JSC, University of Washington, Seattle WA) were used to generate the control rAAV genomes. The resulting constructs were co-transfected with the pDG6 packaging plasmid into HEK293 cells to generate rAAV vectors carrying serotype 6 capsids, that were harvested, enriched, and quantitated as previously described (50).

3. Vector genome quantification

[34] Total DNA was extracted from flash-frozen tissue samples with Tri-Reagent (MRC Inc.), according to manufacturer's instructions. All real-time PCR reactions were performed on a QuantStudio 3 Real Time PCR System (Applied Biosystems, Foster City, CA) in a total volume of 15 μ l, consisting of 5 μ l sample DNA, 10.0 μ l TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.2 μ M of each primer, and 0.1 μ M TaqMan custom probe (Applied Biosystems, Foster City, CA). Reaction conditions were 50° C for 2 minutes, 95° C for 10 minutes, and 40 cycles of [95° C for 15 seconds followed by 60° C for 1 minute. Each sample was analyzed in triplicate for concentration of total murine genomes and of total vector genomes. For vector genome detection by qPCR, the primers used to amplify either the rAAV6-cTnT455-RNR or rAAV6-CK8- μ Dys or rAAV6- Δ CMV-Luc (control vector) were unique to each vector. For the RNR vector the amplicon spanned from the distal region of the cTnT promoter, continuing into the proximal RNR1 subunit. For the μ Dys vector the amplicon was contained within the CK8 regulatory cassette, while the amplicon for the control vector resided within the human growth hormone (hGH) poly-adenylation. hGH Primers: 5'-CACAATCTTGGCTCACTGCAA-3', 5'-GGAGGCTGAGGCAGGAGAA-3', TaqMan Probe: 5'-6FAM-CTCCGCCTCCTGGGTTCAAGCG-MBGNQ-3'; CK8 RC Primers: 5'-CCCAGATGCCTGGTTATAATT-3', 5'-CGGGAACATGGCATGCA-3', TaqMan Probe: 5'-6FAM-CCCCCAACACCTGCTGCCTCT-MBGNQ-3'; cTnT455-RNR1 Primers: 5'-CCCAGTCCCCGCTGAGA-3', 5'-AGGTTCCAGGCGCTGCT-3', TaqMan Probe: 5'-6FAM-ACTCATCAATGTATCTTATCATG-MBGNQ-3'. Results were presented relative to DNA content in each 5 μ l DNA tissue sample to determine vector genomes per ng DNA.

4. Tissue processing and imaging analysis

[35] Tissues were collected and analyzed 5 months post-administration of vectors and compared with age-matched male control vector (rAAV6- Δ CMV-Luc) injected *mdx*^{4cv} and wild-type (WT) mice. Hearts were either snap frozen in liquid nitrogen or were

embedded in Optimal Cutting Temperature (O.C.T.) compound (VWR International) and flash frozen in liquid nitrogen cooled isopentane for histochemical or immunofluorescence analysis. The snap frozen samples were further processed by grinding to a powder under liquid nitrogen in a mortar kept on dry ice for subsequent extraction of nucleic acid and protein.

[36] Heart cross-sections (10 μ m) were co-stained with antibodies raised against alpha 2-laminin (Sigma, rat monoclonal, 1:200), the hinge-1 domain of dystrophin (alexa488 conjugated MANEX1011b, Developmental Studies Hybridoma Bank, University of Iowa, mouse monoclonal, 1:200), the human RRM1 (Abcam, rabbit monoclonal, 1:200), and the human RRM2 (Abcam, rabbit monoclonal, 1:200). Conjugated secondary antibodies (Jackson Immuno, Goat anti-Rabbit) were used at a 1:500 dilution. Slides were mounted using ProLong Gold with DAPI (Thermo Fisher Scientific) and imaged via a Leica SPV confocal microscope. Confocal micrographs covering a majority of the heart left ventricular muscle sections were acquired and montaged via the Fiji toolset (ImageJ) and InDesign (Adobe). For histology, Masson's trichrome staining was used to examine heart cross sections. Briefly, 10- μ m muscle cryosections were sequentially stained in Wiegerts' iron hematoxylin (10 min), 1% Ponceau-acetic acid (5 min), and 1% aniline blue (5 s).

5. Western blotting

[37] Radioimmunoprecipitation analysis buffer (RIPA) supplemented with 5mM EDTA and 3% protease inhibitor cocktail (Sigma, Cat# P8340), was used to extract muscle proteins for 0.5 hour on ice with gentle agitation every 10 min. Total protein concentration was determined using Pierce BCA assay kit (ThermoFisher). Muscle lysates from WT, control *mdx*^{4cv} and treated *mdx*^{4cv} (30 μ g) mice were denatured at 99 degrees Celsius for 10 min, quenched on ice, and separated via gel electrophoresis after loading onto Criterion 4–12% Bis-Tris polyacrylamide gels (BioRad). Overnight protein transfer to 0.45 mm PVDF membranes was performed at constant 43 volts at 4-degrees Celsius in Towbin's buffer containing 20% methanol. Blots were blocked for 1 hour at room temperature in 5% non-fat dry milk for 1 hour before overnight incubation with antibodies raised against the hinge-1 region of dystrophin (Developmental Studies Hybridoma Bank, University of Iowa, 1:300), anti-RRM1 (Abcam, rabbit monoclonal, 1:1,000), anti-RRM2 (Abcam, rabbit monoclonal 1:1,000), and anti-GAPDH (Sigma, Rabbit polyclonal, 1:50,000). Horseradish-peroxidase conjugated secondary antibody staining (1:50,000) was performed for 1 h at room temperature before signal development using Clarity Western ECL substrate (BioRad) and visualization using a Chemidoc MP imaging system (BioRad).

6. Quantification of cardiac [dATP]

[38] Approximately 25 μ g of flash frozen, freshly ground ventricle cardiac tissue was used for direct quantification of intracellular dATP using the HPLC-MS/MS method previously described (51). Briefly, samples were extracted 1-3 days before measurement using a 50% methanol solution. The supernatant was stored at -20°C until ready for injection into the HPLC-MS/MS system. A Waters Xevo-TQ-S mass spectrometer coupled with a Waters Acquity I-Class HPLC was used for the analysis (Milford, MA, USA). Monitoring in negative mode via an electrospray ionization (ESI) was used to acquire MS–MS ions. dATP concentrations were quantified with standards and normalized to tissue weight.

7. Langendorff isolated perfused heart experiments

[39] *Ex-vivo* cardiac function was assessed in Langendorff isolated heart preparations as previously described (48,49,52). Hearts were perfused at a constant pressure of 80mmHg with a modified Krebs-Henseleit (KH) buffer supplemented with glucose and pyruvate. The perfusate contained (in mmol/L): 118 NaCl, 25 NaHCO₃, 5.3 KCl, 2.0 CaCl₂, 1.2 MgSO₄, 0.5 EDTA, 10.0 glucose, and 0.5 pyruvate, equilibrated with 95% O₂ and 5% CO₂ (pH 7.4). Temperature was maintained at 37.5°C throughout the protocol.

Left ventricular (LV) function was monitored via a water-filled balloon inserted into the LV and connected to a pressure transducer. LV systolic pressure (LVSP), end diastolic pressure (EDP), heart rate (HR), and minimum and maximum rate of pressure change in the ventricle ($\pm dP/dt$) were obtained from the attached data acquisition system (PowerLab, ADInstruments, Colorado Springs, CO). After 5 minutes of stabilization, hearts were equilibrated for 10 minutes at spontaneous heart rates and then fixed at a heart rate of ~ 450 bpm with an electrical stimulator (Grass Technologies, Warwick, RI). Pressure-volume relationships (i.e., Frank-Starling curves) were assessed by gradually increasing the volume of the LV balloon. After a 5-minute recovery period, the perfusate was changed to an identical buffer as above except for the addition of 4.0 mmol/L $CaCl_2$ to simulate a high workload (HWL) challenge for 20 minutes.

8. Statistical Analysis

[40] All values are reported as means $\pm$ standard error of the mean (SEM). Starling curves and HWL function were analyzed by two-way repeated measures analysis of variance (ANOVA) followed by Tukey's post hoc analysis. End-point data was analyzed via one-way ANOVA or t-tests as appropriate. Mantel-Cox tests were used to analyze survival curves. Significance was tested at the $P < 0.05$ level.

RESULTS

1. Improvements in baseline cardiac function in vector-treated mdx^{4cv} hearts

[41] As depicted in Figure 1, 22-24 month-old mdx^{4cv} mice were administered one of three treatments: rAAV6-cTnT455-ribonucleotide reductase (RNR; referred to as mdx^{4cv} + RNR); rAAV6-CK8-micro-dystrophin (μ Dys; referred to as mdx^{4cv} + μ Dys), or rAAV6- Δ CMV-Firefly Luciferase control vector (referred to as mdx^{4cv}) at a dose of 2×10^{14} vg/kg. By the end of the 20-week treatment period, both mdx^{4cv} + RNR and mdx^{4cv} + μ Dys mice showed improvements in survival rates compared to mdx^{4cv} mice, although this did not reach statistical significance (Figure 2). At the end of 5 months, an extensive evaluation of *ex-vivo* cardiac function using the Langendorff isolated heart preparation was performed. The isolated heart technique allows for the direct assessment of inherent myocardial function without the confounding effects of neuro-humoral or other systemic variables. An additional cohort of age-matched, untreated C57BL6 mice (WT) were used as comparison controls. At baseline, RPP was significantly decreased in mdx^{4cv} hearts due to an approximate 20% decrease in LVDevP (Figures 3A and 3B). RNR treated mdx^{4cv} mice exhibited a restoration of RPP ($P = 0.0564$) primarily due to a normalization of LVDevP (Figures 3A and 3B). Although μ Dys treated mdx^{4cv} hearts appeared to normalize LVDevP, this did not lead to a significant improvement in RPP (Figures 3A and 3B). Both $+dP/dt$ and $-dP/dt$, an index of ventricular contractility and relaxation, respectively, were decreased 30% in mdx^{4cv} hearts ($P = 0.06$). The $+dP/dt$ was similar to control in both RNR treated mdx^{4cv} and μ Dys treated mdx^{4cv} hearts. However, only RNR treated mdx^{4cv} hearts demonstrated $-dP/dt$ values similar to control levels (Figures 3C and 3D).

2. Positive changes in Frank-Starling mechanics in vector-treated mdx^{4cv} hearts

[42] To evaluate further systolic and diastolic function in vector treated- mdx^{4cv} hearts, the pressure-volume relationship (i.e., Frank-Starling mechanism) in the isolated perfused heart preparation were examined. The left ventricular systolic pressure (LVSP) response to increased preload was significantly improved in both in mdx^{4cv} + RNR and mdx^{4cv} + μ Dys hearts compared to mdx^{4cv} (Figure 4A). However, only RNR treatment improved the diastolic response in mdx^{4cv} hearts, to levels similar to WT (Figure 4B). Both contractility and relaxation (i.e., $+dP/dt$ and $-dP/dt$, respectively) were impaired in mdx^{4cv} compared to age-matched controls (Figures 4D and 4E). Both mdx^{4cv} + RNR and mdx^{4cv} + μ Dys hearts had significantly elevated $+dP/dt$ values above mdx^{4cv} (Figure 4E).

Interestingly, treatment of *mdx*^{4cv} hearts with RNR also significantly improved $-dP/dt$ values (Figure 4D). These data suggest that both RNR and μ Dys treatment can improve systolic function in *mdx*^{4cv} hearts. However, these data showed that only the RNR treatment corrected diastolic dysfunction in *mdx*^{4cv} hearts.

3. Augmented response to increased cardiac workload in treated *mdx*^{4cv} hearts

[43] It was previously reported that RNR overexpression in transgenic or vector-treated mouse hearts elevated baseline function but did not impair the response to an acute physiological increase in cardiac work (48,49). To verify that the improved systolic and diastolic function in RNR treated *mdx*^{4cv} hearts at baseline was not associated with an inability to respond to an increased energetic demand, hearts were stressed with a combination of high calcium and elevated heart rates, via pacing stimulation. As shown in Figures 5A and 5B, *mdx*^{4cv} hearts had a blunted response to the increased workload as both LVDevP and RPP were ~25-30% lower than wild-type hearts. In addition both $+dP/dt$ and $-dP/dt$ were impaired in *mdx*^{4cv} relative to wild-type hearts (Figures 5C and 5D). Systolic parameters in *mdx*^{4cv} + μ Dys hearts were effectively improved and similar to age-matched wild-type hearts for the entire duration of the workload challenge (Figures 5A to 5C). Measures of systolic function significantly increased in *mdx*^{4cv} +RNR hearts during the initial half of the high workload protocol and remained ~15% higher than *mdx*^{4cv} (Figures 5A to 5C). Interestingly, $-dP/dt$ values tended to be elevated only in *mdx*^{4cv} +RNR hearts during the physiological challenge (Figure 5D). These data show that both RNR and μ Dys treatments improve systolic function in *mdx*^{4cv} hearts without compromising cardiac reserve. Combined with the baseline and pressure-volume relationship assessments, these data demonstrate that, in addition to the systolic enhancements, RNR has an added benefit of improving diastolic function.

4. RNR and μ Dys transduction, expression, and cardiomyocyte localization

[44] To evaluate the localization of RNR and micro-dystrophin protein within the hearts of mice, immunofluorescence imaging was performed. As shown in Figure 6, the RNR subunit (Rrm1) was robustly expressed in ventricles of RNR treated mice. The expression of μ Dys appeared to be saturated relative to full-length dystrophin levels, with both being properly localized to the sarcolemma of cardiomyocytes. Evaluations of general muscle histopathology and potential differences in myocardial fibrosis by Masson trichrome staining were also performed, and no discernable difference between treated or untreated *mdx*^{4cv} mice were observed (Figure 7). In addition, neither RNR nor μ Dys treatment significantly altered body weight (BW), heart weight (HW), or the HW to BW ratio (Figure 8). Western blotting was performed to determine the extent of rAAV6-mediated RNR and μ Dys protein expression profiles in ventricular tissue (Figure 9). μ Dys protein expression in ventricular tissue that approached levels similar to wild-type mice were observed, while both human RNR subunits were found to be elevated to comparable levels within ventricular tissue (Figure 9A). To evaluate the relative proportions of dATP concentrations within ventricular tissue, HPLC-MS/MS analysis was performed on ground ventricular tissue from *mdx*^{4cv} and *mdx*^{4cv} +RNR mice. The concentration of dATP within the ventricular tissue obtained from *mdx*^{4cv} mice treated with RNR (0.568 ± 0.22 pmol dATP/mg) was approximately 10-fold higher relative to *mdx*^{4cv} controls (0.051 ± 0.02 pmol dATP/mg) (Figure 9B). For adult wildtype, an average dATP value of 0.021 pmol/mg tissue with a standard deviation of 0.007 was previously reported (51). Additionally, cardiac vector genome data was comparable relative to the vector dose administered (Figure 9C).

5. Micro-dystrophin can prevent loss of strength and fatigue recovery in young *mdx* mouse hind limb muscle

[45] Both groups of young *mdx*^{4cv} mice treated with μ Dys or μ Dys+RNR at 2 weeks generated more force per muscle weight when measured *in vivo* by foot pedal compared to untreated *mdx*^{4cv} controls (Figure 10). Treated mice also recovered more quickly from a fatigue protocol of repeated tetanic contractions.

[46] It will be readily understood that the embodiments, as generally described herein, are exemplary. The following more detailed description of various embodiments is not intended to limit the scope of the present disclosure, but is merely representative of various embodiments. Moreover, the order of the steps or actions of the methods disclosed herein may be changed by those skilled in the art without departing from the scope of the present disclosure. In other words, unless a specific order of steps or actions is required for proper operation of the embodiment, the order or use of specific steps or actions may be modified.

[47] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[48] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The applicants expect skilled artisans to employ such variations as appropriate, and the applicants intend for the various embodiments of the disclosure to be practiced otherwise than specifically described herein. Accordingly, this disclosure includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

[49] Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above-cited references and printed publications are individually incorporated herein by reference in their entirety.

[50] It is to be understood that the embodiments of the present disclosure are illustrative of the principles of the present disclosure. Other modifications that may be employed are within the scope of the disclosure. Thus, by way of example, but not of limitation, alternative configurations of the present disclosure may be utilized in accordance with the teachings herein. Accordingly, the present disclosure is not limited to that precisely as shown and described.

[51] The particulars shown herein are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present disclosure only and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of various embodiments of the disclosure.

[52] Definitions and explanations used in the present disclosure are meant and intended to be controlling in any future construction unless clearly and unambiguously modified in the examples or when application of the meaning renders any construction meaningless or essentially meaningless in cases where the construction of the term would render it meaningless or essentially meaningless, the definition should be taken from Webster's Dictionary, 3rd Edition or a dictionary known to those of ordinary skill in the art, such as the Oxford Dictionary of Biochemistry and Molecular Biology (Ed. Anthony Smith, Oxford University Press, Oxford, 2004).

[53] It will be apparent to those having skill in the art that many changes may be made to the details of the above-described embodiments without departing from the underlying principles of the invention.

CLAIMS

What is claimed is:

1. A method for treating a subject having muscular dystrophy, comprising:
administering to the subject a therapeutically effective amount of a first pharmaceutical composition comprising an RRM1 gene and an RRM2 gene operably coupled to a first regulatory cassette.
2. A method for prophylactically treating a subject at risk of developing muscular dystrophy, comprising:
administering to the subject a therapeutically effective amount of a first pharmaceutical composition comprising an RRM1 gene and an RRM2 gene operably coupled to a first regulatory cassette.
3. The method of claim 1 or 2, wherein the first pharmaceutical composition further comprises a first delivery vehicle.
4. The method of any of claims 1-3, further comprising:
administering to the subject a therapeutically effective amount of a second pharmaceutical composition comprising a micro-dystrophin gene operably coupled to a second regulatory cassette.
5. The method of claim 4, wherein the second pharmaceutical composition further comprises a second delivery vehicle.
6. A method for treating a subject having muscular dystrophy, comprising:
administering to the subject a therapeutically effective amount of a first pharmaceutical composition comprising an RRM1 gene operably coupled to a first regulatory cassette in a first delivery vehicle.
7. The method of claim 6, further comprising:

administering to the subject a therapeutically effective amount of a second pharmaceutical composition comprising an RRM2 gene operably coupled to a second regulatory cassette in a second delivery vehicle.

8. The method of claim 7, further comprising:

administering to the subject a therapeutically effective amount of a third pharmaceutical composition comprising a micro-dystrophin gene operably coupled to a third regulatory cassette in a third delivery vehicle.

9. A method for prophylactically treating a subject at risk of developing muscular dystrophy, comprising:

administering to the subject a therapeutically effective amount of a first pharmaceutical composition comprising an RRM1 gene operably coupled to a first regulatory cassette in a first delivery vehicle.

10. The method of claim 9, further comprising:

administering to the subject a therapeutically effective amount of a second pharmaceutical composition comprising an RRM2 gene operably coupled to a second regulatory cassette in a second delivery vehicle.

11. The method of claim 10, further comprising:

administering to the subject a therapeutically effective amount of a third pharmaceutical composition comprising a micro-dystrophin gene operably coupled to a third regulatory cassette in a third delivery vehicle.

12. The method of claim 10 or 11, wherein the first delivery vehicle and the second delivery vehicle are separate delivery vehicles.

13. The method of claim 11, wherein the first delivery vehicle, the second delivery vehicle and the third delivery vehicles are separate delivery vehicles.

14. The method of any one of claims 1-13, wherein the regulatory cassette is selected from the group consisting of a CK8 promoter and a cardiac troponin T (cTnT) promoter.

15. The method of any one of claims 1-14, wherein the delivery vehicle is selected from the group consisting of an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector.

16. The method of any one of claims 1-15, wherein the muscular dystrophy is selected from at least one of myotonic muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, congenital muscular dystrophy, oculopharyngeal muscular dystrophy, distal muscular dystrophy, and Emery-Dreifuss muscular dystrophy.

17. The method of any one of claims 1-15, wherein the muscular dystrophy is selected from at least one of Duchenne muscular dystrophy and Becker muscular dystrophy.

18. The method of any one of claims 1-17, wherein the delivery vehicle is a recombinant adeno-associated virus type 6 (rAAV6) vector.

19. The method of any one of claims 1-18, wherein the subject is a mammal.

20. The method of any one of claims 1-19, wherein the subject is a human.

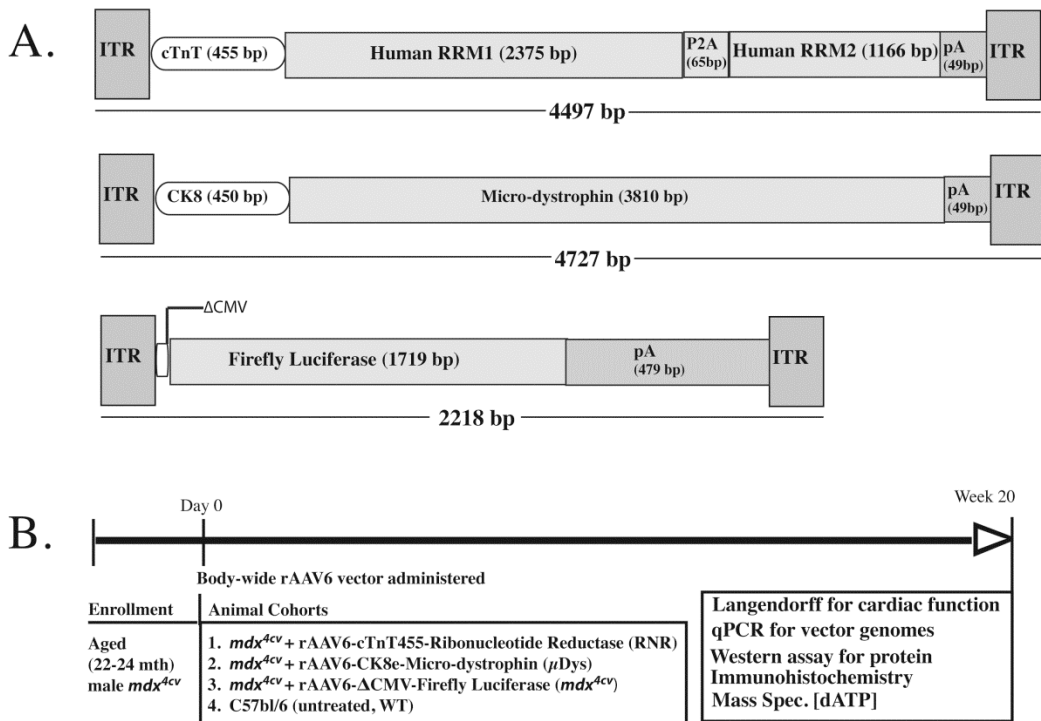
ABSTRACT

The present disclosure relates generally to methods of treating a subject having muscular dystrophy or DMD. The present disclosure also relates generally to methods of prophylactically treating a subject at risk of developing muscular dystrophy or DMD. In some embodiments, the methods may include administering a pharmaceutical composition including an RRM1 gene, an RRM2 gene, and a delivery vehicle to a subject. In another embodiment, the methods may include administering a pharmaceutical composition including an RRM1 gene and an RRM2 gene coupled to a regulatory cassette to a subject. In yet another embodiment, the methods may include administering a pharmaceutical composition including an RRM 1 gene, an RRM2 gene, a regulatory cassette, and a delivery vehicle to a subject.

FIGURES

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Figure 1: Overview of experimental design for the treatment of aged (22-24 mo) *mdx*^{4cv} mice. **(A)** Graphic representation of rAAV6 vectors utilized in the present study. The ribonucleotide reductase vector contains the human cDNA for the RRM1 and RRM2 subunits whose expression is driven by the cardiac specific (cTnT455) regulatory cassette (RC). The human micro-dystrophin (Δ R2-R15/ Δ R18-R22/ Δ CT) vector has expression driven by the CK8 muscle specific RC. The control vector utilized in the present study carries the firefly luciferase transgene whose CMV early promoter/enhancer RC has been deleted. **(B)** Outline of animal enrollment, vector administration, and experimental protocols implemented following a treatment period of 5 months.

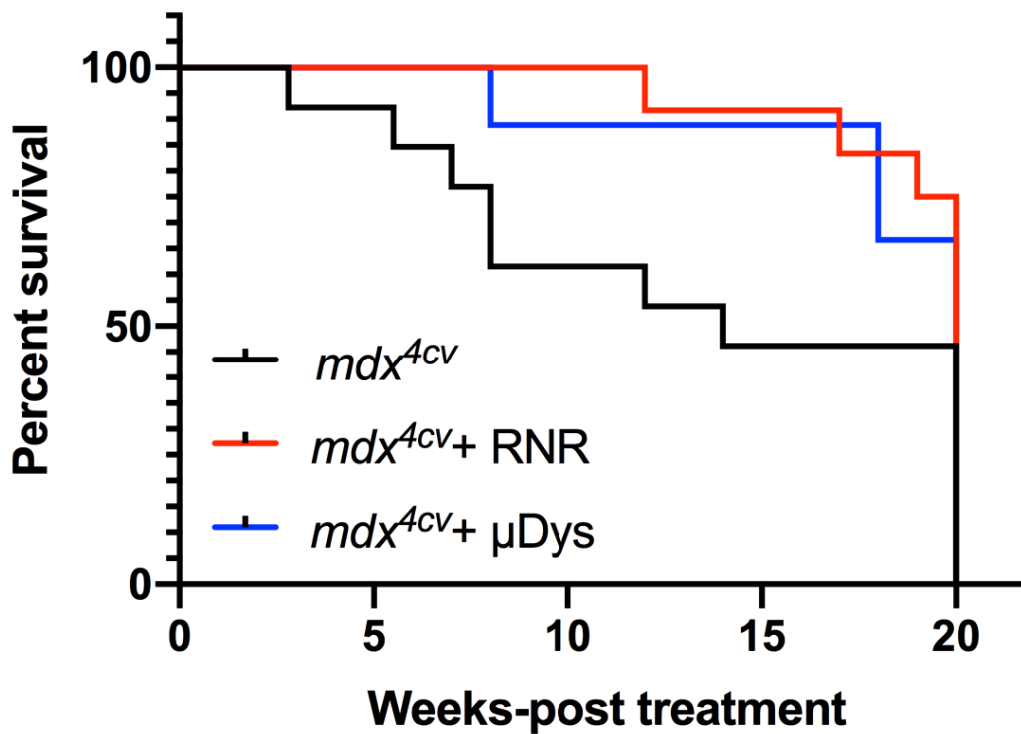


FIGURES

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Figure 2: Survival curves in vector-treated mdx^{4cv} mice.

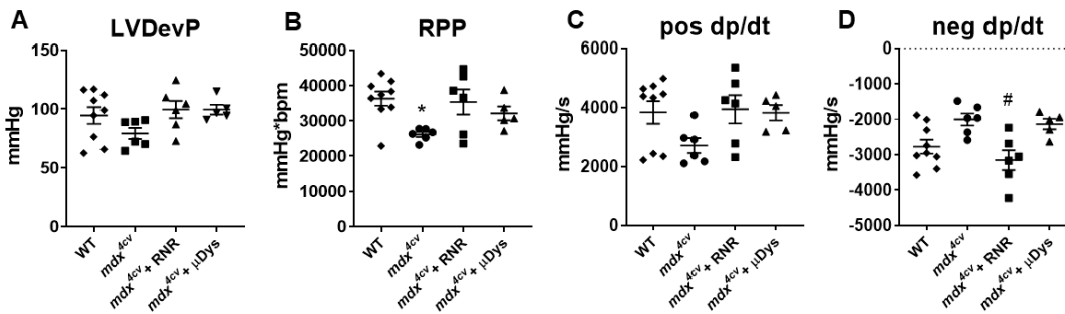
Kaplan Meier analysis was performed on mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). All mice were followed for 20 weeks post injection.



FIGURES

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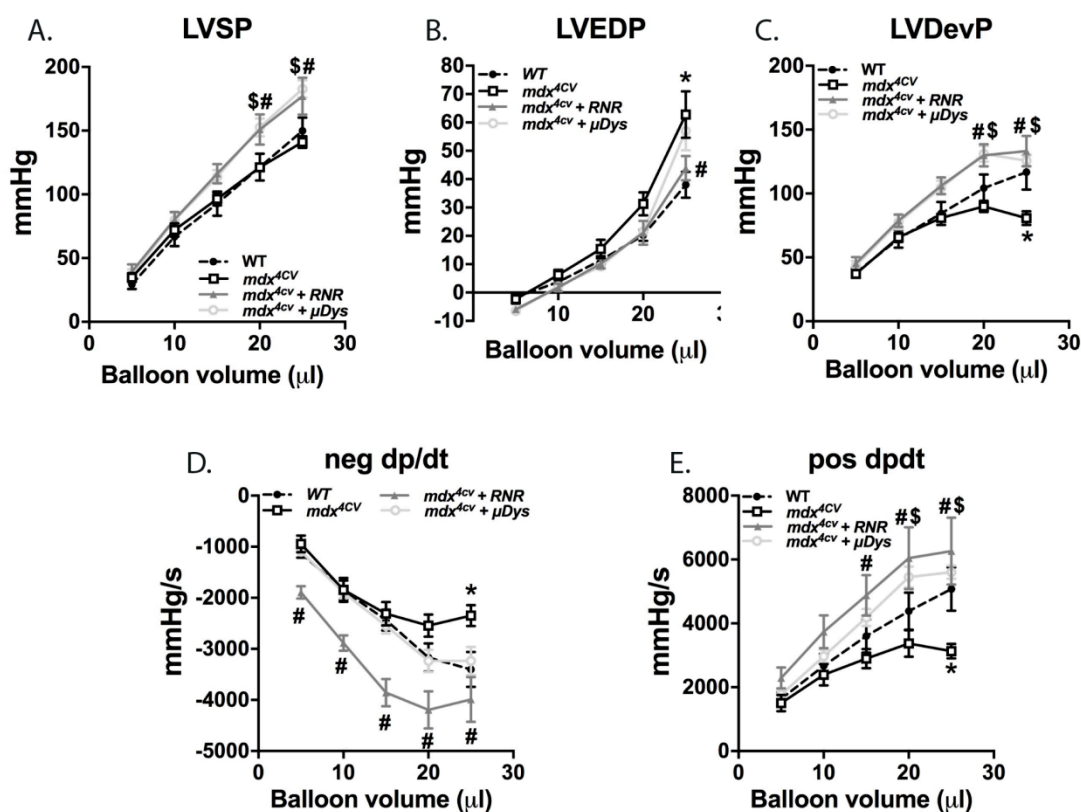
Figure 3: Baseline ex-vivo cardiac function in vector-treated mdx^{4cv} mice. Hearts were isolated from mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). Age-matched, non-diseased, non-treated wild-type mice were used as controls (WT, n=8). All hearts were perfused with a glucose-pyruvate buffer. Functional assessment was performed at spontaneous heart rates. **(A)** Left ventricular developed pressure (LVDevP, the difference between systolic and diastolic pressures). **(B)** Rate pressure product (RPP, the product of LVDevP and HR). **(C)** Negative rate of pressure change calculated by the first derivative of the descending LV pressure wave (-dP/dt), used as an index of ventricular relaxation. **(D)** Positive rate of pressure change calculated by the first derivative of the ascending LV pressure wave (+dP/dt), used as an index of ventricular contractility. * P<0.05 mdx^{4cv} vs. WT; # P<0.05 mdx^{4cv} +RNR vs. mdx^{4cv} .



FIGURES

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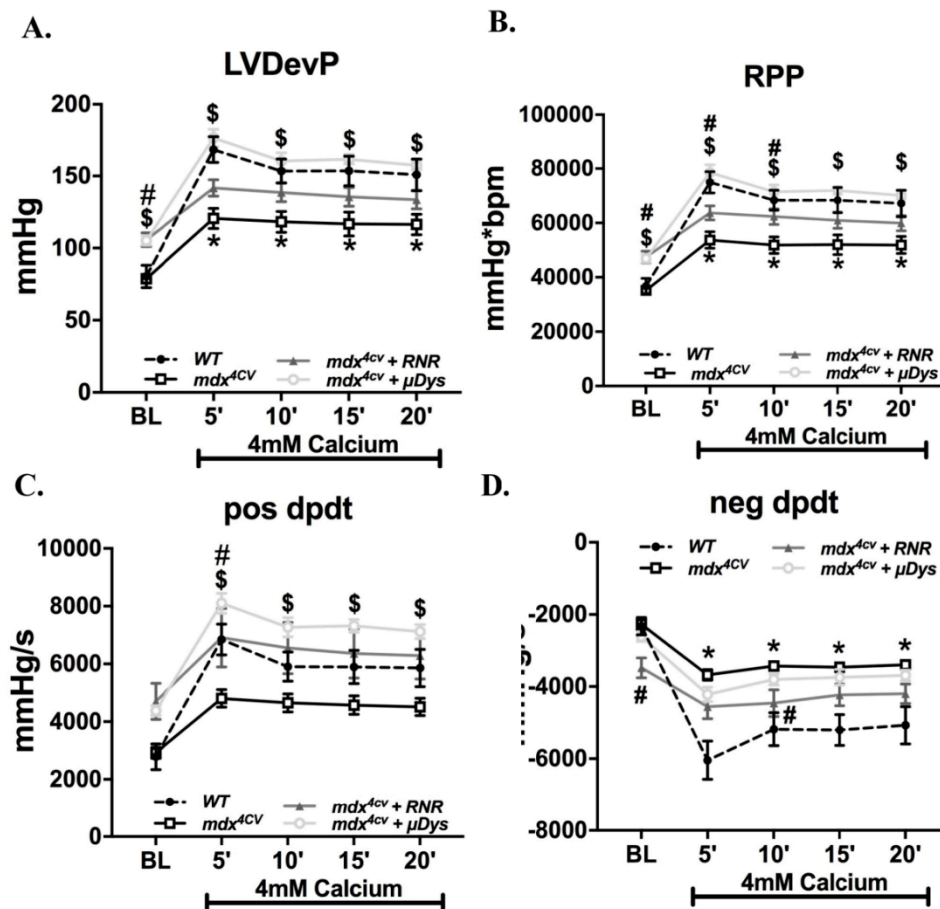
Figure 4: Pressure-volume relationships in isolated perfused hearts from vector-treated mdx^{4cv} mice. Hearts were isolated from mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). Age-matched, non-diseased, non-treated wild-type mice were used as controls (WT, n=8). The pressure-volume relationship (i.e., Frank-Starling mechanism) was evaluated by gradually increasing the volume of the LV balloon. Hearts were paced at 450 bpm throughout the protocol. **(A)** Left ventricular systolic pressure (LVSP). **(B)** LV end-diastolic pressure (LVEDP). **(C)** Left ventricular developed pressure (LVDevP, the difference between systolic and diastolic pressures). **(D)** Negative rate of pressure change calculated by the first derivative of the descending LV pressure wave (-dP/dt), and is used as an index of ventricular relaxation. **(E)** Positive rate of pressure change was calculated by the first derivative of the ascending LV pressure wave (+dP/dt), and is used as an index of ventricular contractility. * P<0.05 mdx^{4cv} vs. WT; # P<0.05 mdx^{4cv} +RNR vs. mdx^{4cv} ; \$ P<0.05 mdx^{4cv} + μ Dys vs. mdx^{4cv} .



FIGURES

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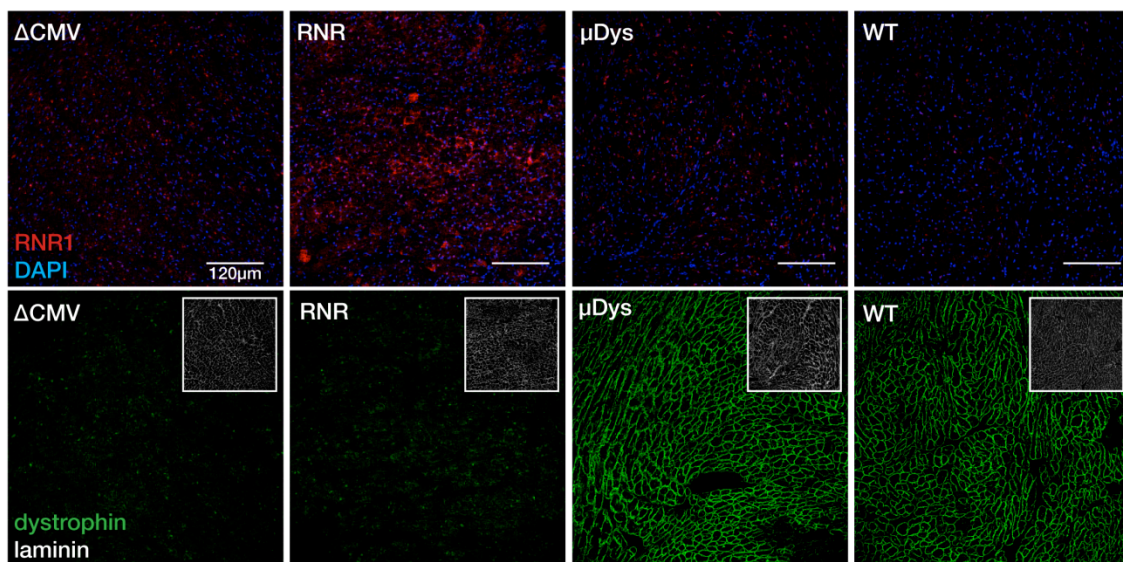
Figure 5: The response of vector-treated mdx^{4cv} mice to high workload challenge in Langendorff isolated heart preparations. Hearts were isolated from mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). Age-matched, non-diseased, non-treated wild-type mice were used as controls (WT, n=4). All hearts were perfused with a glucose-pyruvate buffer containing high calcium (4.0 mmol/L) to simulate a high workload challenge for 20min. Hearts were paced at 450pm throughout the protocol. **(A)** Left ventricular developed pressure (LVDevP, the difference between systolic and diastolic pressures). **(B)** Rate pressure product (RPP, the product of LVDevP and HR). **(C)** Positive rate of pressure change calculated by the first derivative of the ascending LV pressure wave (+dP/dt), is used as an index of ventricular contractility. **(D)** Negative rate of pressure change calculated by the first derivative of the descending LV pressure wave (-dP/dt), is used as an index of ventricular relaxation. * P<0.05 mdx^{4cv} vs. WT; # P<0.05 mdx^{4cv} +RNR vs. mdx^{4cv} ; \$ P<0.05 mdx^{4cv} + μ Dys vs. mdx^{4cv} .



FIGURES

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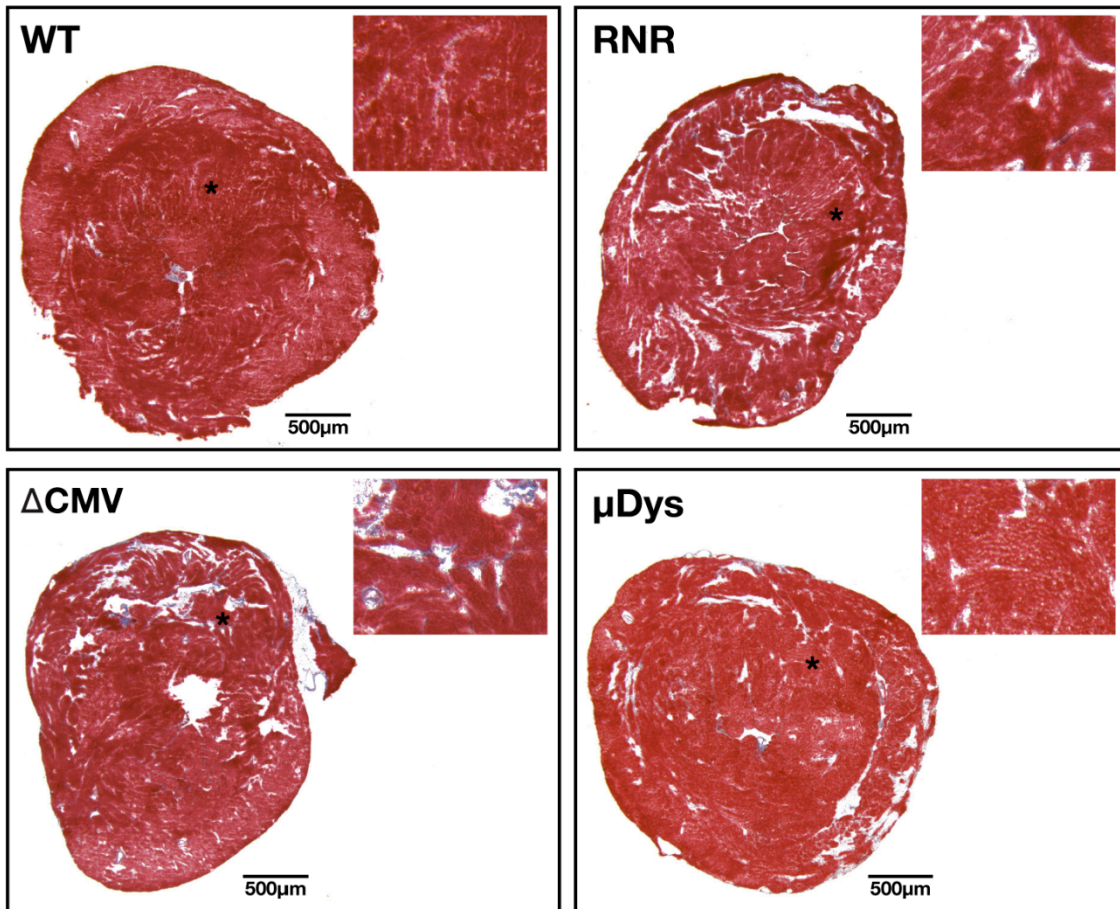
Figure 6: Cardiac transduction following intravenous delivery of ribonucleotide reductase or microdystrophin. At 5-months following vector administration, cryosections were prepared and immunostained with antisera against dystrophin or ribonucleotide reductase. A considerable level of protein is detected for each ribonucleotide reductase subunit-1 (human specific) as indicated by immunofluorescent staining (Red) localized primarily within the cytoplasm of cardiomyocytes with occasional perinuclear accumulation (upper panel). Noteworthy on the lower panel, is the robust level of expression for dystrophin in WT and in aged *mdx*^{4cv} mice treated with AAV6-CK8-microdystrophin (laminin staining, inset image).



FIGURES

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Figure 7: Heart histological staining of mdx^{4cv} mice suggests no morphological alterations from RNR therapy. Representative full-view photomicrographs of Masson trichrome staining of the hearts from mdx^{4cv} mice displaying control vector (Δ CMV), and rAAV6-treated with either RNR or μ Dys from mdx^{4cv} mice. Similarly, 20X enlarged view of the corresponding images (*) is shown.

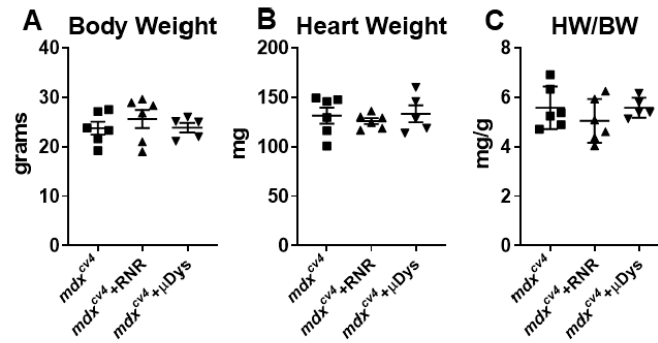


FIGURES

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Figure 8: Physical characteristics of vector-treated mdx^{4cv} mice.

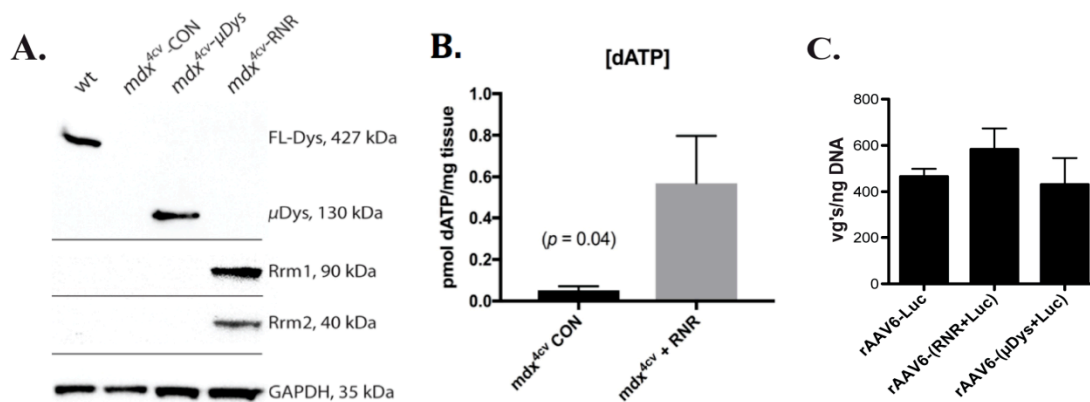
(A) Body weights and (B) heart weights were obtained from mdx^{4cv} mice treated with control vector (mdx^{4cv} , n=6), ribonucleotide reductase (mdx^{4cv} +RNR, n=6), or micro-dystrophin (mdx^{4cv} + μ Dys, n=5). (C) Heart weights (HW) were normalized to body weights (BW) to obtain HW/BW ratio. No statistical differences were noted among the variables.



FIGURES

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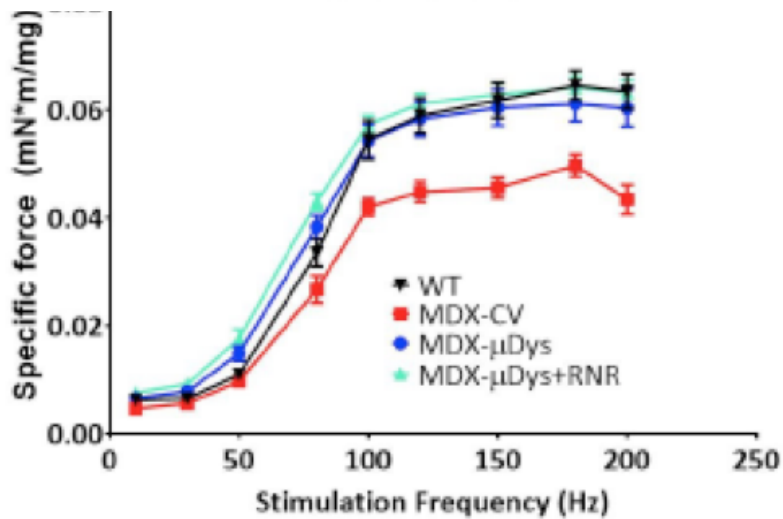
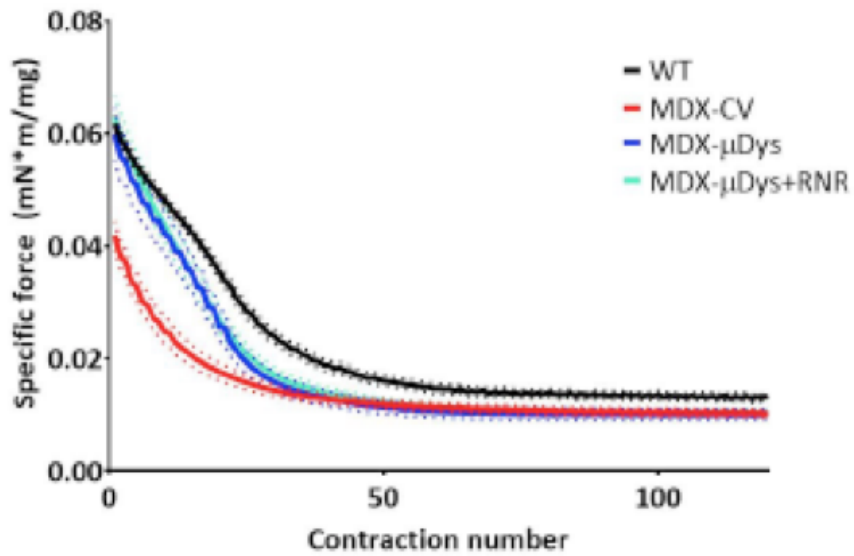
Figure 9: Protein expression levels for μ Dys, RNR and resultant vector genomes due to rAAV6-mediated gene transfer. (A) RNR and μ Dys protein expression detection as revealed by immunoblotting of cardiac whole tissue lysates using either RRM1, RRM2 or anti-dystrophin antibody. (B) HPLC-MS/MS intracellular [dNTP] quantification from methanol extracted cardiac tissue. (C) qPCR analysis of vector genomes from cardiac tissue revealed similar vector genomes being represented for all treated cohorts.



FIGURES

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Figure 10: Comparison of specific force generation per body weight by wild type control mice (WT), mdx^{4cv} mice treated with control vector (MDX), mdx^{4cv} mice treated with micro-dystrophin (MDX+ μ Dys), and mdx^{4cv} mice treated with micro-dystrophin and RNR (MDX+ μ Dys+RNR).



APPENDIX

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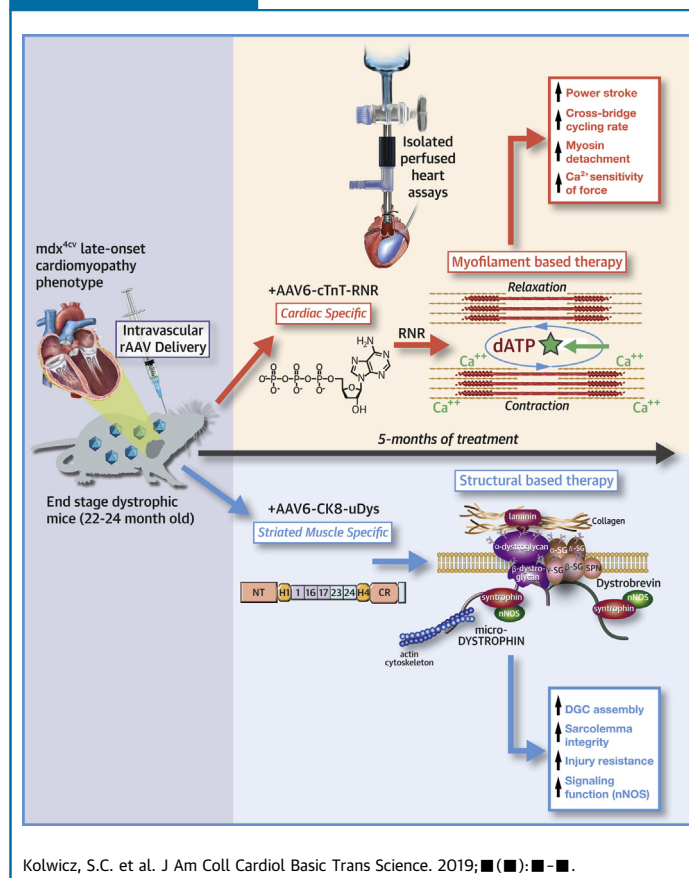
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NEW RESEARCH PAPER

Gene Therapy Rescues Cardiac Dysfunction in Duchenne Muscular Dystrophy Mice by Elevating Cardiomyocyte Deoxy-Adenosine Triphosphate

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VISUAL ABSTRACT



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HIGHLIGHTS

- rAAV vectors increase cardiac-specific expression of RNR and elevate cardiomyocyte 2-deoxy-adenosine triphosphate levels.
- Elevated myocardial RNR and subsequent increase in 2-deoxy-adenosine triphosphate rescues the performance of failing myocardium, an effect that persists long term.
- We show the ability to increase both cardiac baseline function and high workload contractile performance in aged (22- to 24-month old) mdx4cv mice, by high-level muscle-specific expression of either microdystrophin or RNR.
- Five months post-treatment, mice systemically injected with rAAV6 vector carrying a striated muscle-specific regulatory cassette driving expression of microdystrophin in both skeletal and cardiac muscle, exhibited the greatest effect on systolic function. In comparison, mice treated with rAAV6 vector carrying RNR that expresses exclusively in cardiac muscle not only exhibited greatly improved baseline systolic function but also improved diastolic function.
- Importantly, vector-directed over-expression of RNR did not impair cardiac reserve during increased physiological demand in aged mdx4cv hearts.

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ABBREVIATIONS
AND ACRONYMS**CK8** = miniaturized murine creatine kinase regulatory cassette**CMV** = cytomegalovirus**cTnT** = cardiac troponin T**dADP** = deoxy-adenosine diphosphate**dATP** = deoxy-adenosine triphosphate**DMD** = Duchenne muscular dystrophy***mdx*** = mouse muscular dystrophy model**rAAV** = recombinant adeno-associated viral vector**RNR** = ribonucleotide reductase**μDys** = microdystrophin

SUMMARY

Mutations in the gene encoding for dystrophin leads to structural and functional deterioration of cardiomyocytes and is a hallmark of cardiomyopathy in Duchenne muscular dystrophy (DMD) patients. Administration of recombinant adeno-associated viral vectors delivering microdystrophin or ribonucleotide reductase (RNR), under muscle-specific regulatory control, rescues both baseline and high workload-challenged hearts in an aged, DMD mouse model. However, only RNR treatments improved both systolic and diastolic function under those conditions. Cardiac-specific recombinant adeno-associated viral treatment of RNR holds therapeutic promise for improvement of cardiomyopathy in DMD patients. (J Am Coll Cardiol Basic Trans Science 2019;■:■-■) © 2019 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Duchenne muscular dystrophy (DMD) and its milder and allelic form, Becker muscular dystrophy (BMD), are the most frequent muscular dystrophies, occurring once in ~5,000 male births, and are due to mutations in the dystrophin gene (1). DMD patients typically die due to cardiac and respiratory muscle failure; thus, maintenance of adequate function in both cardiac and skeletal muscle is critical for optimal DMD therapy. The primary function of dystrophin is to provide a structural role by mechanically linking the subsarcolemmal cytoskeleton to the extracellular matrix through the dystrophin-glycoprotein complex (DGC) (2). This linkage transmits the forces of contraction to the extracellular matrix and protects muscles from contraction-induced injury (3-7). In addition to a structural or mechanical role, the DGC also serves as a scaffold for cytoplasmic and membrane-associated signaling proteins and ion channels (8-11). The complete absence of dystrophin results in drastic reductions of all DGC components (12-14). Together, an absence of dystrophin and reduction in the DGC components causes membrane destabilization and permeability defects that lead to myofiber degeneration, repeated cycles of degeneration/regeneration, and the gradual replacement of muscle fibers with fibrotic, connective, and adipose tissue.

In contrast, some in-frame deletions, truncations, and missense mutations lead to reduced dystrophin expression associated with milder phenotypes. These pathologies are largely curtailed in mouse (*mdx*) and canine (*cxmd*) models of DMD following the vector-mediated delivery of muscle-specific expression of highly functional miniaturized versions of dystrophin, microdystrophin (μDys) (15-24). In *mdx* mice, muscle pathology is milder than in humans, with the exception of the diaphragm; however, the dystrophic phenotype worsens with increasing age including the development of cardiac dysfunction (25-32). Administration of recombinant adeno-associated viral (rAAV)-mediated μDys therapy in *mdx* mice preceding the onset of cardiomyopathy is highly cardioprotective (33-35). However, when *mdx* mice are treated with μDys at a late stage of cardiomyopathy, such as would be the case for a number of DMD patients, a full rescue of the dysfunctional cardiac phenotype is not achieved (30,35-37).

We have developed a cardiac function-enhancing gene therapy approach that targets myosin in contractile filaments by overexpressing the enzyme ribonucleotide reductase (RNR). RNR converts adenosine diphosphate (ADP) to deoxy-ADP (dADP), which is rapidly converted to deoxy-adenosine triphosphate (dATP) in cells. In numerous in vitro studies, we have shown that dATP increases cross-bridge binding and

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and US Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the JACC: Basic to Translational Science [author instructions page](#).

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cycling, resulting in stronger, faster contraction and faster relaxation (38-45). We have also reported that dATP improves contractile properties of myocardium from end-stage human heart failure (HF) in vitro (42) and dogs with end-stage idiopathic dilated cardiomyopathy (46). In normal rodent muscle, we reported that increases in cardiomyocytes and cardiac function occur with as little as ~1% of the ATP pool in the dATP form (40,47). Similarly, rAAV-mediated delivery of RNR under cardiac-specific regulatory control resulted in enzyme overexpression exclusively in cardiomyocytes and significantly improved left ventricular function without adverse cardiac remodeling in normal and infarcted rodent hearts (48). Our data indicated that dATP could rescue the preload responsiveness of failing hearts, suggesting restoration of the abnormal Frank-Starling Law of the Heart that often occurs in HF.

In the current study, we compare the relative therapeutic capacity of CK8-driven μ Dys or cardiac troponin T (cTnT)-driven RNR, via intravenously administered rAAV vectors in an advanced-age, DMD cardiomyopathy mouse model. We show a restoration of myocardial workload as indicated by rate pressure product (RPP) for baseline function in *mdx*^{4cv} mice treated with RNR. This outcome was primarily attributed to the normalization of left ventricular developed pressure (LVDevP). Although *mdx*^{4cv} mice treated with μ Dys appeared to normalize LVDevP, this did not result in a significant increase in RPP. Upon further evaluation of cardiac function, the pressure-volume relationship revealed that systolic pressure response with increased preload was significantly improved with the treatment of either RNR or μ Dys. However, only RNR treatment resulted in significant improvements in diastolic functional parameters, returning them to values that were similar to wild-type (WT) control hearts. As a further assessment of cardiac function, we tested hearts using a high workload challenge protocol. Both RNR and μ Dys treatments improved systolic function in *mdx*^{4cv} hearts without compromising cardiac reserve. These positive results suggest that targeted expression of RNR within the myocardium can significantly improve contractile performance in an advanced-age model of DMD cardiomyopathy and may have therapeutic implications for DMD patients.

METHODS

ANIMAL EXPERIMENTS. Male WT C57Bl/6J (The Jackson Laboratory, Bar Harbor, Maine) and *mdx*^{4cv} (generated in-house) mice were used for these studies (17). All animals were experimentally manipulated in

accordance with the Institutional Animal Care and Use Committee of the University of Washington. Experimental mice were administered vector at 22 to 24 months of age via the retro-orbital sinus with a 200- μ l bolus injection in Hanks balanced saline solution at a dose of 2×10^{14} vg/kg. All mice were housed in a specific-pathogen free animal care facility using a 12-h light/12-h dark cycle with access to food and water ad libitum.

VECTOR PRODUCTION. rAAV genomes containing the CK8 regulatory cassette (expressed exclusively in skeletal and cardiac muscle) and the human codon optimized (GenScript) μ Dys (Δ R2-15/ Δ R18-22/ Δ CT) (24), followed by the rabbit beta-globin poly-adenylation (pA) signal, were generated using standard cloning techniques. The rAAV genomes containing the cardiac muscle-specific cTnT455 regulatory cassette, the codon optimized human RNR transgene flanked by 100-bp untranslated regions, and the rabbit beta-globin pA were generated as previously described (48). The “dead” rAAV genomes or promoter-less firefly luciferase followed by the human growth hormone (hGH) pA (kindly provided by J.S.C., University of Washington, Seattle, Washington) were used to generate the control rAAV genomes. The resulting constructs were cotransfected with the pDG6 packaging plasmid into HEK293 cells to generate rAAV vectors carrying serotype 6 capsids, which were harvested, enriched, and quantitated as previously described (49).

VECTOR GENOME QUANTIFICATION. Total DNA was extracted from flash-frozen tissue samples with Tri-Reagent (MRC Inc., Cincinnati, Ohio), according to manufacturer’s instructions. All real-time polymerase chain reaction (PCR) reactions were performed on a QuantStudio 3 Real Time PCR System (Applied Biosystems, Foster City, California) in a total volume of 15 μ l, consisting of 5 μ l sample DNA, 10.0 μ l TaqMan Universal PCR Master Mix (Applied Biosystems), 0.2 μ M of each primer, and 0.1 μ M TaqMan custom probe (Applied Biosystems). Reaction conditions were 50° C for 2 min, 95° C for 10 min, and 40 cycles of [95° C for 15 s followed by 60° C for 1 min]. Each sample was analyzed in triplicate for concentration of total murine genomes and of total vector genomes. For vector genome detection by quantitative PCR, the primers used to amplify either the rAAV6-cTnT455-RNR or rAAV6-CK8- μ Dys, or rAAV6- Δ cytomegalovirus(CMV)-Luc (control vector) were unique to each vector. For the RNR vector, the amplicon spanned from the distal region of the cTnT promoter, continuing into the proximal RNR1 subunit. For the μ Dys vector, the amplicon was contained within the CK8 regulatory

cassette, whereas the amplicon for the control vector resided within the hGH pA. hGH primers included: 5'-CACAACTTTGGCTCACTGCAA-3', 5'-GGAGGCTGAG GCAGGAGAA-3'; TaqMan probe: 5'-6FAM-CTCCGCC TCCTGGGTCAAGCG-MBGNQ-3'; CK8 RC primers: 5'-CCCAGATGCCTGGTTATAATT-3', 5'-CGGGAACAT GGCATGCA-3'; TaqMan probe: 5'-6FAM-CCCCCAAC ACCTGCTGCCTCT-MBGNQ-3'; cTnT455-RNR1 primers: 5'-CCCAGTCCCCGCTGAGA-3', 5'-AGGTTCCAGGCGCT GCT-3'; and TaqMan probe: 5'-6FAM-ACTCATCAATG TATCTTATCATG-MBGNQ-3'. Results were presented relative to DNA content in each 5- μ l DNA tissue sample to determine vector genomes per ng DNA.

TISSUE PROCESSING AND IMAGING ANALYSIS. Tissues were collected and analyzed 5 months post-administration of vectors and compared with age-matched male control vector (rAAV6- Δ CMV-Luc) injected *mdx*^{4cv} and WT mice. Hearts were either snap frozen in liquid nitrogen or were embedded in optimal cutting temperature compound (VWR International, Bridgeport, New Jersey) and flash frozen in liquid nitrogen cooled isopentane for histochemical or immunofluorescence analysis. The snap frozen samples were further processed by grinding to a powder under liquid nitrogen in a mortar kept on dry ice for subsequent extraction of nucleic acid and protein.

Heart cross-sections (10 μ m) were co-stained with antibodies raised against alpha 2-laminin (Sigma, St. Louis, Missouri; rat monoclonal, 1:200), the hinge-1 domain of dystrophin (alexa488 conjugated MAN-EX1011b, Developmental Studies Hybridoma Bank, University of Iowa, mouse monoclonal, 1:200), the human RRM1 (Abcam, Cambridge, United Kingdom; rabbit monoclonal, 1:200), and the human RRM2 (Abcam, rabbit monoclonal, 1:200). Conjugated secondary antibodies (Jackson Immuno, Goat anti-Rabbit) were used at a 1:500 dilution. Slides were mounted using ProLong Gold with DAPI (Thermo Fisher Scientific) and imaged via a Leica SPV confocal microscope. Confocal micrographs covering a majority of the heart left ventricular muscle sections were acquired and montaged via the Fiji toolset (ImageJ) and InDesign (Adobe, San Jose, California). For histology, Masson's trichrome staining was used to examine heart cross-sections. Briefly, 10- μ m muscle cryosections were sequentially stained in Wiegerts' iron hematoxylin (10 min), 1% Ponceau-acetic acid (5 min), and 1% aniline blue (5 s).

WESTERN BLOTTING. Radioimmunoprecipitation analysis buffer supplemented with 5 mM ethylenediaminetetraacetic acid and 3% protease inhibitor cocktail (Sigma, Cat# P8340), was used to extract muscle proteins for 0.5 h on ice with gentle agitation

every 10 min. Total protein concentration was determined using Pierce BCA assay kit (Fisher Scientific, Kent, Washington). Muscle lysates from WT, control *mdx*^{4cv}, and treated *mdx*^{4cv} (30 μ g) mice were denatured at 99°C for 10 min, quenched on ice, and separated via gel electrophoresis after loading onto Criterion 4-12% Bis-Tris polyacrylamide gels (BioRad). Overnight protein transfer to 0.45 mm polyvinylidene difluoride membranes was performed at constant 43 volts at 4°C in Towbin's buffer containing 20% methanol. Blots were blocked for 1 h at room temperature in 5% non-fat dry milk for 1 h before overnight incubation with antibodies raised against the hinge-1 region of dystrophin (Developmental Studies Hybridoma Bank, University of Iowa, 1:300), anti-RRM1 (Abcam, rabbit monoclonal, 1:1,000), anti-RRM2 (Abcam, rabbit monoclonal 1:1,000), and anti-GAPDH (Sigma, Rabbit polyclonal, 1:50,000). Horseradish-peroxidase conjugated secondary antibody staining (1:50,000) was performed for 1 h at room temperature before signal development using Clarity Western ECL substrate (BioRad) and visualization using a Chemidoc MP imaging system (BioRad).

QUANTIFICATION OF CARDIAC dATP. Approximately 25 μ g of flash frozen, freshly ground ventricle cardiac tissue was used for direct quantification of intracellular dATP using the high-performance liquid chromatography (HPLC)-with tandem mass spectrometry (MS/MS) method previously described (50). Briefly, samples were extracted 1 to 3 days before measurement using a 50% methanol solution. The supernatant was stored at -20°C until ready for injection into the HPLC-MS/MS system. A Water's Xevo-TQ-S mass spectrometer coupled with a Water's Acquity I-Class HPLC was used for the analysis (Milford, Massachusetts). Monitoring in negative mode via electrospray ionization was used to acquire MS/MS ions. dATP concentrations were quantified with standards and normalized to tissue weight.

LANGENDORFF ISOLATED PERFUSED HEART EXPERIMENTS. Ex vivo cardiac function was assessed in Langendorff isolated heart preparations as previously described (47,48,51). Hearts were perfused at a constant pressure of 80 mm Hg with a modified Krebs-Henseleit buffer supplemented with glucose and pyruvate. The perfusate contained (mmol/l): 118 NaCl, 25 NaHCO₃, 5.3 KCl, 2.0 CaCl₂, 1.2 MgSO₄, 0.5 ethylenediaminetetraacetic acid, 10.0 glucose, and 0.5 pyruvate, equilibrated with 95% O₂ and 5% CO₂ (pH 7.4). Temperature was maintained at 37.5°C throughout the protocol. Left ventricular (LV) function was monitored via a water-filled balloon inserted into the LV and connected to a pressure

transducer. LV systolic pressure (LVSP), end diastolic pressure, heart rate (HR), and minimum and maximum rate of pressure change in the ventricle ($\pm dP/dt$) were obtained from the attached data acquisition system (PowerLab, ADInstruments, Colorado Springs, Colorado). After 5 min of stabilization, hearts were equilibrated for 10 min at spontaneous HRs and then fixed at a HR of ~ 450 beats/min with an electrical stimulator (Grass Technologies, Warwick, Rhode Island). Pressure-volume relationships (i.e., Frank-Starling curves) were assessed by gradually increasing the volume of the LV balloon. After a 5-min recovery period, the perfusate was changed to an identical buffer as above except for the addition of 4.0 mmol/l $CaCl_2$ to simulate a high workload challenge for 20 min.

STATISTICAL ANALYSIS. All values are reported as means $\pm$ SEM. Starling curves and high workload function were analyzed by 2-way repeated measures analysis of variance followed by pairwise comparisons using Tukey's alpha adjustment method. Other endpoint data were analyzed via 1-way analysis of variance or Student's *t*-tests as appropriate. Kaplan-Meier methods were used to analyze survival curves and compared using the log-rank test. Statistical significance was tested at the $p < 0.05$ level. Statistical analyses were completed using Prism 7.0 (GraphPad Software, San Diego, California).

RESULTS

IMPROVEMENTS IN BASELINE CARDIAC FUNCTION IN VECTOR-TREATED *MDX*^{4cv} HEARTS. As depicted in Figure 1, 22- to 24-month-old *mdx*^{4cv} mice were administered 1 of 3 treatments: rAAV6-cTnT455- RNR (referred to as *mdx*^{4cv}+RNR); rAAV6-CK8- μ Dys (referred to as *mdx*^{4cv}+ μ Dys), or rAAV6- Δ CMV-Firefly Luciferase control vector (referred to as *mdx*^{4cv}) at a dose of 2×10^{14} vg/kg. By the end of the 20-week treatment period, both *mdx*^{4cv}+RNR and *mdx*^{4cv}+ μ Dys mice showed improvements in survival rates compared to *mdx*^{4cv} mice, although this did not reach statistical significance (Supplemental Figure 1). At the end of 5 months, an extensive evaluation of ex vivo cardiac function using the Langendorff isolated heart preparation was performed. The isolated heart technique allows for the direct assessment of inherent myocardial function without the confounding effects of neuro-humoral or other systemic variables. An additional cohort of age-matched, untreated C57BL6 mice (WT) was used as comparison control. At baseline, RPP was significantly decreased in *mdx*^{4cv} hearts due to an approximate 20% decrease in LVDevP (Supplemental Figures 1A and 1B). RNR-treated

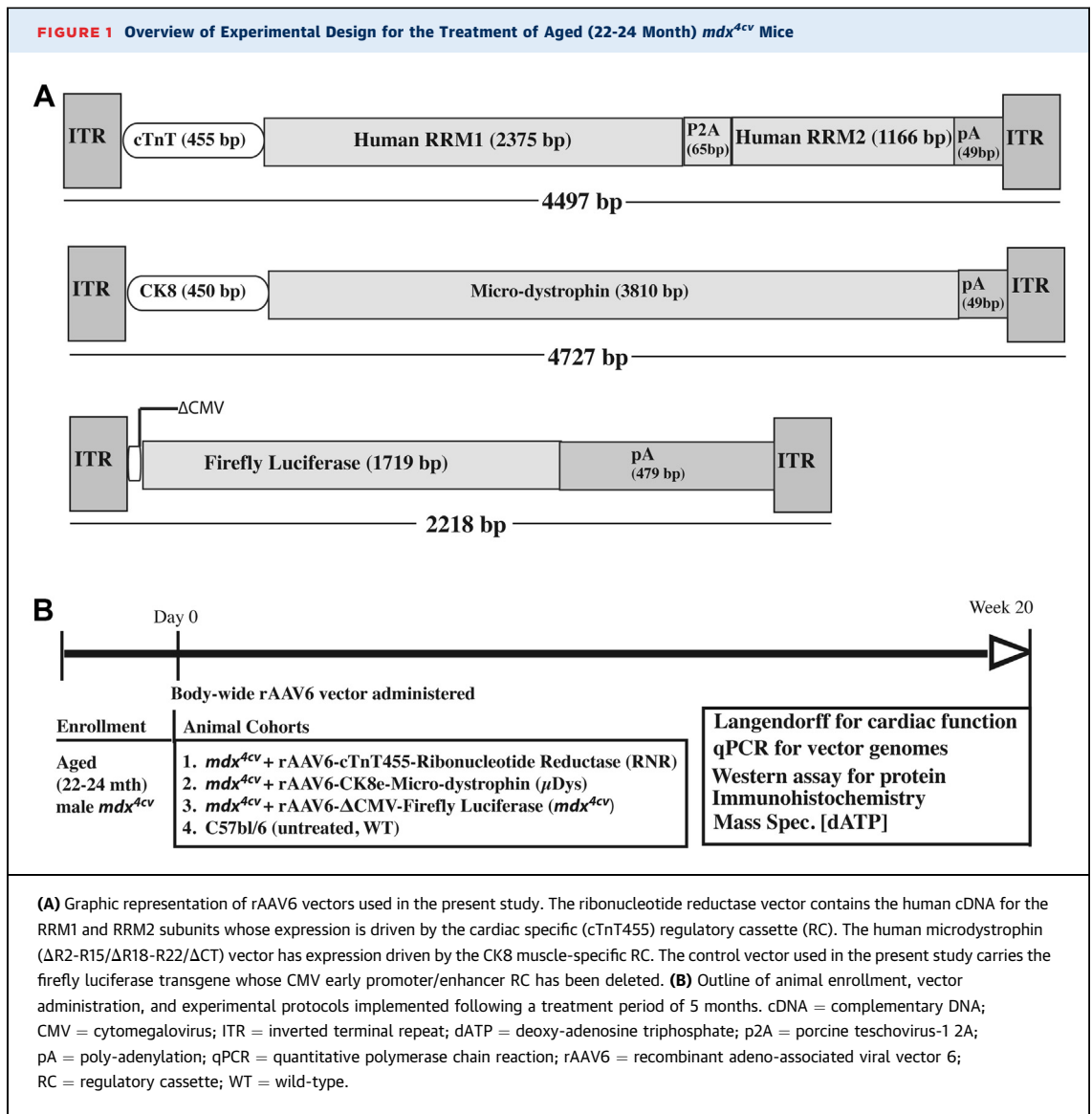
mdx^{4cv} mice exhibited a restoration of RPP ($p = 0.056$) primarily due to a normalization of LVDevP (Supplemental Figures 2A and 2B). Although μ Dys-treated *mdx*^{4cv} hearts appeared to normalize LVDevP, this did not lead to a significant improvement in RPP (Supplemental Figures 2A and 2B). Both $+dP/dt$ and $-dP/dt$, an index of ventricular contractility and relaxation, respectively, were decreased 30% in *mdx*^{4cv} hearts ($p = 0.061$). The $+dP/dt$ was similar to control in both RNR-treated *mdx*^{4cv} and μ Dys-treated *mdx*^{4cv} hearts. However, only RNR-treated *mdx*^{4cv} hearts showed $-dP/dt$ values similar to control levels (Supplemental Figures 2C and 2D).

POSITIVE CHANGES IN FRANK-STARLING MECHANISMS IN VECTOR-TREATED *MDX*^{4cv} HEARTS.

To evaluate further systolic and diastolic function in vector-treated *mdx*^{4cv} hearts, we examined the pressure-volume relationship (i.e., Frank-Starling mechanism) in the isolated perfused heart preparation. The LVSP response to increased preload was significantly improved in both in *mdx*^{4cv}+RNR and *mdx*^{4cv}+ μ Dys hearts compared to *mdx*^{4cv} (Figure 2A). However, only RNR treatment improved the diastolic response in *mdx*^{4cv} hearts, to levels similar to WT (Figure 2B). Both contractility and relaxation (i.e., $+dP/dt$ and $-dP/dt$, respectively) were impaired in *mdx*^{4cv} compared to age-matched control subjects (Figures 2D and 2E). Both *mdx*^{4cv}+RNR and *mdx*^{4cv}+ μ Dys hearts had significantly elevated $+dP/dt$ values above *mdx*^{4cv} (Figure 2D). Interestingly, treatment of *mdx*^{4cv} hearts with RNR also significantly improved $-dP/dt$ values (Figure 2E). All told, these data suggest that both RNR and μ Dys treatment can improve systolic function in *mdx*^{4cv} hearts. However, only the RNR treatment corrected diastolic dysfunction in *mdx*^{4cv} hearts.

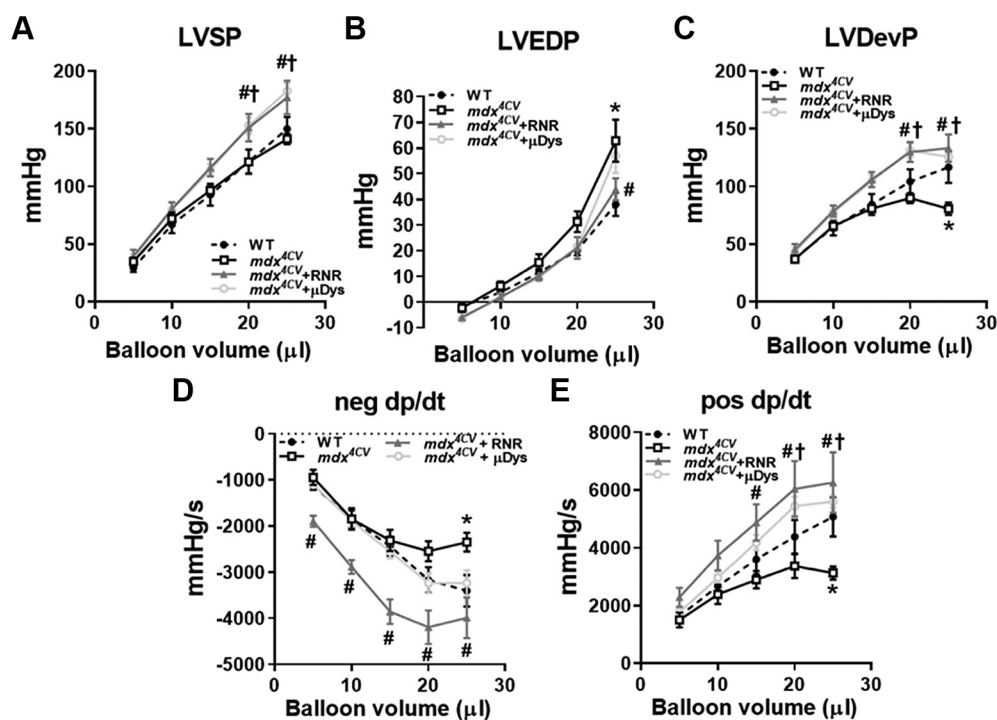
AUGMENTED RESPONSE TO INCREASED CARDIAC WORKLOAD IN TREATED *MDX*^{4cv} HEARTS.

We previously reported that RNR overexpression in transgenic or vector-treated mouse hearts elevated baseline function but did not impair the response to a short-term physiologic increase in cardiac work (47,48). To verify that the improved systolic and diastolic function in RNR-treated *mdx*^{4cv} hearts at baseline was not associated with an inability to respond to an increased energetic demand, we stressed hearts with a combination of high calcium and elevated heart rates, via pacing stimulation. As shown in Figures 3A and 3B, *mdx*^{4cv} hearts had a blunted response to the increased workload as both LVDevP and RPP were $\sim 25\%$ to 30% lower than WT hearts. In addition, both $+dP/dt$ and $-dP/dt$ were impaired in *mdx*^{4cv} relative to WT hearts (Figures 3C and 3D). Systolic



parameters in *mdx*^{4cv}+ μ Dys hearts were effectively improved and similar to age-matched WT hearts for the entire duration of the workload challenge (Figures 3A to 3C). Measures of systolic function significantly increased in *mdx*^{4cv} + RNR hearts during the initial half of the high workload protocol and remained ~15% higher than *mdx*^{4cv} (Figures 3A to 3C). Interestingly, -dP/dt values tended to be elevated only in *mdx*^{4cv} + RNR hearts during the physiologic challenge (Figure 3D). These data show that both RNR and μ Dys treatments improve systolic function in *mdx*^{4cv} hearts without compromising cardiac reserve. Combined with the baseline and pressure-volume relationship assessments, our data show that, in addition to the systolic enhancements, RNR has an added benefit of improving diastolic function.

RNR AND μ Dys TRANSDUCTION, EXPRESSION, AND CARDIOMYOCYTE LOCALIZATION. To evaluate the localization of RNR and μ Dys protein within the hearts of mice, we performed immunofluorescence imaging. As shown in Figure 4, the RNR subunit (Rrm1) was robustly expressed in ventricles of RNR-treated mice. The expression of μ Dys appeared to be saturated relative to full-length dystrophin levels, with both being properly localized to the sarcolemma of cardiomyocytes. We also evaluated general muscle histopathology and potential differences in myocardial fibrosis by Masson trichrome staining, and observed no discernable difference between treated or untreated *mdx*^{4cv} mice (Figure 5). In addition, neither RNR nor μ Dys treatment significantly altered body weight, heart weight, or the heart weight-to-body

FIGURE 2 Pressure-Volume Relationships in Isolated Perfused Hearts From Vector-Treated *mdx*^{4cv} Mice

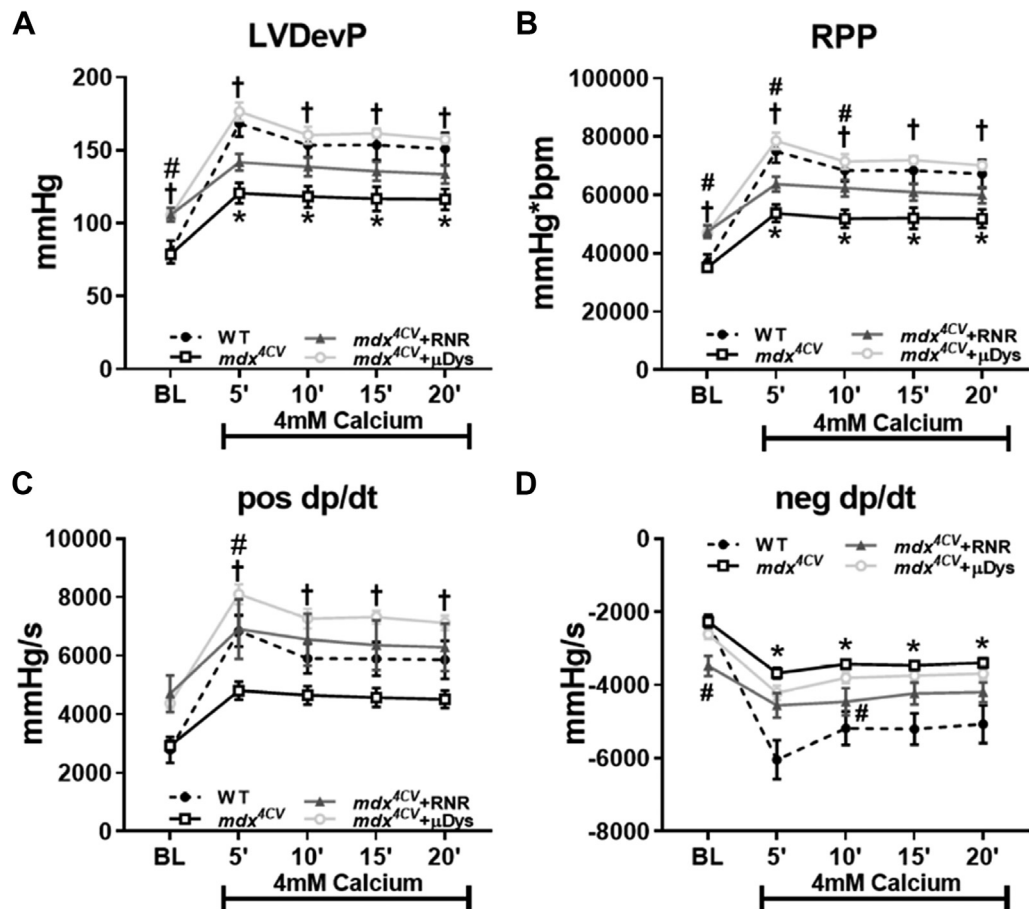
Hearts were isolated from *mdx*^{4cv} mice treated with control vector (*mdx*^{4cv}, *n* = 6), ribonucleotide reductase (*mdx*^{4cv} + RNR, *n* = 6), or microdystrophin (*mdx*^{4cv} + μ Dys, *n* = 5). Age-matched, nondiseased, nontreated wild-type (WT) mice were used as control subjects (*n* = 8). The pressure-volume relationship (i.e., Frank-Starling mechanism) was evaluated by gradually increasing the volume of the left ventricular (LV) balloon. Hearts were paced at 450 beats/min throughout the protocol. (A) Left ventricular systolic pressure (LVSP). (B) LV end-diastolic pressure (LVEDP). (C) LV developed pressure (LVDevP, the difference between systolic and diastolic pressures). (D) Positive (pos) rate of pressure change was calculated by the first derivative of the ascending LV pressure wave (+dP/dt), and is used as an index of ventricular contractility. (E) Negative (neg) rate of pressure change calculated by the first derivative of the descending LV pressure wave (-dP/dt), and is used as an index of ventricular relaxation. * *p* < 0.05 *mdx*^{4cv} versus WT; # *p* < 0.05 *mdx*^{4cv} + RNR versus *mdx*^{4cv}; † *p* < 0.05 *mdx*^{4cv} + μ Dys versus *mdx*^{4cv}.

weight ratio (Supplemental Figure 3). Western blotting was performed to determine the extent of rAAV vector 6-mediated RNR and μ Dys protein expression profiles in ventricular tissue (Figure 6). We observed μ Dys protein expression in ventricular tissue that approached levels similar to WT mice, whereas both human RNR subunits were found to be elevated to comparable levels within ventricular tissue (Figure 6A). To evaluate the relative proportions of dATP concentrations within ventricular tissue, we performed HPLC-MS/MS analysis on ground ventricular tissue from *mdx*^{4cv} and *mdx*^{4cv} + RNR mice. The concentration of dATP within the ventricular tissue obtained from *mdx*^{4cv} mice treated with RNR (0.57 ± 0.22 pmol dATP/mg) was approximately 10-fold higher relative to *mdx*^{4cv} control subjects (0.05 ± 0.02 pmol dATP/mg) (Figure 6B). For adult WT, we previously reported an average dATP value of 0.02 pmol/mg tissue with a SD of 0.01 (50). Additionally,

cardiac vector genome data was comparable relative to the vector dose administered (Figure 6C).

DISCUSSION

In the present study, we used a novel gene therapy approach that targets myosin in the contractile filaments of cardiomyocytes via overexpression of the RNR enzyme to rescue cardiomyopathy in a DMD mouse model. RNR overexpression results in elevated dATP, which can be used by cardiac myosin (in place of ATP), and increases cross-bridge binding and cycling, resulting in stronger, faster contraction and faster relaxation (38-45,52). We developed an rAAV vector that over-expresses RNR selectively in heart muscle via inclusion of a modified cardiac-specific enhancer/promoter derived from the human cTnT gene, which facilitates increases in myocyte contraction and cardiac performance in normal rodent hearts

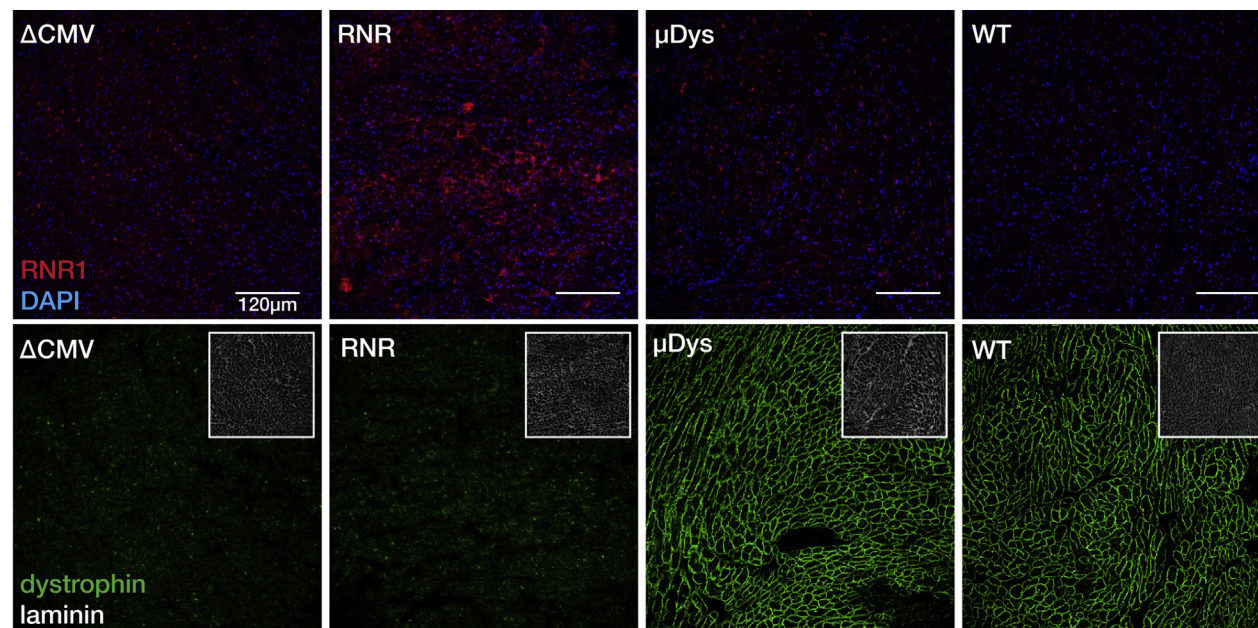
FIGURE 3 The Response of Vector-Treated *mdx*^{4cv} Mice to High Workload Challenge in Langendorff Isolated Heart Preparations

Hearts were isolated from *mdx*^{4cv} mice treated with control vector (*mdx*^{4cv}, *n* = 6), ribonucleotide reductase (*mdx*^{4cv} + RNR, *n* = 6), or microdystrophin (*mdx*^{4cv} + μ Dys, *n* = 5). Age-matched, nondiseased, nontreated wild-type (WT) mice were used as control subjects (*n* = 4). All hearts were perfused with a glucose-pyruvate buffer containing high calcium (4.0 mmol/l) to simulate a high workload challenge for 20 min. Hearts were paced at 450 beats per minute throughout the protocol. (A) LVDevP (the difference between systolic and diastolic pressures). (B) Rate pressure product (RPP, the product of LVDevP and HR). (C) Positive rate of pressure change calculated by the first derivative of the ascending LV pressure wave (+dP/dt), is used as an index of ventricular contractility. (D) Negative rate of pressure change calculated by the first derivative of the descending LV pressure wave (-dP/dt), is used as an index of ventricular relaxation. * *p* < 0.05 *mdx*^{4cv} versus WT; # *p* < 0.05 *mdx*^{4cv} + RNR versus *mdx*^{4cv}; † *p* < 0.05 *mdx*^{4cv} + μ Dys versus *mdx*^{4cv}. Abbreviations as in Figures 1 and 2.

as well as in infarcted rodents and pig hearts (48,53). Importantly, we previously showed that dATP-producing cells deliver it to surrounding myocardium by diffusion through gap junctions (54), such that only a minority of cardiomyocytes needs to be transduced to have global benefits within the heart. We now show that using this vector technology leads to a clear benefit of improving cardiac function in an advanced-age model of DMD cardiomyopathy.

Early manifestations of impaired cardiac dysfunction in *mdx* mice are generally not reported. Khairallah et al.(55) observed a decline in LV function in isolated perfused hearts that coincided with

decreased fatty acid oxidation and increased glucose oxidation. However, perfused heart function in *mdx* mice was maintained at 8 months, despite significant reductions in phosphocreatine levels and free energy availability from ATP hydrolysis (56). The authors surmised that young adult *mdx* hearts, akin to DMD patient hearts, experience right ventricular dilation, LV diastolic deficits, and abnormal energy metabolism. More recently, a declination in cardiac function was not observed until 12 months of age (57) and abnormal in vivo pressure-volume dynamics were observed in 22-month-old *mdx* mice (58). Treatment of young male *mdx* mice with rAAV6 vector delivering

FIGURE 4 Cardiac Transduction Following Intravenous Delivery of Ribonucleotide Reductase or Microdystrophin

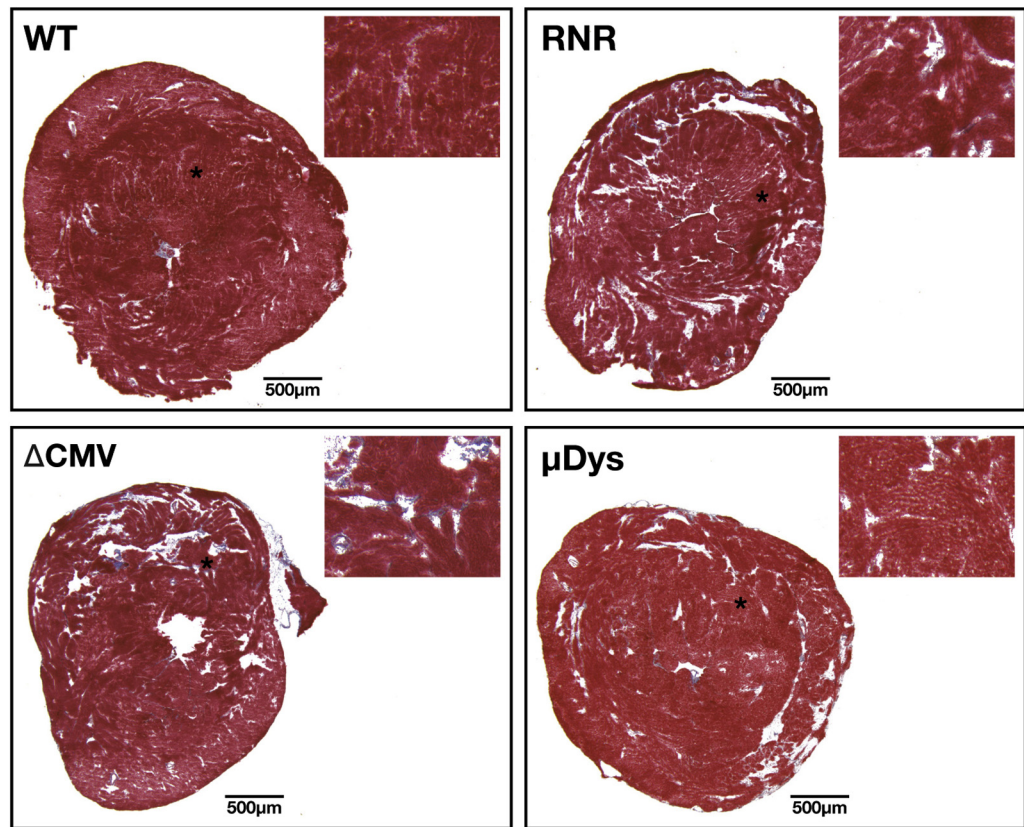
At 5-months after vector administration, cryosections were prepared and immunostained with antisera against dystrophin or ribonucleotide reductase. A considerable level of protein is detected for each ribonucleotide reductase subunit-1 (human specific) as indicated by immunofluorescent staining (**red**) localized primarily within the cytoplasm of cardiomyocytes with occasional perinuclear accumulation (**upper panel**). On the lower panel, the robust level of expression for dystrophin in WT and in aged *mdx*^{4cv} mice treated with AAV6-CK8-μDys (laminin staining, **inset image**). Abbreviations as in [Figures 1 and 2](#).

cytomegalovirus (CMV) promoter/enhancer driven microdystrophin did not correct the impairments in +dP/dt, -dP/dt, or LV systolic pressure when assessed at 5 months of age via hemodynamic analysis (35). However, an improvement was noted for the preload recruited stroke work in *mdx* mice treated with μDys compared to untreated *mdx* or WT control subjects (35).

In our current study, 27-month to 29-month-old mice were subjected to ex vivo assessment of cardiac function using the Langendorff isolated heart preparation, which is a century-old methodology with several advantages and limitations (59). The perfusate used is similar to, but not the same as blood, and situations of physiologic challenge (e.g., Frank-Starling and high calcium) may exaggerate the in vivo situation. However, the procedure remains a simple and reproducible experiment that allows for interrogation of cardiac physiology in the absence of confounding systemic variables and serves as a viable tool to perform cardiac phenotyping in preclinical studies (60). Under baseline conditions and at spontaneous heart rates, we observed a significant reduction in RPP in *mdx*^{4cv} hearts. Decreased systolic and diastolic performance in *mdx*^{4cv} hearts also existed while examining the length-tension

relationship (i.e., Frank-Starling mechanism). Treatment of *mdx*^{4cv} mice with either the RNR or the μDys vector restored systolic pressure development without affecting spontaneous HRs at baseline. Both of the treatments restored the disrupted Frank-Starling response, particularly for systolic function. The RNR treatment also had beneficial effects on the diastolic properties of the *mdx*^{4cv} myocardium. This strongly supports our previous studies where we observed a tendency for overexpression of RNR to improve myocardial diastolic response in young, healthy, transgenic mice (47), mice treated with AAV (48), and in a porcine HF model (53).

In addition to the evaluation of basal cardiac performance, we tested cardiac reserve with a combination of high calcium plus elevated HRs and confirmed a significantly blunted response in 27-month to 29-month-old *mdx*^{4cv} hearts. Consistent with a previous report in young *mdx* mice (35), expressing μDys in aged *mdx*^{4cv} hearts significantly improved systolic performance during increased physiologic demand to a level similar to age-matched healthy control subjects. We previously reported that transgenic or vector-directed overexpression of RNR did not impair cardiac reserve during increased physiologic demand

FIGURE 5 Heart Histologic Staining of *mdx*^{4cv} Mice Suggests no Morphologic Alterations From RNR Therapy

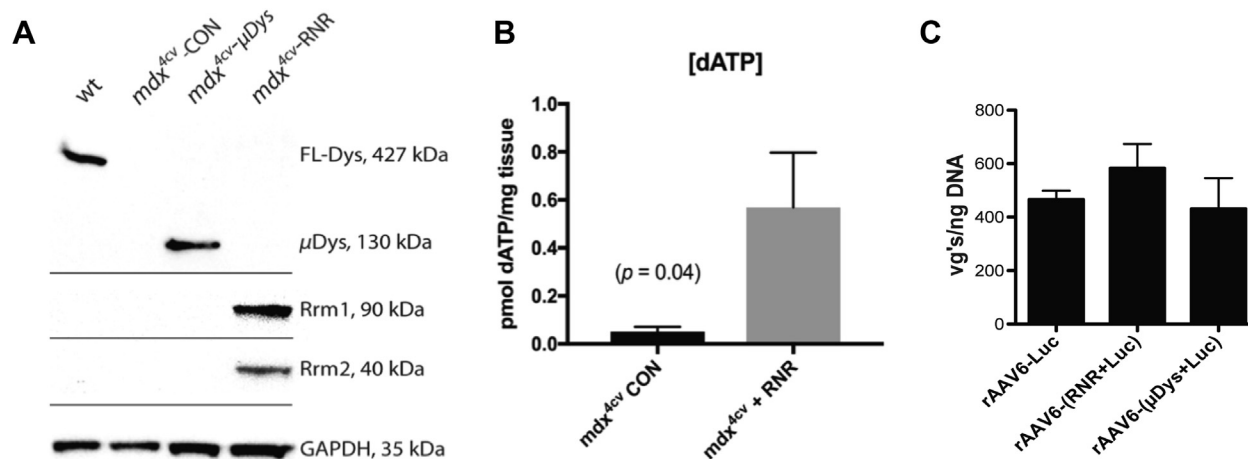
Representative full-view photomicrographs of Masson trichrome staining of the hearts from *mdx*^{4cv} mice displaying control vector (Δ CMV), and rAAV6-treated with either RNR or μ Dys from *mdx*^{4cv} mice. Similarly, a 20 $\times$ enlarged view of the corresponding images (*) is shown. Abbreviations as in [Figures 1 and 2](#).

in young healthy hearts (47,48). Herein, we report that RNR over-expression in *mdx*^{4cv} hearts normalizes both the systolic and diastolic response to an increased cardiac challenge.

Approximately two-thirds of DMD mutations are deletions that span 1 or more exons, often leading to large deletions clustering around 2 mutational hotspots; the most common spanning from exons 45-55 resulting in removal of a central portion of the rod domain inclusive of disrupting the neuronal nitric oxide synthase (nNOS) localization domain. The second most common hotspot spans from exons 3-19, and removes the majority of the amino-terminal actin-binding domain 1 which is essential for dystrophin function. When such deletions interrupt the reading frame, the resultant mutated protein usually expresses at extremely low levels and associates with the loss of ambulation at or before 12 years of age. Even when such deletions produce an in-frame partially functional truncated protein, such as occurs

in BMD, dramatic phenotype diversity can occur. For example, in a previous study, BMD subjects whose out-of-phase deletions, toward the alignment of adjoining spectrin-like repeats, developed dilated cardiomyopathy at nearly a decade earlier than in patients with in-phase deletions (61). In contrast to large deletion mutations, there are reported cases where individual missense mutations lead to X-linked dilated cardiomyopathy (62-64). In one case, a novel missense mutation within exon 9 at nucleotide 1043 resulted in a T279A amino acid change in a highly conserved position of mutation. This mutation resulted in a substitution of a beta-sheet for alpha-helical structure, destabilizing the protein, and leading to the cardiac specific phenotype described by the authors (63).

DMD-related cardiomyopathy usually occurs during middle to late adolescence, but the clinical presentation is deceptive, as patients are wheelchair-bound and are not required to perform increased

FIGURE 6 Protein Expression Levels for μ Dys, RNR, and Resultant Vector Genomes Due to rAAV6-Mediated Gene Transfer

(A) RNR and μ Dys protein expression detection as revealed by immunoblotting of cardiac whole tissue lysates using either RRM1, RRM2, or antidystrophin antibody. (B) HPLC-MS/MS intracellular [dNTP] quantification from methanol extracted cardiac tissue. (C) qPCR analysis of vector genomes from cardiac tissue revealed similar vector genomes being represented for all treated cohorts. dNTP = deoxynucleotide triphosphate; HPLC-MS/MS = high-performance liquid chromatography-tandem mass spectrometry; qPCR = quantitative polymerase chain reaction; other abbreviations as in Figures 1 and 2.

cardiac workload. The diversity of cardiac phenotype in DMD suggests many levels of pathogenic mechanisms. The pathologic heterogeneity of the DMD ventricular myocardium is a consequential result of myocardial atrophy (65,66), compensatory mechanisms leading to cardiac remodeling with ensuing ventricular dilation and fibrosis (67,68). Absence of clear genotype-phenotype correlation in DMD probably results from at least 4 secondary cellular processes including aberrant intracellular Ca^{2+} homeostasis, decreased nitric oxide-cyclic guanosine monophosphate pathways, mitochondrial dysfunction, and increased reactive oxygen species, which individually or collectively influence the clinical phenotype (69). As an indication of the progressive nature of DMD cardiomyopathy, the estimated overall incidence of latent DCM is 25% by 6 years of age and 59% by 10 years of age in DMD patients (70). As such, further evidence suggests latency in cardiac dysfunction at basal levels that becomes more pronounced with an induction of increased physiological demand. Consistent with these observations, an impaired response to beta-adrenergic stimulation by either dobutamine or isoproterenol can be detected in *mdx* mice at 3 to 4 months of age (35,71). Exercise can potentially be a double-edged sword for DMD, where the enthusiasm for potential skeletal muscle benefits can be dampened by the insidious cardiotoxicity from training (72-74). With gene replacement therapy via rAAV-mediated μ Dys in current ongoing clinical trials,

it will be of interest to assess cardiac function to determine whether additional therapeutic support in the form of increased contractility will be beneficial.

Relative expression assays by immunofluorescence and Western blotting indicate robust expression levels for the RNR subunits (RRM1 and RRM2) and μ Dys in the vast majority of cardiomyocytes. However, intracellular variability in RNR protein detection (via immunostaining) was observed for both RRM1 and RRM2, indicating that not all cardiomyocytes were effectively transduced. Nevertheless, we previously have shown that gap junction transport of dATP from transduced cardiomyocytes to adjacent cardiomyocytes occurs (54), which likely accounted for the consistently elevated LV function of mice treated with the rAAV vector that over-expresses RNR. This is in contrast to the anchored sarcolemma localization of μ Dys, which would occur only in transduced muscle cells. Additionally, because muscle-specific regulatory cassettes were used for expression of therapeutic proteins, we can surmise that <1% of protein expression would be generated from non-muscle cell types present in the heart, suggesting that the functional benefit was truly caused by cardiomyocyte transduction. As previously stated, the cTnT regulatory cassette drives RNR expression only in heart muscle, whereas the CK8 regulatory cassette drives μ Dys expression no selectively in both cardiac and skeletal muscle cells, but at only very low background levels in other tissues. This

raises the possibility that the functional benefit of expressing μ Dys in skeletal muscles might increase energetic demands on the heart, thereby partially masking some of the μ Dys-derived cardiac functional benefits (37). Because RNR delivery to DMD mice of advanced age increased cardiac function in the absence of RNR expression in skeletal muscle, and because over-expression of endogenous RNR should be nonimmunogenic, supplemental RNR therapy in conjunction with μ Dys therapy might be beneficial for DMD patients. Future studies are aimed at determining the extent of functional restoration by combining both RNR- and μ Dys-based therapies.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: RNR over-expression represents an emerging therapy for improving cardiac dysfunction via cardiac myosin activation in a variety of clinical situations. Currently, there are 3 ongoing clinical trials using rAAV-mediated variants of μ Dys that have shown broad promise in terms of safety, and in biomarker indicators suggesting pathologic improvements. If cardiac dysfunction is detected in the patients from these clinical studies, our studies in aged, *mdx* mice suggest that combinatorial RNR therapy may be beneficial.

TRANSLATIONAL OUTLOOK: Our novel strategy of vector-mediated, cardiac-specific over-expression of RNR shows promising potential for rescuing both systolic and diastolic function in aged, DMD mice even in the absence of addressing the underlying dystrophin deficiency. Future studies investigating the combined effects of μ Dys and RNR may disclose an improved therapeutic strategy to provide structural and functional improvement of DMD patient hearts.

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KEY WORDS cardiomyopathy, diastolic dysfunction, dystrophin, ribonucleotide reductase, recombinant adeno-associated virus vectors

APPENDIX For supplemental figures, please see the online version of this paper.
