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14. ABSTRACT

Dystrophin protein is absent in patients with DMD, whereas it is variable in molecular weight and abundance in patients with Becker Muscular Dystrophy (BMD). After treating DMD patients with exon skipping drugs to restore dystrophin, the amount of rescued dystrophin in muscle is variable, mirroring what is observed in BMD muscle. To move DMD exon skipping therapies forward, it is imperative that we understand this variability. We recently showed that the pro-inflammatory microRNA, miR-146a, prevents dystrophin production and is much higher in dystrophic versus healthy muscle. In dystrophic mice, we also found miR-146a levels to coincide with worsening of disease state. Treating dystrophic mice with antiinflammatory drugs (that inhibit NF B-driven inflammation) reduces miR-146a and improves disease progression. These data suggests a model where the pro-inflammatory state in dystrophic muscle activates miR-146a, exacerbating DMD disease and leading to progressive pathology.

15. SUBJECT TERMS- Postdoctoral fellowships research, bio-medical research, training, education

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INTRODUCTION

Cutting edge precision medicine drugs which act through a mechanism known as exon skipping are currently in clinical trials for Duchenne muscular dystrophy (DMD). Their goal is to turn DMD, the most common fatal genetic muscle disease, into BMD which is a milder disease caused by different mutations in the same (dystrophin) gene. DMD is caused by mutations that completely prevent dystrophin protein production while BMD is caused by mutations that preserve dystrophin protein. BMD-causing mutations produce a truncated dystrophin protein. The extent of truncated dystrophin in BMD is variable both between adjacent muscle fibers in the same patient, and between different patients. Interestingly, variability of dystrophin protein in BMD mirrors the variability observed in dystrophin rescue in pre-clinical and clinical DMD exon skipping trials. The inconsistency and unevenness in exon skipping dystrophin rescue is a significant and critical barrier to obtaining new drug approval. Highlighting this, on April 25th, an FDA advisory panel voted against accelerated approval of the most promising exon skipping drug, eteplirsen, due to low and uneven dystrophin rescue in a Phase IIb Clinical Trial. Following this, the FDA delayed a final decision about the fate of the drug, before then granting accelerated approval on September 19th. The FDA advisory committee recommendation and the delayed FDA decision to grant eteplirsen accelerated approval highlights the urgent need to characterize the factors responsible for variability in dystrophin restoration in DMD exon skipping. In the proposed work, our major goals are: 1) to understand how an inflammatory microRNA, miR-146a, contributes to variability in dystrophin levels and 2) to move towards development of therapeutic miR-146a inhibition in DMD for which we have a grated patent (US Patent 9,637,738). This application aligns with the DMDRP focus area, "Expansion of existing preclinical translational data in support of a specific therapeutic development path."

Recently, we discovered that an inflammatory microRNA, miR-146a, directly inhibits dystrophin protein production (Fiorillo, Heier et al. 2015). miR-146a negatively correlates with dystrophin levels in BMD muscle and in exon-skipping treated DMD muscle. miR-146a increases with DMD disease severity, while inhibitors of NFκB-driven inflammation significantly reduce miR-146a. Elevated miR-146a is also observed in other inflammatory conditions(Eisenberg, Eran et al. 2007; Panguluri, Bhatnagar et al. 2010; Lu, Yan et al. 2013; Olivieri, Lazzarini et al. 2013), suggesting it plays a distinct role in chronic inflammation. Interestingly, dystrophin reduction has been observed in disorders where both dysregulated NFκB signaling and chronic inflammation are present (Vainzof, Passos-Bueno et al. 1996; Acharyya, Butchbach et al. 2005; Townsend, Daly et al. 2011). Taken together, these data suggest miR-146a is a key mediator of chronic inflammatory signaling, dystrophin production and DMD disease pathogenesis. These converging roles provide a unique opportunity to determine how miR-146a

contributes to DMD disease progression and to explore the potential for therapeutic targeting of miR-146a as a precision medicine strategy in DMD and BMD.

Hypothesis & Objective. We will test the **hypothesis** that miR-146a drives and perpetuates a pro-inflammatory microenvironment in DMD through chronic NF-κB activation and dystrophin reduction. Our major goal is to understand how miR-146a impacts DMD disease progression and to move towards development of therapeutic miR-146a inhibition in DMD and BMD with the goal of increasing dystrophin expression in muscle. This is particularly timely and important in light of the FDA's decision to grant accelerated approval to the most promising exon skipping drug, eteplirsen.

KEYWORDS

Duchenne Muscular Dystrophy (DMD), microRNAs, exon skipping, dystrophin, muscle inflammation, NF-κB, miR-146a

ACCOMPLISHMENTS

a. What were the major goals of the project?

Major Task 1: Mentoring & Committee Meetings	**Months**	**Completion date and/or percentage completion**
Subtask 1: Meet weekly with Primary Mentor	1-24	50% complete, ongoing
Subtask 2: Meet monthly with Co-mentors	1-24	50% complete, ongoing
Subtask 3: Meet every 6 months with mentoring committee	1-24	50% complete, ongoing
Milestone(s) Achieved: Bi-directional evaluation of career development plan and mentorship	24	50% complete, ongoing
Major Task 2: Didactic & Leadership Training	**Months**	**Completion date and/or percentage completion**
Subtask 1: Attend EMBO Female Leaders in Science Course	1-6	Completed May 1st 2017
Subtask 2: Attend The Jackson Laboratory Course: Pathology of Mouse Models of Human Disease	6-12	Not completed, course only allows clinicians and not PhD scientists and did not communicate this in. I plan on looking into an NIH course that does the

		same thing
Subtask 3: Take GWU Course in Immunology	12-24	Changed, completed - I have instead participated in an inter-Departmental workshop on muscle immunology at CNMC in the Fall of 2017.
Milestone (s) Achieved: Comprehensive training in new fields (immunology and comprehensive mouse disease models) and leadership skills achieved.	24	Partially completed, in progress
Major Task 3: Interdisciplinary Seminars	**Months**	**Completion date and/or percentage completion**
Subtask 1: Attend weekly and monthly seminars at CNMC	1-24	50% completed, ongoing
Subtask 2: Attend weekly Partridge Laboratory meetings	1-24	50% completed, ongoing
Milestone (s) Achieved: Present 2x per year at both departmental seminars and laboratory meetings	24	50% completed, ongoing
Major Task 4: Grant Writing and Career Skills	**Months**	**Completion date and/or percentage completion**
Subtask 1: Attend CTSI Grants Enhancement Seminars	1-24	50% completed, ongoing
Subtask 2: Work with CTSI on putting together R01 Submission	12-24	Not yet completed
Milestone (s) Achieved: 3 grant submissions at the end of the award period; R01 in preparation by end of award period.	24	Not yet completed
Major Task 5: Conferences	**Months**	**Completion date and/or percentage completion**
Attend one international conference per year, (Molecular Mechanisms of Muscle Wasting and Disease, PPMD, MDA Scientific Conference).	1-24	September 2017, Attended Ottawa NMD Conference and presented poster; September 2018 attended Molecular Mechanisms of Muscle Wasting and gave oral

		presentation
Milestone(s) Achieved: Presentation at 2 international conferences	24	Completed

Research-Specific Tasks:

Specific Aim 1: Define the role of miR-146a in inflammatory/dystrophic disease etiology		**Note: We decided to perform specific Aim 2 first**
Major Task 1: Determine if miR-146a inhibition (AAV9 miR-146a-sponge) improves *mdx* muscle pathology & function.	**Months**	**Completion date and/or percentage completion**
Subtask 1: AAV9 design and generation; generate AAV9-miR-146a sponge construct Core used: UPenn Vector Core facility	1-6	50% complete, AAVs designed and being tested
Subtask 2: Test the efficacy of 146a-sponge *in vitro* by co-transfection with a Traf6 3′UTR reporter	6-8	Currently being tested with new strategy as Traf6 3'UTR reporter was yielding inconclusive results
Subtask 3: Perform AAV9-miR-146a sponge delivery in mdx and WT mice 2 groups x 12 mice/group	8-12	Not yet completed
Milestone(s) Achieved: Determination of how overexpression or loss of miR-146a affects DMD disease pathogenesis	12	Not yet completed
Major Task 2: Determine if miR-146a overexpression via rAAV9 exacerbates muscle pathology and function in *mdx* mice.	**Months**	**Completion date and/or percentage completion**
Subtask 1: AAV9 design and generation; clone the murine pre-miR-146a sequence into a AAV9-CMV-Luciferase vector (AAV9-miR-146a); Core used: UPenn Vector Core facility	1-6	Not yet completed
Subtask 2: Perform AAV9-miR-146a delivery in mdx and WT mice 2 groups x 12 mice/group	6-12	Not yet completed
Milestone(s) Achieved: Determination of how miR-146a overexpression affects inflammation and functional outcomes in mdx mice; manuscript submission	12	Not yet completed
Major Task 3: Create mdx52/miR-146aKO mice	**Months**	**Completion date and/or percentage**

		completion
Subtask 1: Breed mice and genotype	12-24	25% complete, ongoing; 146a-/- mice were obtained and are being bred
Subtask 2: Perform phenotyping, functional assays	12-24	Not yet completed
Specific Aim 2: Determine if miR-146a impacts PMO-based therapeutic dystrophin rescue and muscle function.		
Major Task 1: *Co-delivery trial: exon-skipping antisense + miR-146a antagomiR (both PMO chemistry)*	**Months**	**CNMC**
Subtask 1: Perform trial 8 mice/group Groups will be: 1) *mdx*, 2) *mdx* + PMO (25 mg/kg/wk IV), 3) *mdx* PMO + CTRL antagomiR (200mg/kg/wk IV), 4) *mdx* PMO + miR-146a antagomiR (200mg/kg/wk IV).	12-18	Dec 2017 Completed, second trial planned
Subtask 2: Perform Phenotyping and end-point assays	12-18	Dec 2017 Completed, second trial planned
Subtask 3: Perform dystrophin quantification and post-sacrifice analysis	18-24	Dec 2017 Completed, second trial planned
Milestone(s) Achieved: Determine outcomes of miR-146a inhibition in exon skipping; manuscript submission	24	Dec 2017 Completed trial, second trial planned, manuscript being prepared

b. What was accomplished under these goals?

Mentoring and Committee Meetings

- I have met my primary mentor weekly, my co-mentors monthly, and have had one committee meeting.

Didactic & Leadership Training

- I have attended the EMBO Women in Science Leadership Course and I have participated in a muscle inflammation workshop within the Center for Genetic Medicine Research at CNMC.

Interdisciplinary Seminars

- I have presented once at a Departmental seminar, once at Partridge Lab Meeting and have additionally conducted my own weekly lab meetings with my lab manager, technician and faculty member Christopher Heier.

Grant Writing and Career Skills

- I have attended grants enhancement seminars, and will begin attending Departmental Seminars for grant writing

- I am in the process of putting together a foundation grant submission (November 2018) the resubmission of an NIH K01 grant (March 2019).

Conferences

- I have attended the Ottawa Neuromuscular Diseases Conference (Sept 2017) and the Ascona Molecular Mechanisms of Muscle Wasting and Disease Conference (Sept 2018).

Research:

Specific Aim 1: Define the role of miR-146a in inflammatory/dystrophic disease etiology

- We have designed inhibitor sequences for miR-146a inhibition under control of a U6 promoter. We are currently subcloning this into an AAV cloning vector and will work with the Vector Core and Jeff Chamberlain to produce AAV to inject into mice.

- We have created a Luciferase construct with 5xmiR-146a binding sites to check for the efficiency of miR-146a inhibition in vitro.

- We have started to cross mdx52 mice to miR-146a$^{-/-}$ mice

Specific Aim 2: Determine if miR-146a impacts PMO-based therapeutic dystrophin rescue and muscle function.

- We have performed Trial from Aim 2 (data below)

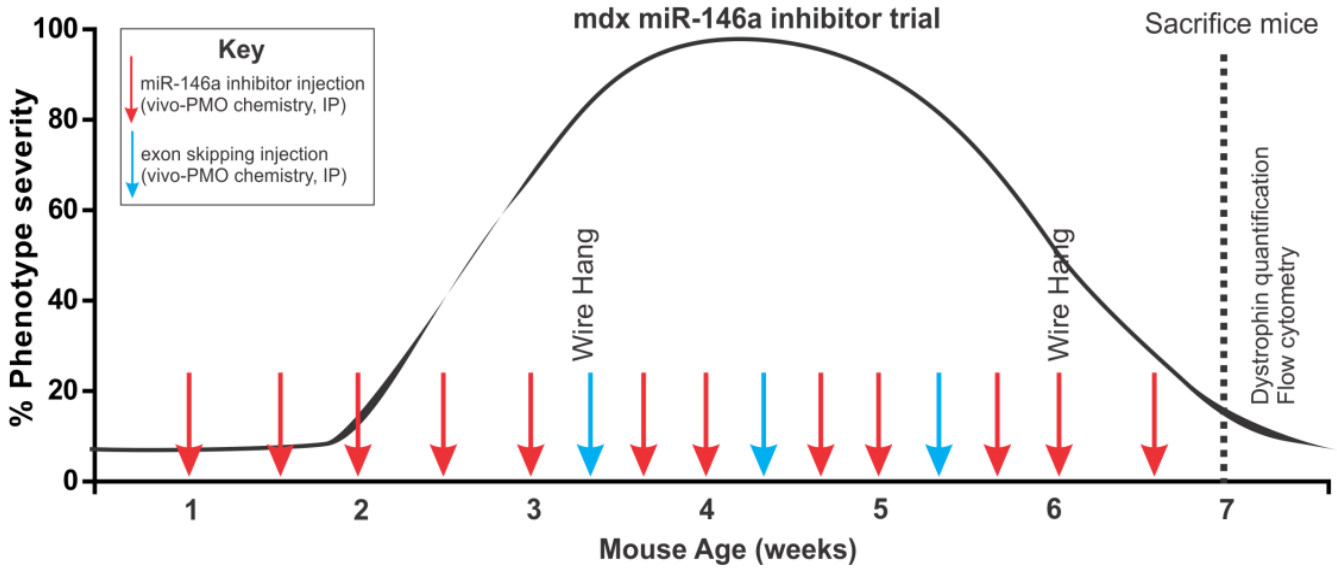

Figure 1. Experimental design of exon skipping + miR-146a inhibitor study.

Aim 2. *Study design:* We started with Aim 2 to determine the feasibility of miR-146a inhibition as a potential therapeutic, testing the hypothesis that combining miR-146 inhibition (miR-146aI) with exon skipping would enhance dystrophin restoration. Because miR-146a is upregulated from birth in *mdx* mice, we chose to perform inhibitor injections from 1 week of age (12.5 mg/kg, vivo-morpholino chemistry, intraperitoneal injection route). miR-146aI injections were performed bi-weekly from week 1 to week 7 (12 injections total). At week 3 we additionally injected an exon-skipping vivo-PMO into both CTRL and miR-146aI-injected mdx mice; these injections were continued from week 1-3 (12.5 mg/kg, 1 injection per week, 3 injections total). This delivery program was chosen to induce a moderate amount of exon skipping such that we could measure any potential improvements via miR-146aI injection. Saline-injected mdx and WT mice were used as control and received one saline injection for each injection experimental groups received. As we are still setting up and procuring the functional equipment in our animal facility, our functional measurement of muscle strength was limited to a wire hang test which was performed at week 3 and week 6. The full experimental plan is illustrated in Figure 1.

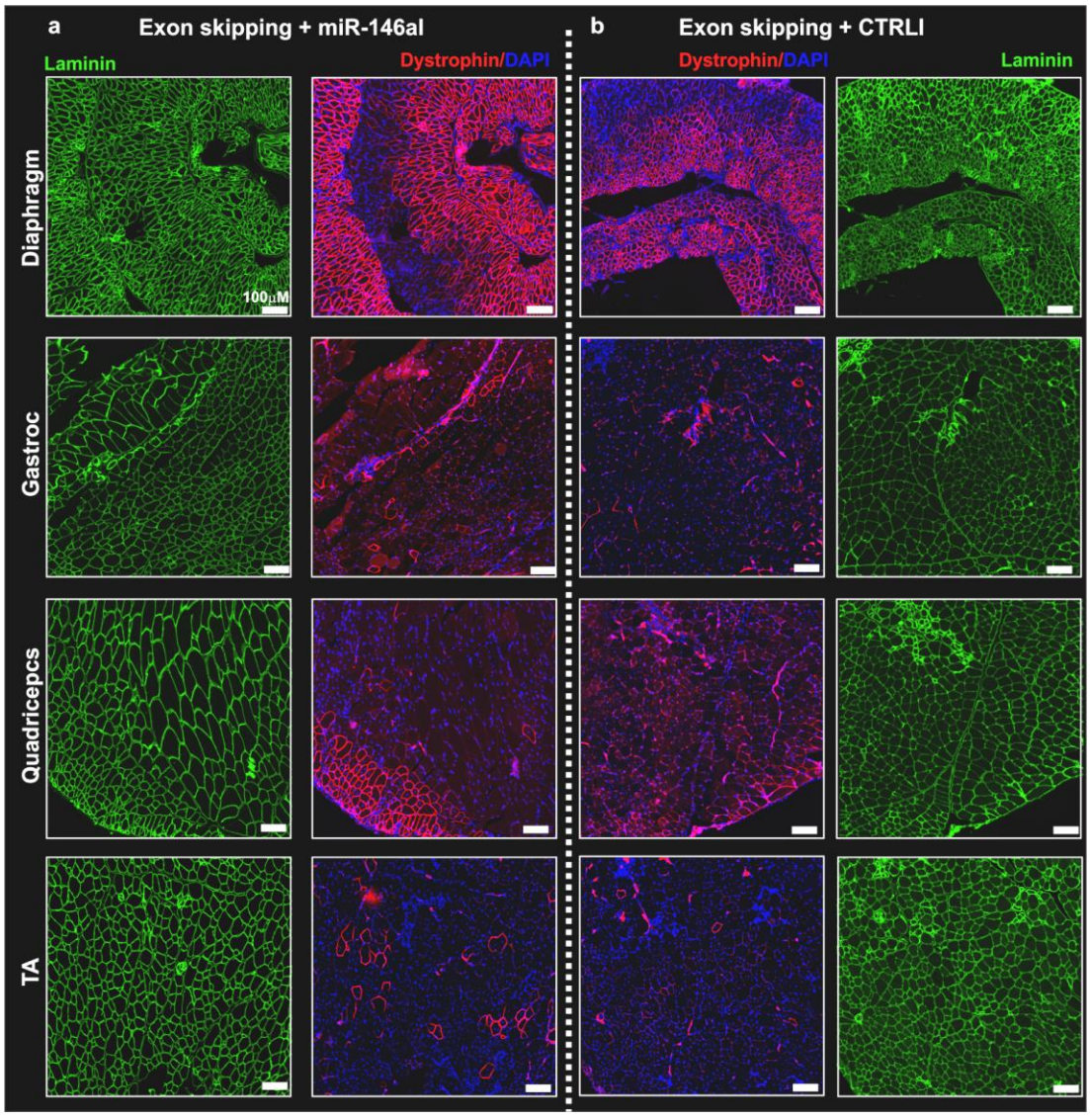

Figure 2. Exon skipping + miR-16aI administration increases dystrophin rescue in skeletal muscles. *Mdx* mouse trial was performed as described in the text. Images of entire muscle cryosections (8uM) were taken using the Olympus VS-120 Virtual Scanning Microscope at 20x magnification. A) Muscles from exon skipping + miR-146aI-injected *mdx* mice. Laminin staining (green) for the designated muscles is shown on the left and Dystrophin (red) and DAPI (blue) staining on the right. B) Muscles from exon skipping + CTRLI-injected *mdx* mice. Dystrophin (red) and DAPI (blue) staining shown to the left and Laminin (green) staining on the right. (n=4 per group with 6-8 more currently being analyzed; scale bare represents 100□M).

Results: We assessed the levels of dystrophin in *mdx* mice treated with exon skipping and either miR-146aI or CTLRI in the diaphragm, gastrocnemius, quadriceps and tibialis anterior (Figure 1). The diaphragm muscle showed the most dystrophin rescue overall, which is as

expected for IP route of delivery (Wu, Lu et al. 2012). As shown by immunofluorescence miR-146a inhibition increased dystrophin rescue in all muscles measured. We additionally analyzed the heart and found that both CTLRI and miR-146aI-injected mice had sporadic revertant fibers (Figure 2a,b), but miR-146aI-injected mice show modest dystrophin rescue in the heart while CTRLI-injected mice did not (Figure 2c,d). This is particularly interesting and relevant as previous reports on exon skipping vivo-morpholinos found no apparent dystrophin rescue in the heart via IP injection (Wu, Lu et al. 2012).

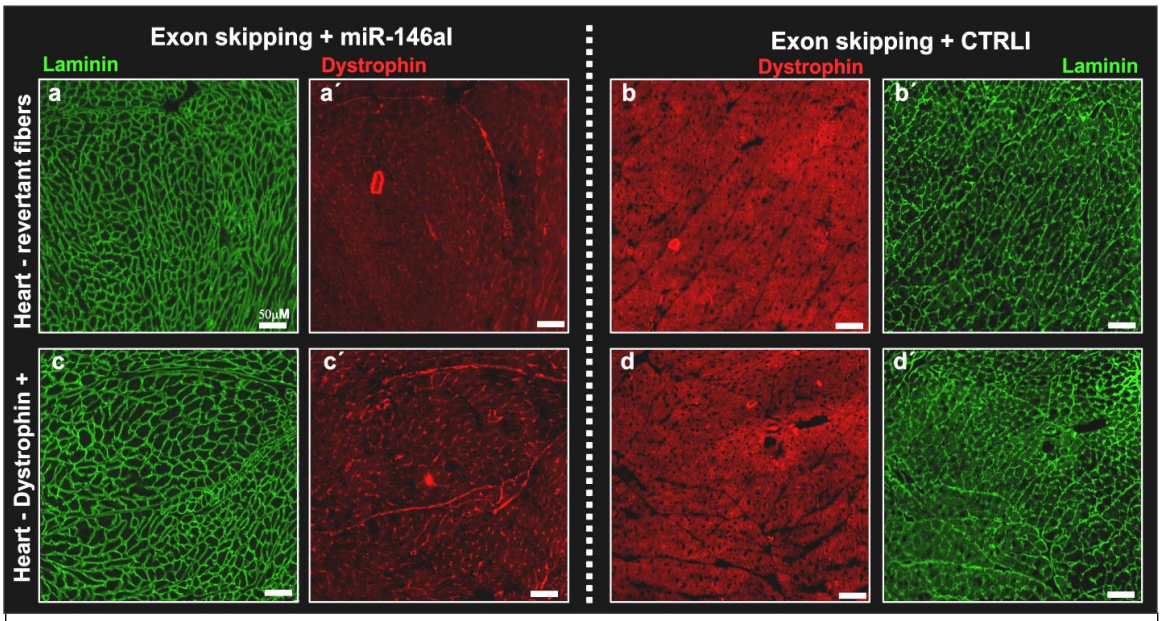

Figure 3. Exon skipping + miR-16aI administration increases dystrophin rescue in heart. Images of entire heart cryosections (8uM) were taken using the Olympus VS-120 Virtual Scanning Microscope at 20x magnification Laminin staining is shown in green and dystrophin staining in red. A & A') Heart sections from exon skipping + miR-146aI-injected mdx mice show revertant fibers. B & B') Heart sections from exon skipping + CTRLI-injected mdx mice show revertant fibers. C & C') miR-146aI injected mice show modest dystrophin in the heart. D&D') No apparent dystrophin rescue seen in CTLRI-injected mice. (n=4 per; scale bar represents 50μM).

As a secondary outcome measure, we analyzed muscle strength using a simple wire hang test (Aartsma-Rus and van Putten 2014). At 6 weeks of age, when mice had received 3 doses of exon skipping and 5 weeks of CTRL or inhibitor injections, miR-146a inhibition resulted in a significant improvement in the hang time (Figure 4b). Interestingly, at 3 weeks of age, when mice had received 2 weeks of inhibitor dosing, but before mice received exon skipping injections, miR-146aI-treated mice showed improved hang time (Figure 4a). This data suggests that independent of exon skipping, miR-146a inhibition may have potential to improve the mdx phenotype.

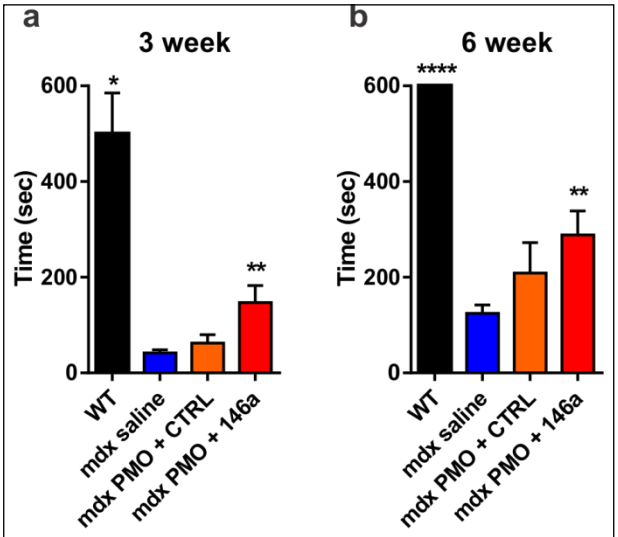

Figure 4. miR-146aI administration improves muscle strength. A wire hang test was performed in all 4 treatment groups at 3 (A) weeks (before exon skipping administration) and at 6 weeks (B). In both cases miR-146aI administration significantly increased hang time in mdx mice.

References:

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Townsend, D., M. Daly, et al. (2011). "Age-dependent dystrophin loss and genetic reconstitution establish a molecular link between dystrophin and heart performance during aging." Mol Ther **19**(10): 1821-1825.
Vainzof, M., M. R. Passos-Bueno, et al. (1996). "The sarcoglycan complex in the six autosomal recessive limb-girdle muscular dystrophies." Hum Mol Genet **5**(12): 1963-1969.
Wu, B., P. Lu, et al. (2012). "Long-term rescue of dystrophin expression and improvement in muscle pathology and function in dystrophic mdx mice by peptide-conjugated morpholino." Am J Pathol **181**(2): 392-400.

c. What opportunities for training and professional development has this project provided?

This project has enabled me to travel to Ottawa, Canada and Ascona, Switzerland to present my work to leaders in the field and make valuable professional connections and collaborations. Additionally, I have hired two lab members, who have been able to develop their skills in working with mdx mice and microRNAs. Further, I have participated in Muscle Inflammation Workshops within our department to increase our knowledge of the interactions between immune cells and muscle

d. How were the results disseminated to the communities of interest?

I have participated in two Duchenne Muscular Dystrophy charity events where I educated members of the community about the research I conduct – these include Dining Away Duchenne (September 2017), A Night of Irish Treasures (March 2018).

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

During the next reporting period, I will perform AAV experiment in mice, finish generating mdx/146a-/- mice, and perform a second trial for exon skipping + miR-146a inhibition (the details are listed in Changes/Problems).

IMPACT

a. What was the impact on the development of the principal discipline of the project?

This work shows that miR-146a inhibition may increase the efficiency of exon skipping in Duchenne Muscular Dystrophy. This work has also led to an unexpected provisional patent that describes the use of microRNAs inhibition as a means to decrease muscle inflammation in dystrophic muscle U.S. Provisional Application Serial No. 62/679,529; Filed June 1, 2018.

b. What was the impact on other disciplines?

We have observed that inflammatory microRNAs, including miR-146a are elevated in other inflammatory muscle disorders including myositis.\

c. What was the impact on technology transfer?

See a - U.S. Provisional Application Serial No. 62/679,529; Filed June 1, 2018.

CHANGES/PROBLEMS

a. Changes in approach and reasons for change.

In a second trial in mdx mice we will use an AAV vectors to co-delivery both a U7snRNP containing a sequence to skip exon 23 in mdx mice and also a U6-driven sponge to inhibit miR-146a. The reason for this change is that in the initial study, we only achieved efficient delivery to the diaphragm. After talking with Dr. Jeff Chamberlain and consulting my mentoring team, we have decided that taking this strategy will yield less variable results. We are currently subcloning these constructs.

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

None to report.

c. Changes that had a significant impact on expenditures.

None to report.

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

None to report.

PRODUCTS

a. Publications, conference papers and presentations

Journal Publications:

Fiorillo, A. A., C. B. Tully, et al. (2018). "Muscle miRNAome shows suppression of chronic inflammatory miRNAs with both prednisone and vamorolone." Physiol Genomics. Acknowledgement of federal support: Yes.

Books:

Omics Approaches to Understanding Muscle Biology - APS Methods in Physiology series; in submission.

b. Inventions, patent applications and/or licenses

U.S. Provisional Application Serial No. 62/679,529; Filed June 1, 2018.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. What individuals have worked on the project?

Name	Christopher Heier
Project Role	Collaborating Faculty
Researcher Identifier	ERA commons ID: cheier
Nearest person month worked	12
Contribution to Project:	Dr. Heier performed microRNA arrays and data analysis that led to finding miR-146a as a marker of steroid drug efficacy in mdx mice. He has also provided intellectual contribution to many of the planned experiments
Funding Support:	NIH R00 R00-HL-130035, Foundation to Eradicate Duchenne

b. Has there been a change in the active other support of the PD/PIs or senior/key personnel since the last reporting period?

Dr. Fiorillo was awarded a startup package from the CRI at Children's National Medical Center is now an Assistant Professor within the Center for Genetic Medicine Research at Children's National Medical Center.

c. What other organizations were involved as partners?

Organization Name: Foundation to Eradicate Duchenne
Location of Organization: P.O Box 2371; Alexandria, VA 22301

d. Partner's contribution to the project

Financial support

APPENDICES:

a. Book chapter – being prepared for submission

Title: Approaches to studying the microRNA-ome in skeletal muscle

Authors: Alyson A. Fiorillo and Christopher R. Heier

Importance of miRNAs in muscle health, function and disease

Muscle is a highly plastic tissue that needs to rapidly undergo dramatic changes in gene expression patterns in order to maintain homeostasis. This requires a delicate balance between satellite cell proliferation, myotube formation and differentiation, and muscle degeneration/regeneration. The disruption of these pathways drives muscle disorders and diseases; this includes dystrophies, inflammatory myopathies, sarcopenia and cachexia. Thus, identifying factors that regulate muscle gene expression programs is essential to understanding muscle health and function and may uncover new therapeutic targets. Since their discovery (Lee, Feinbaum et al. 1993; Wightman, Ha et al. 1993; Reinhart, Slack et al. 2000) it has become well established that miRNAs (miRNAs) are key regulatory factors which fine tune gene expression patterns in all cell and tissue types. As we begin to gain new insight into the function of miRNAs, their essential role as post-transcriptional regulatory elements that drive proper muscle function has become increasingly apparent. As has been observed in the X-linked genetic diseases Duchenne and Becker muscular dystrophy (DMD and BMD, respectively), the chronic dysregulation of miRNAs can exacerbate disease (Eisenberg, Eran et al. 2007; Greco, De Simone et al. 2009; Cacchiarelli, Martone et al. 2010; Cacchiarelli, Incitti et al. 2011; Cacchiarelli, Legnini et al. 2011; Liu, Williams et al. 2012; Fiorillo, Heier et al. 2015; Fiorillo, Tully et al. 2018). In this chapter we will explore the role of miRNAs in skeletal muscle, and the importance of harnessing the power of miRNA profiling to understand how different perturbations to muscle (i.e. exercise, injury, or genetic defects) affect the muscle miRNAome and how the miRNAome, in turn, can yield valuable information about the overall health of muscle.

miRNAs – an introduction

miRNAs are small (~22 nucleotide) non-coding RNAs that primarily function by binding to the 3′ untranslated region (UTR) of a messenger RNA (mRNA) transcript from a target gene. The binding between a miRNA and the 3′ UTR of a gene regulates downregulates protein expression from that transcript by either inhibiting protein translation (imperfect complementary binding), or by promoting mRNA decay (perfect complementary binding) (Valencia-Sanchez, Liu et al. 2006).

In most organisms there are only a small fraction of miRNAs as compared to the number of mRNAs and proteins. In the human genome there are <20,000 protein-coding genes (Ezkurdia, Juan et al. 2014) while the number of annotated miRNAs is approximately 2,700 (from miRbase V22, http://www.mirbase.org/), although a recent publication suggests this number may actually be as high as 3,700 (Londin, Loher et al. 2015). miRNAs are very stable and highly conserved across species – these factors contribute to their appeal as disease-specific biomarkers (Lewis, Burge et al. 2005; Mitchell, Parkin et al. 2008). miRNAs are estimated to regulate approximately >60-70% of the mammalian genome (Friedman, Farh et al. 2009), and are commonly dysregulated in disease which makes them attractive therapeutic targets (Krutzfeldt, Rajewsky et al. 2005).

The basic miRNA processing pathway is illustrated in **Figure 1** and is reviewed in (Kim, Han et al. 2009; Davis-Dusenbery and Hata 2010). Mature miRNAs are ~22 nucleotides (nt) in length, however, they originate from much longer transcripts. The first step in the miRNA processing pathway is the cleavage of the primary transcript (termed pri-miRNA) by the enzyme Drosha – this yields a pre-miRNA hairpin structure that is approximately 70-100nt in length. The pre-miR translocates from the nucleus to the cytoplasm by Exportin 5 (Kim, Han et al. 2009) and is cleaved, by Dicer in combination with trans-activation response RNA-binding protein (TRBP), into two mature miRNAs of ~22 nt with short 3′ overhangs (Kim, Han et al. 2009). These two mature miRNAs are termed the -3p and -5p strands. Dicer transfers the duplex to one of the four human Argonaute (AGO) proteins, requiring AGO to undergo conformation changes to enable binding of the duplex (Wang, Juranek et al. 2009). Strand selection subsequently occurs where one of the strands (called the passenger strand) is discarded. This

allows for activation of the RNA induced silencing complex (RISC) containing AGO and one of the mature miRNAs (called the guide strand or leading strand) and the presentation of the seed sequence of the miRNA (this is most often nucleotides #2-8 of the miRNA). This seed sequence can subsequently interact with its target mRNA, typically within the 3′UTR of the target transcript. Each miRNA can have hundreds or thousands of targets, and thus represents a complex system of "fine tuning" gene expression patterns (Chi, Zang et al. 2009; Hafner, Landthaler et al. 2010). Binding of a miRNA to its target most commonly causes translational repression of that transcript, but sometimes it triggers mRNA decay. This depends on wither the miRNA sequence is an imperfect match (translational repression) or perfect match (mRNA decay) to the target mRNA sequence (Djuranovic, Nahvi et al. 2012; Meijer, Kong et al. 2013).

The selection of the guide and passenger strand from the original pre-miRNA can differ between tissues, and the ratio can also change depending on the disease state (Bang, Batkai et al. 2014; Meijer, Smith et al. 2014). It is also important to understand the historical nomenclature to describe the two mature miRNAs produced from the pre-miRNA (i.e. miR* vs. miR-3p/-5p). A comprehensive guide for the miRNA nomenclature is summarized in (Pritchard, Cheng et al. 2012).

miRNAs in muscle

Described as "micromanagers of gene expression" (Bartel and Chen 2004), it is not surprising that miRNAs play a key role in modulating all the complex regulatory circuits involved in skeletal muscle formation, maintenance, physiological and pathological signaling programs (Guller and Russell 2010; Ballarino, Morlando et al. 2016). Some miRNAs are ubiquitously expressed while others are expressed in a tissue-specific manner (Lee and Ambros 2001; Lagos-Quintana, Rauhut et al. 2002). miRNAs that are either exclusively expressed or enriched in striated muscle have been are referred to as myomiRs (McCarthy 2008) and includes eight miRNAs: miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486 and miR-499 (Sempere, Freemantle et al. 2004; McCarthy and Esser 2007; van Rooij, Sutherland et al. 2007; van Rooij, Quiat et al. 2009; Small, O'Rourke et al. 2010). myomiRs are

expressed in both skeletal and cardiac muscle with two exceptions: miR-208a is expressed only in cardiac muscle while, miR-206 is exclusively expressed in skeletal muscle. Here, we will focus on skeletal muscle myomiRs.

Several myomiRs are encoded by DNA within introns of muscle-specific genes, which facilitate their expression specifically within muscle. For example, muscle-specific miR-208b and miR-499 are encoded within introns of the myosin heavy chain genes *MYH7* and *MYH7B*, respectively (van Rooij, Sutherland et al. 2007; van Rooij, Quiat et al. 2009), while miR-486 is considered muscle-enriched and is located within an intron of ankyrin 1 (*ANK1*) (Small, O'Rourke et al. 2010). The other myomiRs are organized into bicistronic clusters and transcribed together [90]. For example, miR-1-2 and miR-133a-1 are encoded within an intron of the mindbomb E3 ubiquitin ligase 1 *(MIB1*) gene, while miR-1-1 and miR-133a-2 are found in the intron of a muscle-enriched gene encoded by open reading frame 166 on chromosome 20 (C20orf166). In contrast, a cluster consisting of miR-206 and miR-133b resides in an intergenic or non-protein coding space on chromosome 6. Regardless of their genomic locus, the transcriptional regulation of all of myomiRs is known to be driven by myogenic regulatory factors (MRFs) such as MYOD, MYOG, MYF5, MYF6, and SRF (Rao, Kumar et al. 2006; Rosenberg, Georges et al. 2006).

It is important to note that a subset of miRNAs, whose expression is not restricted to muscle, also play key roles in muscle signaling and development. These miRNAs include miR-26a (Dey, Gagan et al. 2012), miR-27b (Crist, Montarras et al. 2009), miR-29 (Wei, He et al. 2013), miR-125b (Ge, Sun et al. 2011), miR-155 (Seok, Tatsuguchi et al. 2011), miR-128a (Motohashi, Alexander et al. 2013), miR-181(Naguibneva, Ameyar-Zazoua et al. 2006), miR-24 (Sun, Zhang et al. 2008), miR-378 (Gagan, Dey et al. 2011), miR221/222 (Cardinali, Castellani et al. 2009), and miR-214 (Shi, Lu et al. 2013). In a disease state the balance of miRNAs is dysregulated: miRNAs not normally found in muscle are increased (Eisenberg, Eran et al. 2007; Fiorillo, Heier et al. 2015; Fiorillo, Tully et al. 2018), while miRNAs that are critical to maintaining muscle homeostasis are decreased (McCarthy and Esser 2007). This has been demonstrated in Duchenne Muscular Dystrophy (DMD) where both myomiRs and non-

myomiRs are highly dysregulated. Specifically, studies have shown regeneration miRNAs are elevated (miR-31, miR-34c, miR-206, miR-335, miR-449, and miR-494) (Greco, De Simone et al. 2009; Cacchiarelli, Martone et al. 2010) and degenerative miRNAs are downregulated (miR-1, miR-29c, and miR-135a) (Greco, De Simone et al. 2009) which is linked to an increase in fibrosis. Further, several studies have found that inflammatory miRNAs are elevated in DMD including miR-222, miR-223, miR-146a, miR-146b, miR-382, miR-320a, miR-142-5p, 142-3p, miR-301a, miR-324-3p, miR-455-5p, miR-455-3p, miR-497 and miR-652 (Greco, De Simone et al. 2009; Cacchiarelli, Martone et al. 2010; Fiorillo, Heier et al. 2015; Fiorillo, Tully et al. 2018). Deregulation of miRNA expression is a common feature of several skeletal muscle disorders (Eisenberg, Eran et al. 2007), and specific treatment regimens (e.g. anti-inflammatories, exon skipping) bring some of these dysregulated miRNAs back towards homeostasis (Cacchiarelli, Martone et al. 2010; Fiorillo, Heier et al. 2015; Fiorillo, Tully et al. 2018). A detailed view of how miRNAs affect normal and diseased muscle is summarized in **Figure 2**.

miRNA Profiling

One advantage to profiling miRNAs is that they are much more stable than mRNAs and can be recovered from formalin-fixed paraffin embedded sections or other sources that typically show low overall RNA quality. Consistent sample processing and RNA extraction methods are critically important to the quality of results from miRNA profiling, however. With that in mind, samples should be handled with care to ensure consistent results and to avoid possible miRNA degradation(Ibberson, Benes et al. 2009). In the following sections we will discuss the major methods of miRNA isolation and profiling, as well as the advantages and disadvantages of each method.

Sample Types and extraction methods

Muscle biopsies or animal muscles

When working with either human muscle biopsies or muscles from another species (i.e. rat, mouse, dog) we recommend freezing muscle in liquid nitrogen-cooled isopentane (also called 2-

methylbutane) and storing at -80^{o}C for later use. This method preserves the integrity of the muscle tissue for sectioning and also allows for RNA and protein extraction. If the muscle is only being analyzed for RNA or protein, it can be directly flash frozen by placing the dissected muscle in a cryogenic tube and placing this directly in liquid nitrogen for at least one minute. Samples should be stored at -80^{o}C until RNA extraction.

Since skeletal muscle is a highly fibrous tissue, the most critical step in the process is the disruption of all the cells. Preparation for homogenization should be carried out on dry ice, or under liquid nitrogen cooled conditions. There are a few ways to homogenize the frozen tissue. The first, most labor-intensive way is to use a mortar and pestle cooled with liquid nitrogen to crush the muscle into a fine powder. Another way is to use a combination of tissue crushing using a Liquid Nitrogen Cooled Mini Mortar and Pestle Set (Bel-Art), followed by directly homogenizing the crushed tissues in the specific reagent being used for RNA extraction (i.e. TRIzol, QIAzol reagents) with a TissueRuptor or equivalent hand-held homogenizer with disposable probes (Qiagen). Alternatively, frozen samples can put directly into the RNA extraction reagent and homogenized with a hand-held homogenizer. Our laboratories have found that the combination of crushing the muscle sample with Liquid Nitrogen Cooled Mini Mortar and Pestle Set and homogenizing in the RNA extraction reagent (TRIzol) results in the highest yield and highest quality RNA.

Muscle cell lines

If using muscle cell lines for miRNA profiling, the procedure is much easier and less labor intensive. Prior to RNA extraction, media and cell debris should be rinsed off of cells with PBS and then cells can either be frozen for later use (-80^{o}C) or RNA extraction can be immediately performed by pipetting the RNA extract reagent (i.e. TRIzol) directly onto cells in plates or dishes. Differentiated myotubes grown in 6-well plates typically provide a concentration of 200-500 ng/ul of RNA (when resuspended in 25-30µL of RNase-free water), which is more than enough to work with for profiling experiments.

Additional considerations

Before beginning RNA extraction it is important to apply RNase inhibitor spray to the laboratory bench and to utilize RNase-free water, tubes, and equipment to prevent RNA degradation. For RNA extraction, the most common reagents and kits utilize chemical extraction with concentrated chaotropic salts such as guanidine thiocyanate (TRIzol and QIAzol). As an additional step to enrich for miRNAs there are commercially available kits available (miRNeasy, Qiagen; *mir*Vana, Ambion; and PureLink, Thermo Fisher Scientific). These kits add a solid-phase extraction procedure on silica columns. However, if you have limited samples and plan on analyzing both miRNA and mRNA, we recommend skipping these miRNA enrichment steps since the columns will only bind small RNAs and mRNAs will be lost in the wash steps. To increase the yield of miRNA recovered using these methods, we recommend performing the isopropanol precipitation step at -20^0C overnight or over the weekend before performing the final ethanol wash and RNA resuspension.

Unlike mRNA profiling, formalin-fixed paraffin embedded tissue (FFPE) can be utilized for miRNA profiling (Doleshal, Magotra et al. 2008). mRNA becomes fragmented in FFPE samples, however miRNA is quite stable, largely because it is less subject to RNase-mediated degradation (Aryani and Denecke 2015). This is a clear advantage of utilizing miRNA profiling in a clinical sample cohort where oftentimes FFPE samples are the only sample type available. The procedure for recovering miRNAs from this sample type begins with cutting FFPE samples into thick sections (~20 µm). Depending on the width of the cross sections as little as 1 section (i.e. for a muscle sample taken at autopsy) and as many as four 20 µm sections (for a small muscle biopsy), or up to 35 mg of unsectioned muscle, can be used. Following deparaffinization and protease treatment (Xi, Nakajima et al. 2007; Doleshal, Magotra et al. 2008) miRNA can be recovered from these tissues by commercially available kits such as RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific), High Pure miRNA isolation kit (Roche), or miRNeasy FFPE kit (Qiagen).

Sample heterogeneity

Muscle tissues contain a heterogeneous population of cell types (Yablonka-Reuveni and Nameroff 1987). Depending on the experimental setup and question, it may be necessary to purify a specific cell type from muscle tissue (i.e. satellite cells, macrophages, differentiated muscle) or purify a specific area from a muscle section (i.e. damaged area, high inflammation area). This can be achieved by using fluorescence-activated cell sorting (FACS), myofiber isolation, or laser capture microdissection (LCM) (Iyer-Pascuzzi and Benfey 2010; Gautam and Sarkar 2015). In FACS a specific cell type is selected from a mixture of live cells using a fluorescently labeled antibody against a specific cell marker, most often a receptor, and miRNA profiling is subsequently performed (Iyer-Pascuzzi and Benfey 2010; Coll, El Taghdouini et al. 2015). Another way to use this method is to purify genetically labeled cells from a transgenic mouse (Lobo, Karsten et al. 2006). Alternatively, LCM can be used on histological sections of dissected muscle to cut out a specific area of interest and purify RNA from these specific regions within the sections. This may be particularly useful if your specimen has variable pathology, such as in studies on the effects of local injury (e.g. notexin injection) or in assessing different stages of muscle development.

Concentration and quality assessment

Once RNA is extracted, it is important to check quality and concentration. As a general rule, each milligram of tissue should yield about 1ug of total RNA. All profiling methods discussed here can use total RNA, so it is not absolutely necessary to assess miRNA quantity separately. The most simplistic way to determine overall RNA quality and quantity is using spectrophotometry (i.e. Nanodrop). RNA has a maximum absorbance at 260 nm, and this reading will yield the RNA concentration. Historically, the ratio of absorbance at 260 to the absorbance at 280 nm has been used as a measure of purity, with a ratio of 2.0 denoting a pure RNA sample. For profiling experiments, RNA that has a purity of 1.7 or higher can generally be used. Once RNA concentrations are determined, it is common practice to dilute all samples to the same concentration. For a more in depth analysis of RNA quality, the Bioanalyzer 2100 (Agilent) can be used with the small RNA chip. This chip can selectively quantify

miRNAs in absolute amounts [pg/μL] and as a relative percentage of small RNA [%]. However, the estimation of miRNA abundance by this method may only be accurate when overall RNA integrity is very high (Pritchard, Kroh et al. 2012). It is also possible to assess miRNA extraction efficiency by incorporating a "spike in" synthetic miRNA from another species (most commonly *C. elegans*) at an early step in the RNA isolation (Kroh, Parkin et al. 2010). Quantification of these spike-in miRNAs in the RNA recovered from muscle tissues/cell lines can serve as an RNA extraction efficiency control, though other normalization methods are preferred for downstream miRNA quantification.

miRNA profiling methods

There are three well-defined approaches for miRNA profiling: quantitative reverse transcription PCR (qRT-PCR), hybridization-based methods, and RNA-sequencing. Deciding which method to choose will need to be based on your experimental goals and your available resources. **Figure 3** summarizes the basic miRNA profiling platforms.

qRT-PCR-based methods

Lower density quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) arrays have emerged as a first-line method of choice by many for miRNA profiling. This is because of the reproducibility of their data in subsequent single-gene qPCR experiments, their increased sensitivity (dynamic range of seven log10 units), and their ability to utilize a small amount of input RNA (as little as 25 picograms). Whereas other platforms require much greater starting material (Chen, Gelfond et al. 2009), it has been demonstrated that this method can detect miRNA content at the single-cell level (Mestdagh, Feys et al. 2008).

As miRNAs are too small to utilize standard methods to perform qRT-PCR methods, the reverse transcription reaction has been modified to generate complementary DNA (cDNA) that can be subsequently used in qRT-PCR. There are two main methods of priming. Chen et al. developed a technique using stem-loop reverse transcription (RT) priming to synthesize cDNA from miRNA, followed

by Taqman Real Time PCR Analysis (Chen, Ridzon et al. 2005). In the reverse transcription reaction, the stem-loop primer serves to prime the cDNA reaction as well as extend the size of the mature miRNA: it consists of a constant region that forms a stem loop and a variable six- nucleotide extension. The stem-loop portion of the primer extends the 18-22 nucleotide miRNA to >60 nucleotides to allow for traditional PCR in subsequent steps. The six-nucleotide extension is the reverse complement of the last 6 nucleotides on the 3′ end of the miRNA of interest and provides specificity. A second method to convert mature miRNA to cDNA involves the addition of a poly (A) tail using E. coli poly (A) polymerase, and then performing qPCR using a miRNA-specific forward primer and a universal poly(T) adaptor reverse primer (Shi, Sun et al. 2012).

To perform large scale miRNA profiling using these methods, the RT-PCR primers are pooled together, and thus convert all target miRNAs into cDNA. This is followed by performing qRT-PCR with pre-plated PCR primers that are distributed across multiwell plates, or microfluidic cards containing nanoliter-scale wells. There is also an option for a pre-amplification step, adding around 10-14 cycles of amplification before samples are run on qPCR array plates. Examples of commercially available qRT-PCR arrays include TaqMan Low Density miRNA Array Cards (stem-loop priming, Thermo Fisher Scientific) and miScript miRNA PCR Arrays (poly(A) priming, Qiagen).

Data analysis for qRT-PCR arrays is performed using a method commonly known as the $\Delta\Delta$Ct method, enabling the comparison of groups by using one group as the normalizer (Livak and Schmittgen 2001). This calculation assumes that the amplification product in qPCR doubles after every PCR cycle. The abundance, then, is inversely related to the cycle (Ct) number in which you detect the signal from your amplicon. In basic terms, the $\Delta\Delta$Ct is calculated 1) from normalizing the cycle in which your target amplifies to the cycle in which your housekeeping reference RNA amplified (called ΔCt), and 2) from then normalizing your “treatment” sample to your “control” sample (called $\Delta\Delta$Ct). More specifically, since each cycle of PCR represents a doubling of your miRNA target, this is all actually done in logarithmic space where the value at each cycle (Ct) is actually 2^Ct. The ΔCt is equal to Ct_{miRNA} minus

$Ct_{Housekeeping}$, and $\Delta\Delta Ct$ is equal to ΔCt_{Test} minus $\Delta Ct_{Control}$. To get a final, relative quantification, the $\Delta\Delta Ct$ result is converted back into a linear space by the equation $2^{-\Delta\Delta Ct}$. Different platforms have different software for performing this relative quantification in parallel and in an automated fashion. It is imperative, however, that amplification curves for each individual miRNA assay are checked before formalizing your results and/or using global normalization methods, as these programs don't always flag improper amplification in the "noise" range. Not performing the proper quality checks can therefore lead to the false appearance that the miRNA expression in one group is significantly different, when in reality the Ct values from the miRNAs or from miRNAs included as normalization controls were background noise. This process of quality checks can add labor to the profiling analysis; however it can also increase confidence and insight by allowing one to directly view the amplification curve data for each individual result.

In terms of data normalization, there are a few strategies that can be used: 1) normalization to stably expressed endogenous reference small RNAs, 2) global normalization to the average of all Ct values from array experiments, and 3) normalization to an external spike-in synthetic oligonucleotide. The first strategy, normalization to reference small RNAs, commonly uses small nuclear/nucleolar RNAs such as snoRNAs or RNU6A and 6B. For best results, the geometric mean of multiple control genes should be used in order to minimize effects of any variation or noise in the individual reference RNAs. As housekeeping RNAs can still be affected by certain diseases or conditions (Gee, Buffa et al. 2011), this is always a good idea for qPCR-based gene expression analyses and is often included as an automated option in expression analysis software. If normalization control RNAs are suspected to be changing or are not well-defined in a sample type, several statistical programs exist that can analyze profiling data within an experiment and rank candidate reference genes in order of stability. These include BestKeeper (Pfaffl, Tichopad et al. 2004), GeNorm (Vandesompele, De Preter et al. 2002), and NormFinder (Andersen, Jensen et al. 2004). These programs also take into account intra- and intergroup variability. Alternatively to using specific endogenous controls, another common method is to use global normalization where the mean expression value of all miRNAs is used as the normalization

factor. Care should be taken to quality check each individual miRNA using this approach. This global normalization method has been reported to reduce technical variation and provide an accurate appreciation of biological change (Mestdagh, Van Vlierberghe et al. 2009). A third normalization method is to normalize to a spike-in synthetic oligonucleotide. This method is frequently used as a processing control to measure miRNA extraction efficiency, and in some instances it may be used for gene expression when overall miRNA content is expected to be quite different in control vs. treatment samples (Roberts, Coenen-Stass et al. 2014). However it is less favorable than the other methods for normalizing gene expression, as a spike-in method only corrects for either extraction efficiency or reverse transcription efficiency and not for variability in expression within the original biological sample.

Hybridization-based Methods

miRNA microarrays

Microarrays were the first technology to be utilized for high-throughput analysis of miRNA expression. They are capable of screening hundreds of target sequences within a single sample, including both pre-cursor and mature miRNAs. The design of miRNA arrays is different from that of other spotted microarrays, however the differing characteristics of miRNAs must be taken into account. First, mature miRNAs are not polyadenylated, meaning that a label (biotin, fluorescence) cannot be incorporated into miRNAs via oligo (dT)-primed reverse transcription. Second, there is minimal sequence available on miRNAs to enable hybridization since miRNAs are typically only 18-22nt in length. This means probe design must also be restricted to this length. This is challenging because, similar to PCR, designing such short hybridization probes leads to variable melting temperature (T_m) caused by highly variable GC-richness, (i.e. 70% GC-rich vs. 20% GC rich miRNAs require very different hybridization temperatures) meaning that conditions cannot be optimized for all miRNAs being detected by the array.

Carlo M. Croce and colleagues described the first miRNA profiling array (Liu, Calin et al. 2004) which measured miRNA precursors rather than mature miRNAs. This method utilizes biotin labeling of miRNAs via RT-PCR, priming the reaction with oligo d(T) primers to turn poly(A) tailed transcripts into

biotinylated cDNA as miRNA precursors are polyadenylated similar to protein coding transcripts. In this method labeled miRNAs are hybridized to a slide spotted with 40nt oligo probes specific for all human and murine pre-miRNAs. Hybridized miRNAs are detected after adding a streptavidin-conjugated Alexa Fluor 647 label, read on a hybridization chip reader.

A handful of the techniques to perform miRNA arrays involve an enzyme-mediated labeling of miRNAs. The first described mature miRNA Array method utilizes a T4 RNA ligase to couple the 3′ end of miRNAs to a fluorescent (Cy3)-modified dinucleotide while the 5' end hybridizes to capture probes that are spotted onto slides (Thomson, Parker et al. 2004). A reference oligonucleotide for all mature miRNAs labeled with Cy5 is included for normalization. This method requires a dephosphorylation step to remove the 5′ phosphate from miRNAs before hybridization; without this step miRNAs are at risk for circularization. Another method of probe labeling is poly(A) extension 3' end using poly(A) polymerase (Goff, Yang et al. 2005), following which a fluorophore-conjugated oligonucleotide is ligated to the miRNA using a splinted or bridged ligation technique (Maroney, Chamnongpol et al. 2008; Git, Dvinge et al. 2010). Here, ligation of an oligo to the 3' end of a miRNA is facilitated by a second oligonucleotide (the "bridge oligo") that hybridizes to both the 3' end of the miRNA and the 5' end of the "capture" oligonucleotide. This technique avoids the issue of circularization; however, poly (A) tailing is generally variable in how many A's are added to the miRNA, which could create variable hybridization properties between miRNAs.

Another method is to modify the miRNAs after they are hybridized to their respective probes. Nelson and colleagues developed a miRNA Array platform using RNA-primed, array-based, Klenow Enzyme (RAKE) to label miRNAs post-hybridization (Nelson, Baldwin et al. 2004). Here, DNA capture probes bound to slides have a spacer sequence with three thymidine bases adjacent to the miRNA-binding region. After miRNA hybridization, the microarray is treated with DNA exonuclease I, which degrades the probes not bound by miRNAs. The Klenow fragment of DNA polymerase I, an enzyme that can use RNA to prime its activity, is added with biotinylated dATP, which becomes incorporated complementary to the three thymidines in the capture probe. The amount of capture miRNA is

subsequently determined by the addition a fluorescently-conjugated streptavidin. Similar approaches have been adapted by others (Yeung, Bennasser et al. 2005; Berezikov, van Tetering et al. 2006).

Like PCR, variable melting temperature (T_m) of the GC-rich content of miRNAs is an obstacle for miRNA hybridization probes. To help reduce Tm and make hybridization of miRNAs to capture probes more consistent, substituting Locked Nucleic Acid (LNA)-modified probes in place of DNA probes has proven to be useful (Castoldi, Schmidt et al. 2006) as these synthetic RNA/DNA molecules increase the overall thermo stability of oligonucleotide probes.

Normalization of miRNA hybridization arrays can be performed in a variety of ways as reviewed in (Pradervand, Weber et al. 2009). One common normalization method is to use the global miRNA expression as the normalizer; which applies the assumption that specific miRNAs in a sample may vary, but the overall miRNA content stays the same (Bissels, Wild et al. 2009; Risso, Massa et al. 2009). This method has been well validated and utilized in the literature. Other methods for data normalization that are described by Hua et al. (Hua, Tu et al. 2008) include quantile normalization, and variance stabilizing normalization (VSN). The goal of the quantile method is to make the distribution of probe intensities for each array in a set of arrays the same. VSN assumes that most miRNAs are not differentially expressed. Both of these normalization methods are available as part of the open source Bioconductor bioinformatics software (https://www.bioconductor.org/). It is recommended, however, that you discuss your normalization method with your bioinformatics team to choose the best normalization method for your data set.

The advantage of miRNA arrays are the number of samples that can be analyzed at once, that one array can cover more miRNAs from more species, and that many institutions already have microarray core facilities which can run them. The limitations of this method are: it requires more starting material than qRT-PCR-based methods, it has lower sensitivity and specificity than other methods making it difficult to discern miRNAs with similar sequences (e.g. miR-146a-5p vs. miR-146b-5p), and it does not enable the absolute quantification of miRNA abundance. Rather, using this

technology is an effective tool for comparing “healthy” to “diseased” muscle or non-exercised to exercised muscle.

Nanostring nCounter gene expression system

A more recent development in hybridization-based miRNA profiling technology is the NanoString nCounter, which applies a unique color-coded “fingerprint” to miRNA-specific probes (Geiss, Bumgarner et al. 2008). This technology utilizes a biotin-labeled 3’ capture probe and a 5’ reporter probe with a color code that is unique to each miRNA. The result is the formation of a tripartite structure consisting of a miRNA in between two bound probes. Unbound probes are removed via affinity purification and bound complexes are immobilized onto a streptavidin-coated slide, followed by imaging and counting of bound reporters. Data analysis can be performed using nSolver™, a software provided by NanoString Technologies. This technique has high sensitivity, high specificity, avoids amplification bias, and has the advantage of being able to discriminate between similar miRNA variants. It is, however, quite expensive compared with other methods and the instrument required to run it is less broadly available.

miRNA sequencing

The development of next generation sequencing platforms provides us with powerful but complex methods to profile miRNA expression. Major strengths of these platforms include their ability to detect novel miRNAs, and their ability to precisely identify miRNAs down to single base-pair differences. This can provide the ability to differentiate between similar miRNAs within the same “family”, and between isomiR’s which possess slight modifications of the reference miRNA sequence. Major disadvantages of these platforms center around their cost, and the advanced computational bioinformatics resources needed to properly analyze the large data sets produced. Additionally, upstream biases are inherently introduced to the datasets through library preparation methods which preferentially select for specific types of RNAs.

A general pipeline for an RNA-seq project runs as follows: sample production, RNA isolation, RNA sub-type selection, library preparation, quality checks, next-generation sequencing, data quality

checks, alignment of sequencing data to the genome, data visualization and analysis. Initially, RNA isolation and quantification can be performed similarly to messenger RNA experiments, as high-quality miRNA can be easily isolated from muscle samples using total RNA isolation methods and reagents such as TRIzol. Subsequently, miRNA isolation kits such as miRNeasy (QIAGEN) are commonly used in order to both enrich for miRNA and further purify the samples for subsequent enzymatic reactions. Library preparation is performed after miRNA isolation and consists of ligating linker sequences to the miRNAs, followed by reverse transcription to convert these into cDNAs, then PCR amplification of these cDNAs, and subsequent purification through gel-based size selection. Once the library preparation is complete, the miRNA-seq reaction can be performed using any of several next-generation sequencing platforms. Examples of these platforms include high throughput sequencers such as the HiSeq 2000 (Illumina) and SOLiD (ABI), as well smaller scale sequencers such as Ion Torrent (Invitrogen) and MiSeq (Illumina).

Note, the miRNA-seq workflow becomes much more complex than qPCR-based methods due to the added steps of library preparation and the subsequent computational bioinformatics steps needed to analyze and interpret the data. Some tool suites have been developed to help streamline the data analysis process. These include publicly available, open-access suites such as the Galaxy Mississipi tool suite (https://mississippi.snv.jussieu.fr/), as well as platform-specific tool suites provided by vendors. A brief description of the steps and general concepts follows. First, compressed sequencing data files may need to be converted to file types used for analyses (.fastq file types). Adapter or linker sequences which were ligated to the miRNAs during library preparation then need to be "trimmed" from the sequencing data to isolate the miRNA sequences alone and the data needs to be quality checked, these can be performed using software tools such as miRge (Baras, Mitchell et al. 2015) and CutAdapt (Chen, Khaleel et al. 2014). Once data clears quality checks, it needs to be aligned to a reference genome or RNA library so that the miRNAs can be properly recognized, positioned and subsequently quantified or visualized. This alignment is performed using a genome alignment tool such as Bowtie (Langmead 2010) or sRbowtie. Quantification can then be performed to detect differences in the

expression levels of miRNAs, using software such as DESeq2 (Love, Huber et al. 2014) or DEXUS (Klambauer, Unterthiner et al. 2013). In order to identify novel miRNAs within a tissue or sample, further tools such as miRDeep2 (Mackowiak 2011), miRanalyzer (Hackenberg, Sturm et al. 2009), or novoMiRank (Backes, Meder et al. 2016) are needed. The purpose of these tools is to reduce false positive identification of other small RNA species as miRNAs, and they can integrate information such as related pre-miRNA sequences or predicted secondary structures.

During RNA isolation and selection, different methods can be used to select for specific subsets or general types of small RNA species. For example, a method known as CLIP-seq uses biochemical techniques to immunoprecipitate miRNA species bound by specific proteins such as Argonaute (AGO), a protein component of the RNA-induced silencing complex (RISC). Integrating biochemical techniques such as this has the benefit of removing some non-miRNA small RNA species and of providing additional biological insight, however it also removes unbound miRNAs from the sample. Alternatively, strictly size-based approaches to enrich samples for miRNAs can be used, however additional small RNAs are then included in the sequencing analysis which can complicate the data analysis. Computational approaches need to be subsequently applied to help minimize incorrect identification of such small RNAs as miRNAs.

Future developments in next-generation sequencing will enhance the abilities of miRNA-seq, improve data storage, and make the technologies involved more broadly accessible. This provides another advantage for next-generation sequencing approaches – whereas qPCR- and hybridization-based technologies are largely static, the technologies and analysis capabilities of sequencing are constantly evolving. Moving forward, new technologies in single molecule sequencing (SMS) are being developed with the potential to improve both speed and data bias in comparison to current platforms (Kapranov, Ozsolak et al. 2012). Post-acquisition, storage of the very large data sets and files produced by next-generation sequencing represents a substantial problem for the field. Advances in computing storage, in data compression, and in minimizing or modifying the type of data files needed for long-term memory storage will all help to address this issue. From the data analysis side,

improvements to software and algorithms are frequently being made at a rapid pace. Because of this, available programs can quickly become out of date. Before planning and executing a next-generation sequencing project, it is thus important to consult with a computational bioinformatics expert and the recent literature to plan your project according to the most trusted analysis workflows and software.

miRNA databases

Once data is generated from profiling, there are a few tools that will help in data interpretation. The most utilized and comprehensive general database is miRBase (www.miRbase.org, v22) (Griffiths-Jones, Grocock et al. 2006; Griffiths-Jones, Saini et al. 2008). miRBase provides information on the predicted hairpin portion of the miRNA transcript (pre-miR), on its genomic locus, and on the mature miRNA sequence. It provides comprehensive information for all annotated miRNAs, including nomenclature, sequence data, predicted gene targets, and validated targets of each miRNA. There are also other tools that help with miRNA target prediction, with mining the literature for validated targets, and with determining the functional significance of miRNAs identified in your profiling data. Some useful databases include: 1) TargetScan (Agarwal, Bell et al. 2015), DIANA-TarBase (Vlachos, Paraskevopoulou et al. 2015) MiRTarBase (Hsu, Lin et al. 2011; Chou, Shrestha et al. 2018), and miRDB (Wang 2008; Wong and Wang 2015) which provide target prediction, 2) miRWalk 7.0 (Dweep and Gretz 2015; Parveen, Gretz et al. 2016) which provides validated miRNA-target interactions and miRNA binding sites, and 3) DIANA miRPath (Vlachos, Zagganas et al. 2015) and miR2Disease (Jiang, Wang et al. 2009) which integrate miRNA profiling data with relevant pathways and diseases. We also recommend using the UCSC Genome Browser to look at potential transcription factors that regulate the miRNAs from your profiling data, as this can give further clues into the pathways affected by experimental conditions (Kent, Sugnet et al. 2002). A comprehensive review of these databases has been reviewed elsewhere (Vlachos and Hatzigeorgiou 2013; Riffo-Campos, Riquelme et al. 2016).

Future Directions

As miRNAs are a relatively new class of biomolecule, they represent an exciting area of research that we are only in the early stages of understanding. As several of the technologies we have described are already well established due to redundancy with mRNA techniques, here we will focus on one emerging technique and several exciting applications that are rapidly evolving. Digital qPCR will be discussed as a newer technique which enables sensitive and absolute, as opposed to relative, quantification of miRNAs. Next, serum miRNA biomarkers have the potential to improve diagnosis, improve translation of preclinical studies, and improve decisions on clinical drug approvals. Finally, as a newer class of biomolecule that is dysregulated in disease, miRNAs provide us with an exciting new class of therapeutic targets.

The concept of digital PCR was first developed in 1992 (Sykes, Neoh et al. 1992), (then termed PCR with limiting dilution) and later defined as digital PCR (Vogelstein and Kinzler 1999) when utilized to quantify cancer-causing mutations. While this technology was usurped by the development of quantitative real time PCR, it is now reemerging due to the recent development of better instruments and chemistry which have made it a simpler and more practical technique. Digital PCR, in comparison to real-time PCR, provides absolute quantification of the target miRNA based on partitioning of individual molecules into thousands of replicate reactions at low dilution. This results in zero or one target miRNA in most reactions. At the end of the PCR reaction the absolute concentration of the miRNA (in copies/µl) is determined by Poisson statistical analysis of the number of positive and negative reactions. The main advantages of digital PCR are: 1) the ability to determine absolute quantification without external references (Bustin and Nolan 2004); 2) no requirement for an endogenous control for normalization, and 3) a high degree of sensitivity and precision as compared to qPCR. Because of its high sensitivity, digital PCR may enable researchers to use serum or other biofluids “neat” in order to accurately determine the physiological concentration of a given miRNA. As we learn more about the muscle miRNAome, research is shifting towards the use of serum biomarkers as surrogate measures for molecular changes in skeletal muscle. Through this evolution, digital PCR may become an increasingly powerful tool to detect low copy number miRNAs that can accurately

predict and reflect in the overall condition of skeletal muscle as it relates to disease, exercise, aging, and overall health.

A particularly exciting area of miRNA research is the development of serum miRNAs as minimally-invasive biomarkers, which has the potential to improve the diagnosis, monitoring, and treatment of muscle diseases. Loosely defined, a biomarker is a measurement that reflects a biological activity such as a disease or a drug response. The discovery that miRNAs are present and highly stable in patient serum provides a new class of highly quantifiable molecule which can be objectively and routinely measured (Mitchell, Parkin et al. 2008). Whereas serum proteins are relatively unstable and degrade during freeze-thaw cycles, serum can be repeatedly freeze-thawed at least six times or even exposed to RNases with no discernable effect on miRNA levels. In muscle diseases, miRNAs are released by damaged muscle into serum in a manner that may help to predict disease state or pharmacodynamic response. For instance, myomiRs are muscle-specific miRNAs and are released from damaged muscle. Accordingly, myomiRs are detected at significantly increased levels in serum from muscular dystrophy patients versus healthy volunteers, and can further differentiate milder BMD patients from more severe DMD patients (Li, Li et al. 2014). In addition to myomiRs, pathology-specific miRNAs are upregulated in certain disease states and may provide mechanism-defined biomarkers. For example, inflammatory miRNAs such as miR-146a are increased in dystrophic muscle of both BMD/DMD patients and animal models, and patient serum levels of these same miRNAs are found to predict a response to anti-inflammatory drugs in other diseases (Fiorillo, Heier et al. 2015; Heier, Fiorillo et al. 2016; Fiorillo, Tully et al. 2018). Moving forward, a major goal is to develop serum miRNAs into objective and minimally invasive surrogate outcome measures in clinical drug trials. Essentially, this will require serum miRNAs to be validated and a biological rationale built which enables them to be used as substitutes for clinically meaningful endpoints which predict the effectiveness of therapies.

In addition to monitoring disease, miRNAs provide us with exciting new strategies to treat disease. Two basic strategies for miRNA-based therapeutics involve either the addition of a miRNA exogenously to improve pathology, or the inhibition of a pathological miRNA. The first of these is a bit

more straightforward. If a decrease in a particular miRNA is found to drive disease pathology, for example by resulting in upregulation of a pathological protein normally repressed by that miRNA, then replacing that miRNA with a “miRNA mimic” may improve pathology. The second strategy typically involves developing an anti-sense oligo to inhibit a miRNA whose presence or upregulation is found to promote pathology. A strength of this strategy is that, since it is targeting an RNA-based molecule, highly specific drugs can be designed that target the specific nucleic acid sequences of that miRNA. Towards this end, a diverse number of strategies and chemistries are being pursued to ultimately produce a new generation of miRNA-based therapeutics. These include antagomiRs which are antisense oligos complementary to the miRNA (Krutzfeldt, Rajewsky et al. 2005), optimized “Tough Decoy” inhibitors (Bak, Hollensen et al. 2013; Hollensen, Bak et al. 2013), miRNA “sponges” which present decoy miRNA binding sites (Ebert, Neilson et al. 2007), and “target protectors” or “masks” which bind 3’ UTRs to block miRNA binding without disrupting expression of the target (Choi, Giraldez et al. 2007). In recent years, at least nine miRNA-based therapeutics have entered preclinical or clinical development, as reviewed in (Christopher, Kaur et al. 2016). Moving forward, it will be interesting to see how these early trials and next-generation drugs perform as we uncover the full potential of miRNA-based therapeutics.

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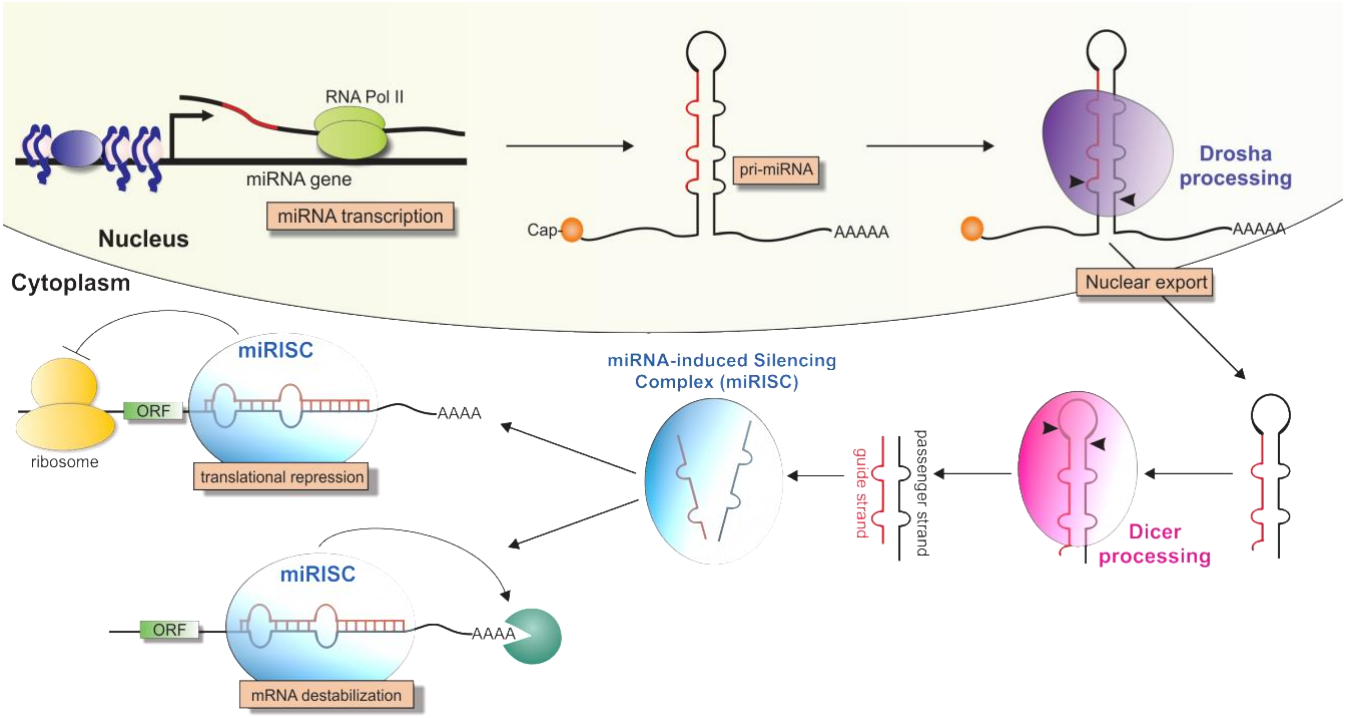

Figure 1. Schematic of miRNA Biogenesis. miRNAs are transcribed by RNA Polymerase II (RNA Pol II) into a pri-miR, which possesses both a poly(A) tail and a 5′ Cap. The enzyme Drosha cleaves the pri-miRNA into a 70-100 nucleotides (nt) hairpin structure termed the pre-miR. The pre-miR translocates into the cytoplasm (via Exportin 5) and is further cleaved by Dicer into two mature miRNAs that are approximately 22nt. These miRNAs are transferred to the AGO proteins to form the miRNA-induced Silencing Complex (miRISC). Strand selection occurs, where the dominant strand termed “guide strand”(shown in red throughout the schematic) remains and the non-active strand or“passenger strand (shown in black throughout the schematic) is discarded. The RISC complex is then activated and engages the seed sequences of the miRNA with the 3’UTR of the target mRNA leading to translational repression or mRNA destabilization and decay.

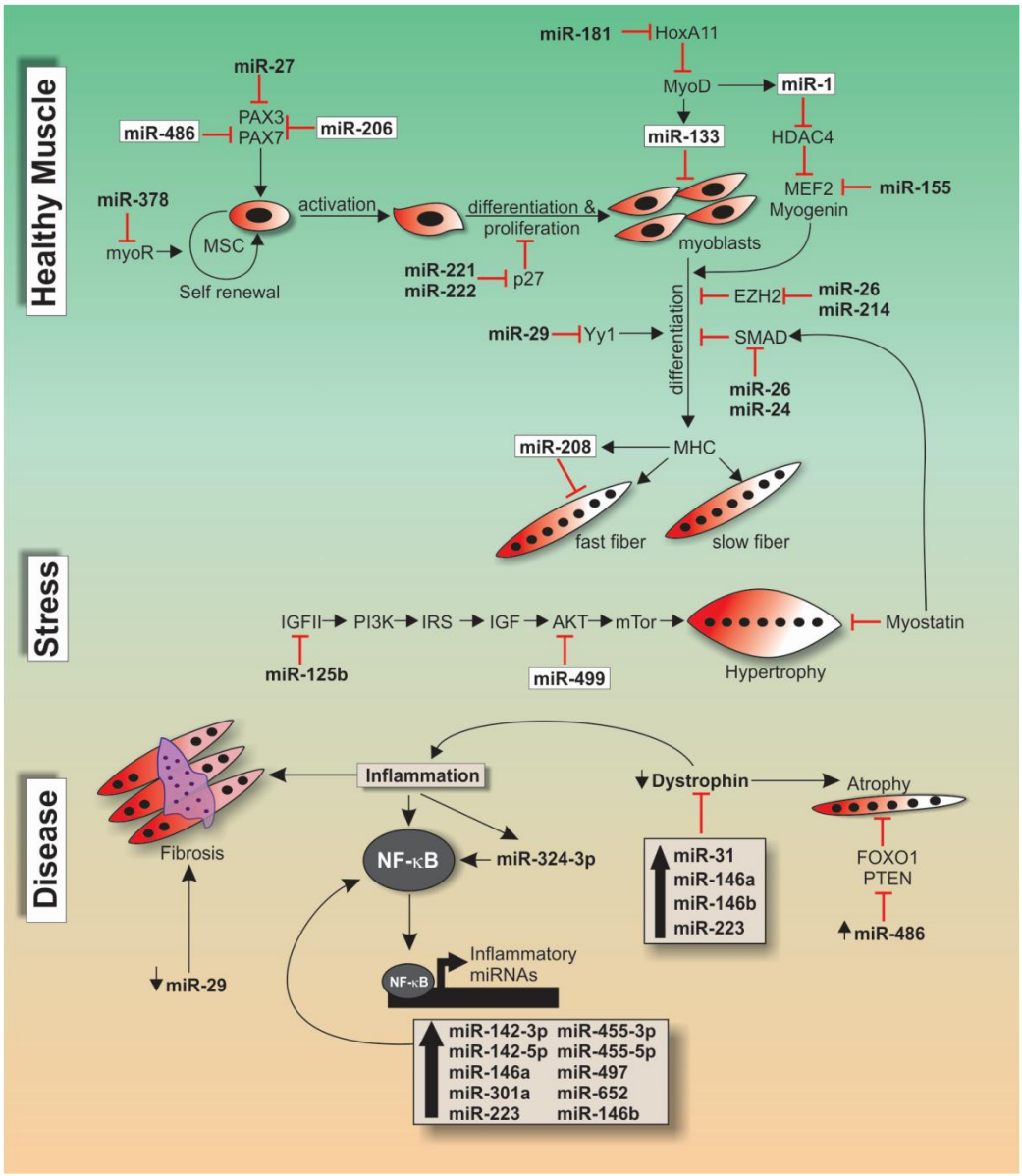

Figure 2. miRNA-mediated regulation of skeletal muscle signaling in healthy muscle, stressed or exercised muscle and disease. Schematic represents gene regulation by miRNAs known to be involved in muscle development and muscle growth (hypertrophy). The bottom two panels illustrate how dysregulated miRNAs in disease feed into different pathways (inflammation, atrophy, fibrosis) to exacerbate disease. Red bars represent inhibition. Canonical myomiRs are shown in white boxes. Adapted from (Ballarino, Morlando et al. 2016) and (Fiorillo, Tully et al. 2018).

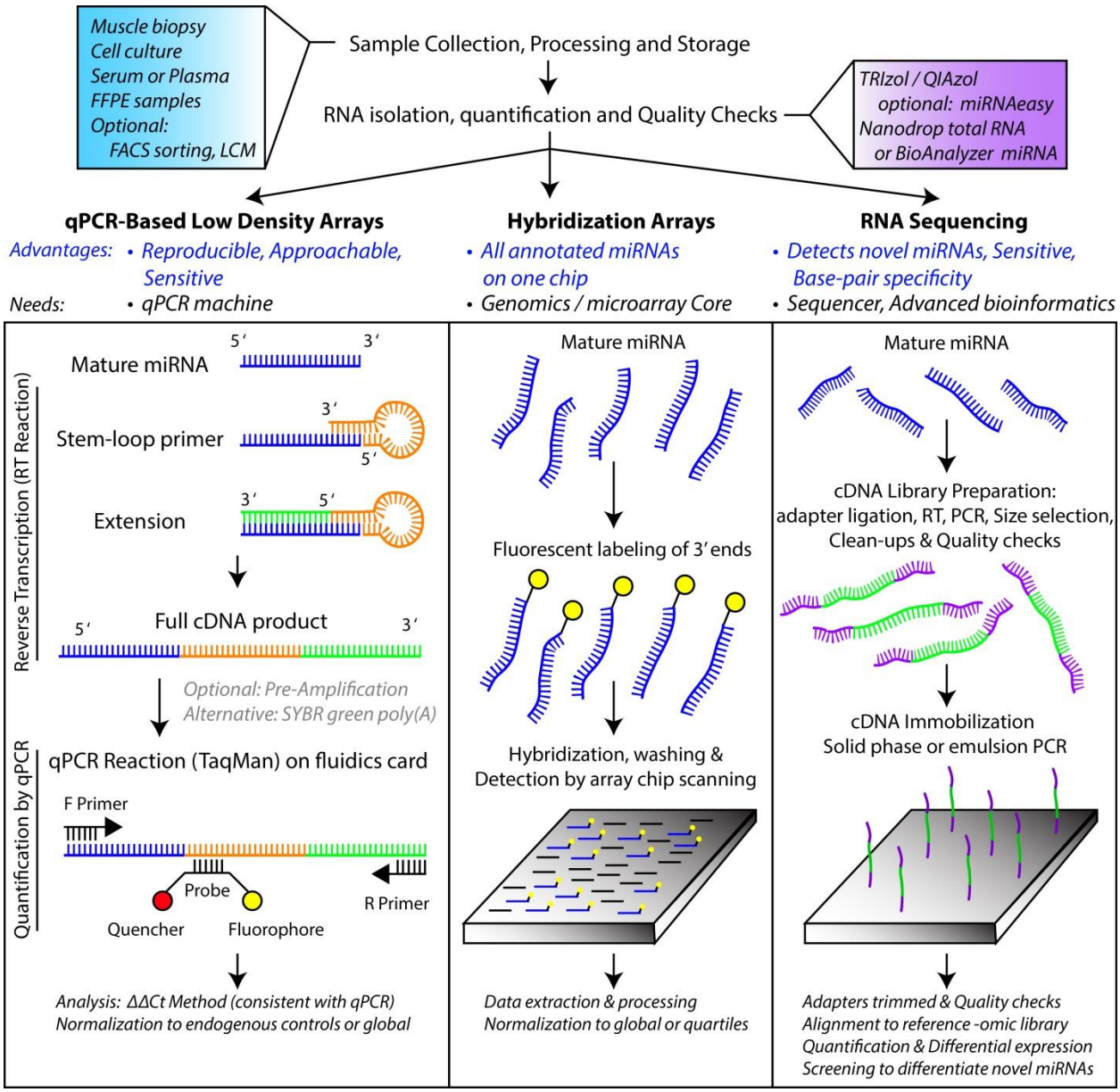

Figure 3. miRNA profiling platforms. Schematic summarizes the overall methodologies for miRNA profiling of muscle or related samples. Each of the columns presents the concepts and processes involved in one of three main profiling methods: qPCR-Based Low Density Arrays (stem-loop RT method depicted here), hybridization arrays, and RNA sequencing. The qPCR-based methods are very approachable for those with prior experience in single gene qPCR. Concepts behind the stem-loop primer RT method are depicted here, an approach that is used by low density microfluidics cards to assay approximately 750 miRNAs per set. Next, hybridization arrays are widely available through genomics cores and can simultaneously assay hundreds of pre-annotated miRNAs from multiple species on a single miRNA gene array chip. Finally, RNA sequencing provides a highly precise method with the ability to detect novel miRNAs or highly similar miRNAs at single base-pair specificity.

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RESEARCH ARTICLE | *-Omic Approaches to Understanding Muscle Biology*

Muscle miRNAome shows suppression of chronic inflammatory miRNAs with both prednisone and vamorolone

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Fiorillo AA, Tully CB, Damsker JM, Nagaraju K, Hoffman EP, Heier CR. Muscle miRNAome shows suppression of chronic inflammatory miRNAs with both prednisone and vamorolone. *Physiol Genomics* 50: 735–745, 2018. First published June 8, 2018; doi: 10.1152/physiolgenomics.00134.2017.—Corticosteroids are highly prescribed and effective anti-inflammatory drugs but the burden of side effects with chronic use significantly detracts from patient quality of life, particularly in children. Developing safer steroids amenable to long-term use is an important goal for treatment of chronic inflammatory diseases such as Duchenne muscular dystrophy (DMD). We have developed vamorolone (VBP15), a first-in-class dissociative glucocorticoid receptor (GR) ligand that shows the anti-inflammatory efficacy of corticosteroids without key steroid side effects in animal models. miRNAs are increasingly recognized as key regulators of inflammatory responses. To define effects of prednisolone and vamorolone on the muscle miRNAome, we performed a preclinical discovery study in the *mdx* mouse model of DMD. miRNAs associated with inflammation were highly elevated in *mdx* muscle. Both vamorolone and prednisolone returned these toward wild-type levels (miR-142-5p, miR-142-3p, miR-146a, miR-301a, miR-324-3p, miR-455-5p, miR-455-3p, miR-497, miR-652). Effects of vamorolone were largely limited to reduction of proinflammatory miRNAs. In contrast, prednisolone activated a separate group of miRNAs associated with steroid side effects and a noncoding RNA cluster homologous to human chromosome 14q32. Effects were validated for inflammatory miRNAs in a second, independent preclinical study. For the anti-inflammatory miRNA signature, bioinformatic analyses showed all of these miRNAs are directly regulated by, or in turn activate, the inflammatory transcription factor NF-κB. Moving forward miR-146a and miR-142 are of particular interest as biomarkers or novel drug targets. These data validate NF-κB signaling as a target of dissociative GR-ligand efficacy in vivo and provide new insight into miRNA signaling in chronic inflammation.

Duchenne muscular dystrophy; inflammation; miRNA; muscle; steroids

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a lethal genetic disease with pediatric onset and is characterized by progressive muscle degeneration with chronic inflammation. The current DMD standard of care is chronic treatment with high-dose corticosteroids (prednisone, deflazacort). Prednisone and deflazacort both increase DMD patient strength, prolong ambulation, and reduce scoliosis, however their long-term use is associated with many side effects that negatively impact patient quality of life (1). Side effects noted as particular concerns to children with DMD and their families include stunted growth, bone fragility, mood disturbances, and weight gain. Accordingly, the development of effective drugs that are safer than corticosteroids is an important goal for DMD and other chronic disorders currently treated with steroids.

To develop an improved drug, it is important to dissect how prednisone works at the molecular level. The drug target of prednisone is the glucocorticoid receptor (GR). Once activated by prednisone the GR exerts its effects by *1*) binding to other proteins to affect their functions, and *2*) moving into the nucleus where it directly binds to DNA promoters to affect gene expression through glucocorticoid response elements (GREs). Many anti-inflammatory effects of prednisone are believed to be caused by GR protein interactions, where the GR inhibits the inflammatory transcription factor NF-κB (58). However, some have hypothesized that prednisone efficacy in DMD is mediated through other functions, either by the direct actions of the GR in binding to DNA to activate GREs in gene promoters (57) or by gross physiological effects such as growth stunting (20). Moving forward, it is important to determine which of these GR properties can be selectively activated, as well as which GR properties are expendable versus which properties are essential for efficacy in treating DMD and other chronic disorders. Vamorolone (VBP15) is a first-in-class dissociative steroid that binds the GR with high affinity (23). Data to date on individual gene targets suggest that vamorolone/GR complexes retain many protein-binding activities of prednisone/GR complexes (e.g., NF-κB inhibition), but vamorolone/GR complexes do not activate gene targets as do prednisone/GR complexes (e.g., GRE transactivation). Thus, vamorolone loses transactivation (gene transcription) activities associated with side effect profiles of corticosteroids, while maintaining anti-inflammatory activities associated with efficacy.

Recently, miRNAs have emerged as a promising new class of biomarkers and therapeutic targets. Specific proinflamma-

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tory microRNAs are becoming increasingly implicated in chronic inflammatory states, as reviewed in Ref. 64. miRNAs are relatively stable, are highly conserved across species, and because miRNAs are not translated into a protein product, their expression can be directly correlated to function. Typically, miRNAs exert their functions by binding to the 3′ untranslated region of mRNA and either inhibiting their translation or promoting mRNA decay, thereby downregulating corresponding protein expression (80). Their stability and conservation across species contribute to their appeal as biomarkers (33, 46). miRNAs are also becoming increasingly attractive therapeutic targets (31).

Here, we analyze expression of the miRNAome in *mdx* dystrophic muscle to dissect molecular signatures that drive effects of contrasting drug treatments at the genomic level in vivo. We are developing vamorolone (VBP15) as a dissociative GR ligand and have previously reported that it shows efficacy similar to prednisone in the *mdx* mouse model of DMD, in the absence of traditional steroid side effects in the *mdx* mouse (23). Here we utilize a more holistic, -omic approach to study the larger scale molecular effects of chronic prednisolone and vamorolone treatment in vivo. This approach enables us to dissect the molecular pathways that are shared versus differentiated for these two GR ligands, which share efficacy but are differentiated in safety profiles at the organismal level (23). We identify a key set of nine miRNAs that are all elevated by muscular dystrophy disease and return toward healthy wild-type levels upon treatment with both drugs. All nine miRNAs are directly activated by or in turn activate the inflammatory transcription factor NF-κB. These data provide a key group of miRNAs for the development of novel biomarkers and therapies, while also validating chronic inflammatory NF-κB signaling pathways as a target of dissociative steroid efficacy in vivo.

MATERIALS AND METHODS

Animal care. All mouse studies were performed in adherence to the NIH Guide for the Care and Use of Laboratory Animals. All experiments were conducted according to protocols that were within the guidelines and approval of the Institutional Animal Care and Use Committee of Children's National Medical Center. All *mdx* (C57BL/10ScSn-Dmd<*mdx*>/J) and wild-type control (C57BL/10ScSnJ) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Drug dosing and mouse muscle samples. Archival muscle samples (diaphragm) from two separate preclinical studies were obtained, with each trial showing a significant benefit from both prednisolone and vamorolone drug treatments (23). Prednisolone was used because it is the active form of prednisone, the current DMD standard of care. The first "discovery set" of diaphragm muscles was from a prophylactic trial design where 2-wk-old (postnatal day 15) *mdx* or wild-type control mice received oral dosing for 6 wk with vehicle (cherry syrup), prednisolone (5 mg/kg), or vamorolone (15 mg/kg) as previously reported (23). The second "validation set" of diaphragm muscles was from an extended trial in older mice where *mdx* or wild-type mice were subjected to treadmill running to unmask mild phenotypes. Mice in this validation set were treated with either vehicle, prednisolone (5 mg/kg), or vamorolone (45 mg/kg) for 4 mo beginning at 2 mo of age (23). At the end point of each trial, diaphragm muscles were harvested and frozen in liquid nitrogen-cooled isopentane.

TaqMan miRNA low-density arrays. We extracted total RNA from five diaphragm muscles per treatment group in the discovery set of samples from 8-wk-old mice. RNA was extracted using a modified TRIzol protocol with isopropanol precipitation at −20°C overnight. This RNA was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher, Carlsbad, CA), with miRNA-specific Megaplex RT Primers, Rodent Pools Set version 3.0 (Thermo Fisher). Levels of each of the miRNAs were profiled in the discovery set using TaqMan Array Rodent MiRNA A+B Cards Set v3.0 (Thermo Fisher).

Quantitative RT-PCR of individual miRNAs. Specific miRNAs were quantified in the validation set of samples using individual TaqMan assays specific for each miRNA (Thermo Fisher) according to the manufacturer's protocol. Assay IDs used include 000468, 001346, 002455, 001280, 000528, 002352, 000464, 002509, and 002248. Total RNA was converted to cDNA using multiplexed RT primers and High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). The cDNA was preamplified using TaqMan PreAmp Master Mix (Thermo Fisher). The miRNAs were then quantified using individual TaqMan assays on an ABI QuantStudio 7 Real-Time PCR machine (Applied Biosystems, Foster City, CA).

Statistical analysis of miRNA expression. In the discovery set experiments, levels of each miRNA were quantified in TaqMan low-density arrays (TLDAs) using Thermo Fisher Cloud software with the Relative Quantification Application (Thermo Fisher) tool. Data were analyzed by ANOVA with post hoc comparison of each group to the *mdx* vehicle-treated group. To identify focus miRNAs for study relating to drug efficacy, we selected all miRNAs that showed a significant difference for all three groups (wild-type, prednisolone, and vamorolone) compared with *mdx* vehicle. A P value of $P \leq 0.05$ was set as the significance threshold, without adjustment for multiple comparisons. To reduce false-positive discovery in this setting, we used an evidence-based approach to identify efficacy-associated miRNAs where *1*) results from the multiple groups were cross-referenced, and *2*) all candidate miRNAs identified in this discovery set were then assayed in a separate validation set of mice. For the validation set experiments, we assayed levels of each individual miRNA in samples from a second, independent preclinical trial in adult *mdx* mice. Data from individual TaqMan Assays were quantified using QuantStudio Real-Time PCR version 1.3 software (Applied Biosystems). The levels of all miRNAs were normalized to the geometric mean of multiple control genes (60, 70). Comparison of groups was made by ANOVA with Holm-Sidak post hoc test comparing each group to *mdx* vehicle.

Bioinformatics. We examined the regulation of each miRNA gene promoter to gain insight into the mechanisms of response to treatment. This was done by examining promoter binding by the inflammatory transcription factor nuclear factor-kappa B (NF-κB, or RELA) or by the GR (NR3C1) using chromatin immunoprecipitation sequencing (ChIP-seq) data. For both NF-κB and the GR, ChIP-seq data from the Encyclopedia of DNA Elements (ENCODE) were queried for physical binding to DNA loci encoding the human homologue of each miRNA target of interest (29, 41). In addition, we examined the following histone modifications, which are enriched at regulatory elements such as promoters or enhancers: histone H3K4 trimethylation (found near promoters), H3K4 monomethylation (found near regulatory elements), and H3K27 acetylation (found near active regulatory elements). For each of these analyses, we used UC Santa Cruz Genome Browser Release 4 (https://genome.ucsc.edu/index.html) with alignment to the GRCh37/hg19 genome build. Each ChIP-seq data set was analyzed using the ENCODE Regulation Super-Track listed under the Regulation menu. Binding by NF-κB or GR was assayed using the Txn Factor ChIP Track. In regions bound by each transcription factor, DNA motifs recognized by that transcription factor were identified through the Factorbook repository within this track. Consensus motif sequence logo pictograms for each transcription factor were also visualized through Factorbook. Histone modifications were examined using the Layered H3K4Me1, Layered H3K4Me3, and Layered H2K27Ac Tracks. Raw data images for visualization of gene loci and ChIP-seq data were obtained using the PDF/PS function in the View menu of the genome browser.

Binding by NF-κB was queried in ChIP-seq data sets produced using TNF-induced lymphocyte cell lines (GM10847, GM12878, GM12891, GM12892, GM15510, GM18505, GM18526, GM18951, GM19099, and GM19193) with ChIP-seq performed using an antibody to an NF-κB subunit (RELA). For the GR, we queried ChIP-seq data sets produced using dexamethasone-treated lung epithelial (A549) and endometrial (ECC-1) cell lines with ChIP-seq performed using an antibody to the GR (NR3C1). Histone modifications were queried in ChIP-seq data sets produced using lymphoblast (GM12878), stem (H1-hESC), myoblast (human skeletal muscle myoblasts), endothelial (human umbilical vein endothelial cells), lymphoblast (K562), keratinocyte (normal human epidermal keratinocyte), and lung fibroblast (normal human lung fibroblast) cell lines using antibodies specific to each histone modification.

To visualize behavior of miRNAs within individual mice for each group, we generated heat map images. Heat maps were generated using relative quantification values of TLDA data exported from the Relative Quantification Application of the Thermo Fisher Cloud software (Thermo Fisher) tool. Heat maps of miRNA expression were produced using Hierarchical Clustering Explorer Version 3.5 (http://www.cs.umd.edu/hcil/multi-cluster/) produced by the Human Computer Interaction Laboratory (University of Maryland, College Park, MD).

RESULTS

Discovery of miRNAome responses to muscular dystrophy disease and treatment. To examine miRNA expression, we utilized TaqMan low-density quantitative PCR array cards to profile ~750 miRNAs within diaphragm muscle from a discovery set of mice. Samples for this experiment came from a trial that utilized a prophylactic approach (23). Briefly, *mdx* mice undergo a stage of peak severity characterized by widespread inflammation and necrosis from ~3–8 wk of age, followed by a recovery stage where mice show milder phenotypes (25). For this prophylactic preclinical trial, treatments were initiated in mice at postnatal day 15 to treat before and during the stage of peak severity. The discovery set of samples consisted of diaphragm from 8-wk-old untreated wild type (vehicle), untreated *mdx* (vehicle), prednisolone-treated *mdx* (5 mg/kg), and vamorolone-treated *mdx* (15 mg/kg) mice. Prednisolone was used here because it is the active form of prednisone, the current DMD standard of care. We chose to examine the diaphragm because respiratory function is important for DMD outcomes, because it is a severely affected muscle in *mdx* that is more comparable to DMD (65) and because diaphragm muscles are more evenly stressed between mice than purely voluntary muscles of the leg. Previously, in these same mice, we found that both prednisolone and vamorolone successfully improved grip strength, muscle pathology, and diaphragm inflammation (23). Additionally, we found in these same mice that prednisolone caused traditional steroid side effects of stunted growth, immunosuppression, and bone loss, whereas the dissociative steroid vamorolone successfully avoided these side effects. Here, using TLDA quantitative PCR array cards we detected ~500 miRNAs expressed in the diaphragm muscle from all groups.

We found that expression levels of 202 miRNAs showed a significant difference in at least one of the groups compared with the untreated *mdx* group (Supplemental Table S1; Supplemental Material for this article is available online at the Journal website). Comparing untreated *mdx* to wild-type mice, expression levels of 136 miRNAs were significantly different in dystrophic muscle. Treatment of *mdx* mice with the glucocorticoid prednisolone caused a significant change in 76 miRNAs in dystrophic muscle. In contrast, treatment with the dissociative steroid vamorolone only affected about half as many miRNAs, with expression of 41 miRNAs changed in *mdx* muscle.

To identify a set of efficacy miRNA markers associated with both the muscular dystrophy disease process and a healthy response to treatment, we queried which miRNAs were different in all three groups (wild type, prednisolone, and vamorolone) compared with untreated *mdx* (Fig. 1). Using this approach, we identified a focus set of nine miRNAs (Fig. 1, *A* and *B*). All nine of these miRNAs were increased in muscular dystrophy and returned toward healthy wild-type levels as a result of treatment with both drugs (Table 1). Of these nine miRNAs, three have previously been found to be dysregulated in muscle disorders (miR-146a, miR-142-3p and miR-142-5p; 17, 28, 40). Interestingly, all nine of these miRNAs are involved in inflammatory signaling pathways and at least seven of them have been found to be upregulated in other inflammatory disorders (6, 9, 13–15, 18, 21, 28, 32, 34–36, 39, 40, 42, 44, 49–51, 59, 61, 67, 68, 71, 74, 75, 82); see Table 1 for miRNA-specific references).

We next compared effects of the two drugs to examine consequences of their differing chemistries on genomic miRNA regulation and steroid side effects. Prednisolone, a traditional glucocorticoid, both activated and inhibited expression of miRNAs (Fig. 1, *C* and *D*). Treatment with prednisolone produced a significant increase in nine miRNAs ($P \leq 0.05$), eight of which were unique. Interestingly, none of the miRNAs queried in the TLDA cards were significantly increased in response to vamorolone treatment. These data are consistent with the more selective dissociative chemistry of vamorolone, which can inhibit inflammatory signaling without activation of individual GR-regulated genes. Additionally, these data identify a set of prednisone-specific miRNAs whose activation is consistent with the activation of glucocorticoid side effects observed in these same mice.

Efficacy miRNA responses are conserved in an independent validation trial. Having identified nine inflammatory miRNAs of interest in the discovery set of mice, we next sought to both validate the miRNA markers that we found and to expand upon our results to determine which ones are of utility in other disease stages or trial designs commonly studied in *mdx* literature (23, 25). To do this, we obtained a validation set of samples from a separate, independent trial using a different trial design characterized by a prolonged treatment regimen in older *mdx* mice. Samples were obtained at a trial end point of 6 mo. At this stage, *mdx* mice show less variability however they have gone through a recovery stage, which results in milder phenotypes that typically require treadmill exercise to unmask. Here, we used gene-specific quantitative RT-PCR to detect expression levels of each individual inflammatory miRNA within diaphragm muscle from the validation set. Differences in the trial design for the validation set of samples versus the previous discovery set include the age of mice (6 mo old), the stage of *mdx* disease (characterized by a phenotypic recovery and increased fibrosis), the added exercise protocol (treadmill running), the length of treatment (4 mo), and the dose (45 mg/kg) of vamorolone (23).

Upon miRNA analysis of the validation set, we found all nine miRNAs showed a conserved response to disease and/or

drug treatments (Fig. 2). One miRNA, miR-301a, showed a roughly twofold increase with disease ($P = 0.002$) but did not respond to drug treatment at this age. One other, miR-324-3p, showed a ~30%–50% decrease in response to both prednisolone ($P = 0.0005$) and vamorolone ($P = 0.02$) but did not show a difference between *mdx* and wild-type mice at this age. The other seven miRNAs all showed both a significant increase with muscular dystrophy ($P \leq 0.005$), and a significant decrease toward healthy wild-type levels in response to both drugs ($P \leq 0.05$). This confirmed that overall, inflammatory miRNAs can be reduced by vamorolone and prednisone at different stages of dystrophy.

Efficacy associated miRNAs indicate NF-κB inhibition mechanism. We queried both NF-κB and GR transcription factor ChIP-seq data from ENCODE and the established literature to gain insight into the transcriptional regulation of each miRNA (Fig. 3). We found that the DNA promoters of eight out of the nine identified miRNAs contain one or more DNA sites that are bound by the inflammatory transcription factor NF-κB (74). This is supported by previous reports demonstrating NF-κB-specific regulation of miR-146a (68), miR-301a (35), and miR-455-3p (48). The other miRNA, miR-324-3p, is regulated by signal transducer and activator of transcription 6 and in turn activates NF-κB, thereby participating in inflammatory NF-κB signaling as well (13). In contrast, only one miRNA, miR-497, had a DNA promoter site bound by the GR; however, it also possessed an NF-κB binding site with its promoter region (Fig. 3). In addition to being regulated by NF-κB, when inappropriately expressed these miRNAs are associated with chronic inflammation, muscle wasting, fibrosis, and adipocyte formation (5, 6, 10, 24, 35, 49, 51, 54, 66, 67, 72, 76; refer to Fig. 3).

One of the most interesting miRNAs in the ChIP-seq analysis was miR-142, which we found is in a DNA locus surrounded by at least 13 DNA elements bound directly by NF-κB (Fig. 3, *A* and *B*). All 13 of these NF-κB binding sites overlapped with corresponding ChIP-seq data that detect histone modifications, which correspond to active regions of transcription regulation (H3K4me3, H3K27Ac, and H3K4me1). Together, these data indicate that the upregulation of NF-κB-activated miRNAs is a signature of dystrophic muscle and that inhibition of chronic NF-κB signaling is a signature shared by two distinctly different but effective steroidal drugs.

Prednisone activates miRNAs associated with side effects and the 14q32 locus. Next, we queried ChIP-seq data and established literature to gain insight into the set of miRNAs that we found were specifically activated by prednisolone

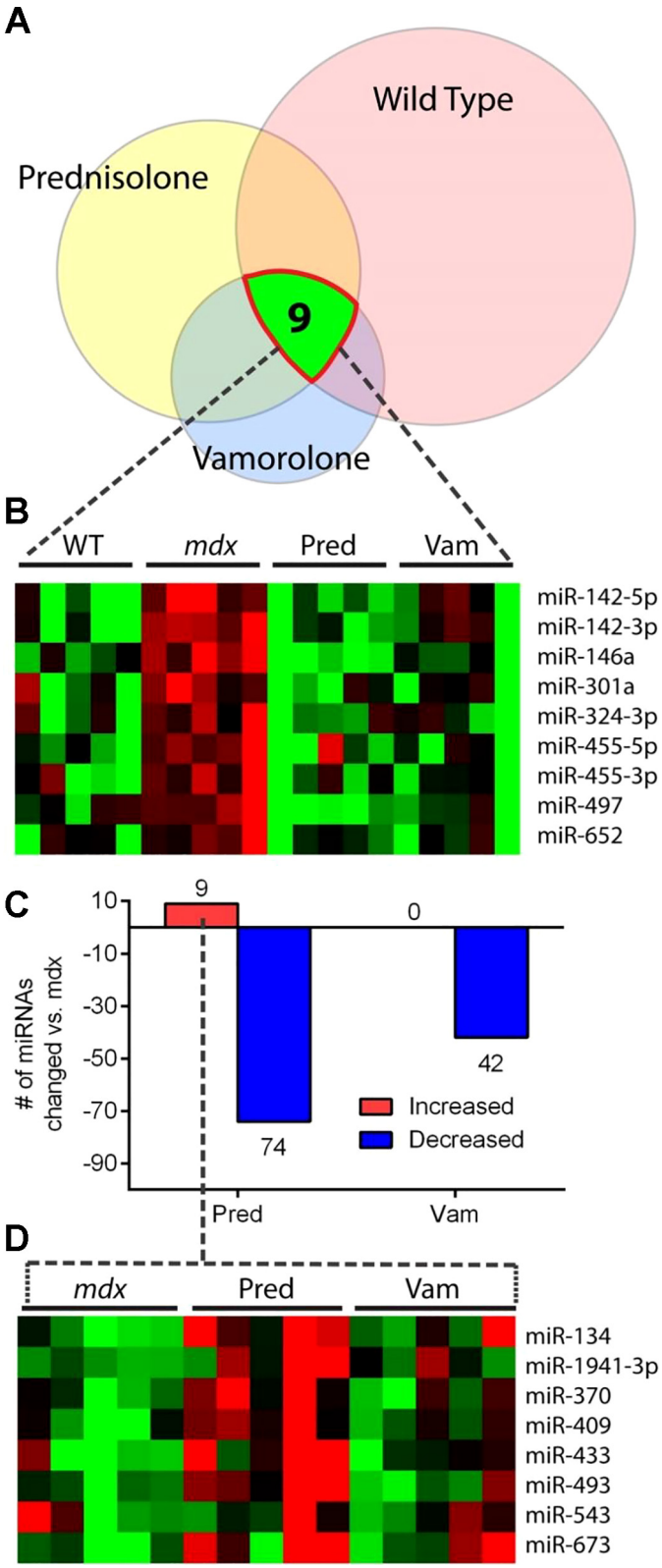


Fig. 1. Summary of muscle miRNA changes discovered in response to dystrophy and its treatment. Expression of the miRNAome was quantified in diaphragm muscle of mice from a discovery set of mice ($n = 5$ mice per group). Groups included WT (vehicle), *mdx* (vehicle), *mdx* treated with prednisolone (5 mg/kg), and *mdx* treated with vamorolone (15 mg/kg), with mice treated from 2 to 8 wk of age in a prophylactic trial design. *A*: a Venn diagram illustrates the proportion of miRNAs that are significantly different than untreated *mdx* muscle in the WT, prednisolone, and vamorolone groups. The nine miRNAs that were significantly different in all three groups vs. untreated *mdx*, highlighted here, were chosen as a focus set of efficacy-associated miRNAs. *B*: heat map visualization of the expression of the nine efficacy associated miRNA markers within each individual mouse. *C*: bar graph showing the number of miRNAs that significantly increased or decreased in response to either of the drug treatments. *D*: heat map of the eight unique miRNAs that were increased by prednisolone. Heat map: red, increased; green, decreased. Pred, prednisolone; Vam, vamorolone; WT, wild type.

Table 1. *Nine miRNAs are elevated by dystrophy and respond to both drugs in the discovery set of diaphragm muscle*

miRNA	WT, %	*Mdx*, %	Pred, %	Vam, %	miRNA Function Disease Associations	Sources
142-5p	43 ± 20**	100 ± 23	44 ± 11**	61 ± 23*	DC homeostasis; resolution of acute inflammation/IBD, Alzheimer's, LGMD2D, DMD	14, 40, 44, 59, 75
142-3p	49 ± 19***	100 ± 10	50 ± 13***	65 ± 20**	Innate immunity; DC homeostasis; resolution of acute inflammation/SLE, LGMD2B/2D, DMD	18, 28, 44, 59
146a	56 ± 10***	100 ± 20	39 ± 6***	52 ± 12***	Stages inflammation/IBD, LGMD2A/2B, Myositis, MM, wasting, HF, MG, Alzheimer's, MS	9, 15, 34, 36, 39, 49, 51, 68, 71
301a	81 ± 15*	100 ± 11	70 ± 18*	77 ± 12*	NF-κB positive feedback loop/IBD	21, 35
324-3p	75 ± 17*	100 ± 19	71 ± 14*	75 ± 13*	Induces and activates NF-κB/IBD	6, 13, 21
455-5p	61 ± 11*	100 ± 17	64 ± 30*	64 ± 15*	TGF-β Signaling/FSHD, LGMD2A, nemaline myopathy	15, 67
455-3p	63 ± 21*	100 ± 24	62 ± 18*	61 ± 14*	Innate immunity; cartilage development/IBD, Alzheimer's	32, 50, 74, 82
497	72 ± 12*	100 ± 22	49 ± 11***	65 ± 11**	NF-κB feedback mechanism via IKKβ	42
652	66 ± 18*	100 ± 30	62 ± 14*	64 ± 18*	Inflammatory signals in immune cells	61

Values are expressed as % expression in comparison to *mdx* vehicle. DC, dendritic cell; DMD, Duchenne muscular dystrophy; FSHD, facioscapulohumeral muscular dystrophy; HF, heart failure; IBD, inflammatory bowel disease; LGMD2D, limb girdle muscular dystrophy type 2D; MG, myasthenia gravis; MM, Miyoshi myopathy; MS, multiple sclerosis; NF-κB, an inflammatory transcription factor named nuclear factor kappa-light-chain-enhancer of activated B cells; Pred, prednisolone; SLE, systemic sclerosis; TGF, transforming growth factor; Vam, vamorolone; WT, wild type. *$P \leq 0.05$, *$P \leq 0.005$, ***$P \leq 0.0005$. ANOVA with post hoc comparison to *mdx* vehicle.

(Fig. 4). Here, we focused on the six miRNAs conserved between mice and humans and on pathways relevant to DMD or steroids. Interestingly, we found that all six of these miRNAs (miR-134, miR-370, miR-409, miR-433, miR-493, miR-543) are transcribed from the same miRNA cluster on mouse chromosome 12F1. This locus is well conserved and has been extensively documented in humans where it resides on chromosome 14q32 (63). Our analysis thus focused specifically on the homologous human cluster (Fig. 4, *A* and *B*). We found that this 14q32 cluster contains seven GR-DNA binding sites and is devoid of NF-κB binding sites (74). At least six of these GR binding sites corresponded to active regulatory enhancer elements. miR-543 has been previously shown to be upregulated by pharmacological glucocorticoids (11). This locus has also been observed to be upregulated by acute stress consistent with upregulation of cortisol, the body's natural glucocorticoid (43). Examining the functional roles of miRNAs upregulated by prednisolone, we found that all are upregulated in states consistent with known side effects of prednisolone treatment. These include insulin resistance (27, 73, 81), behavior changes (43, 81), fibrosis (45, 69), increased risk for heart failure (22, 77), stress (43, 84), and hypertension (11). Together, these data indicate that prednisone specifically activated a separate set of miRNAs in a manner that is consistent with the negative side effects of currently prescribed steroids.

DISCUSSION

Our data identify a set of miRNAs that are elevated by dystrophic disease and that respond to treatment with both prednisolone and the dissociative steroid vamorolone. Of the nine miRNAs we identified, three have been previously found to be elevated in DMD or *mdx* muscle (miR-146a, miR-142-3p, and miR-142-5p), whereas the elevated expression of six others in dystrophic muscle are novel. Analyzing the regulation and functions of these nine miRNAs reveals that all nine are

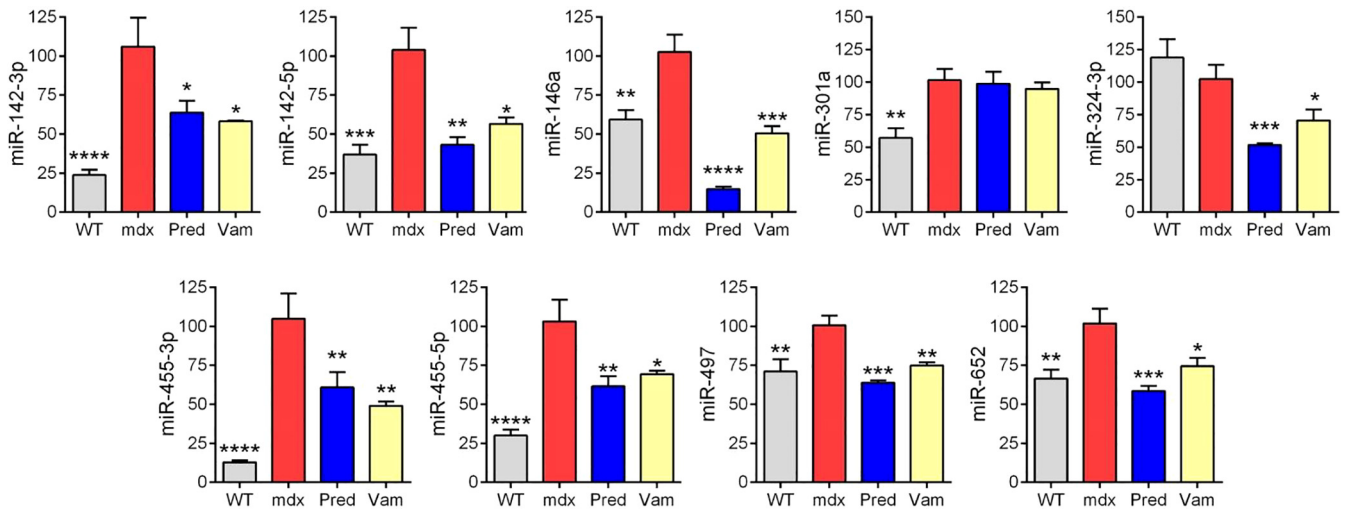
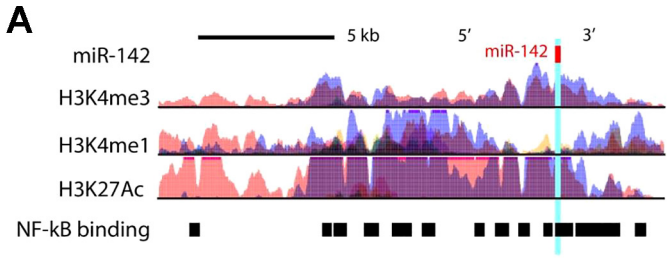


Fig. 2. Behavior of efficacy miRNA signature is maintained in an independent validation trial. A validation set of samples was obtained from a second, independent *mdx* trial performed at a different stage of the *mdx* disease. Mice received daily oral vehicle, prednisolone (5 mg/kg), or vamorolone (45 mg/kg) for 4 mo, with treadmill running to unmask *mdx* phenotypes and muscle harvested at 6 mo of age. The nine miRNAs identified as associated with efficacy in the TLDA arrays were quantified in diaphragm muscle using quantitative RT-PCR in this second set of mice. (Values are graphed as % of untreated *mdx* expression levels; 1 outlier removed from miR-455-5p after significant Grubb's test; $n = 5$ per group; ANOVA with post hoc comparison to *mdx* vehicle; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.0005$). TLDA, Taqman low-density array.



NF-kB motif	a	G	G	G	g	a	T	T	T	C	C	a	.	.	.
miR-142 site 1	T	G	G	G	G	C	T	T	T	C	C	A	A	G	G
miR-142 site 2	A	G	G	G	G	C	T	T	T	C	A	A	A	A	T
miR-142 site 3	A	G	G	T	A	T	T	T	T	C	C	G	G	G	A
miR-142 site 5	G	G	G	G	A	C	T	T	C	C	T	A	G	A	G
miR-142 site 12	A	G	G	G	G	A	T	T	C	T	C	A	C	G	T

C

miRNA	Promoter Regulation	Pathophysiological Roles	Sources
142-5p	NF-κB	ϕ-induced Inflammation and fibrosis; Neuroinflammation	(66, 74)
142-3p	NF-κB	Inflammatory program of MoDCs; Neuroinflammation	(74, 76)
146a	NF-κB	Serum biomarker of inflammation; senescence	(24, 49, 51, 68)
301a	NF-κB	Pro-inflammatory, targets NKRF in NF-κB positive feedback loop	(35, 74)
324-3p	IL4, STAT6	Pro-Inflammatory; Induces NF-κB; pro-fibrotic ϕ pathway	(6, 10, 13)
455-5p	NF-κB	Muscle atrophy, adipocyte formation	(72, 74)
455-3p	NF-κB, TWEAK	Inflammatory muscle wasting; resides in intron of *COL27A1*	(48, 51, 67, 74)
497	NF-κB, GR	Pulmonary fibrogenesis	(5, 74)
652	NF-κB	Acute coronary syndrome marker	(54, 74)

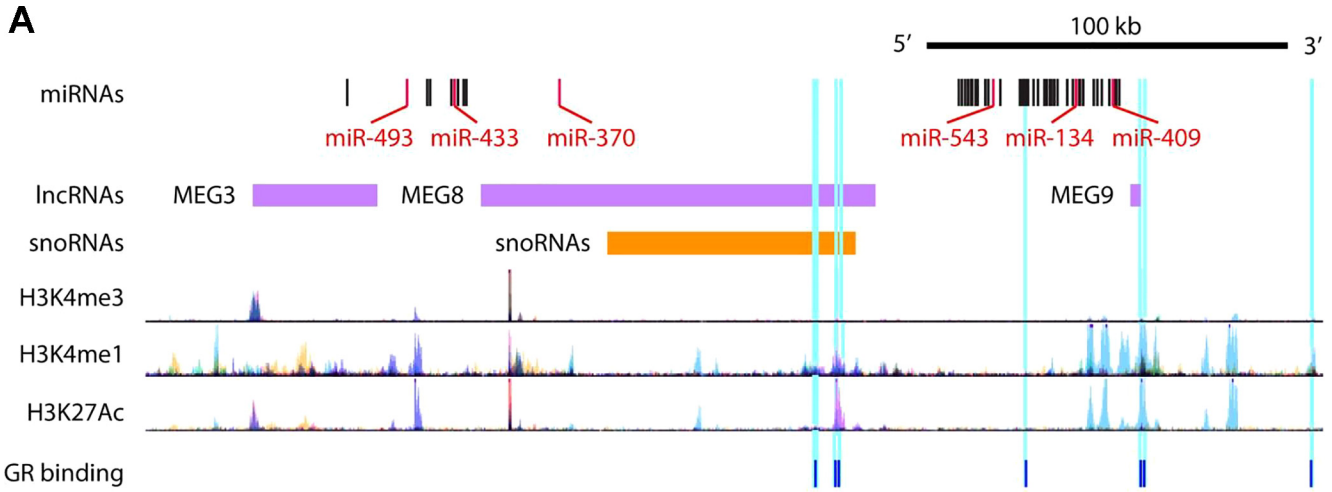
Fig. 3. Promoter analysis of miRNAs indicates NF-κB signaling is a shared target of effective drugs. Transcription factor (NF-κB, GR) binding sites and histone (H3) modifications that mark regulatory regions were examined using ChIP-seq data from ENCODE. DNA-binding motifs for each transcription factor were identified through the Factorbook repository. *A*: schematic of the gene locus for miR-142, illustrating the binding site of 13 neighboring DNA loci that are bound directly by NF-κB. Corresponding epigenetic modification maps are provided showing the location of histone modifications associated with active promoters (H3K4me3) and poised/active enhancers (H3K4me1 and H3K27Ac) in the immediate vicinity of miR-142. *B*: sequence logo pictogram of base frequency at NF-κB binding sites, with the consensus NF-κB motif provided immediately below. Also provided are five representative NF-κB binding site sequences near miR-142, listed in order from the 5′ to 3′ direction. *C*: summary of promoter analysis and literature data indicating each miRNA and known factors or conditions associated with its transcriptional regulation. ChIP-seq, chromatin immunoprecipitation sequencing; COL27A1, collagen type 27 alpha 1 chain; ENCODE, Encyclopedia of DNA Elements; GR, glucocorticoid receptor; IL4, interleukin 4; MoDCs, monocyte-induced dendritic cells; φ, macrophage; NKRF, NF-κB-repressing factor; STAT6, signal transducer and activator of transcription 6; TWEAK, TNF-like weak inducer of apoptosis.

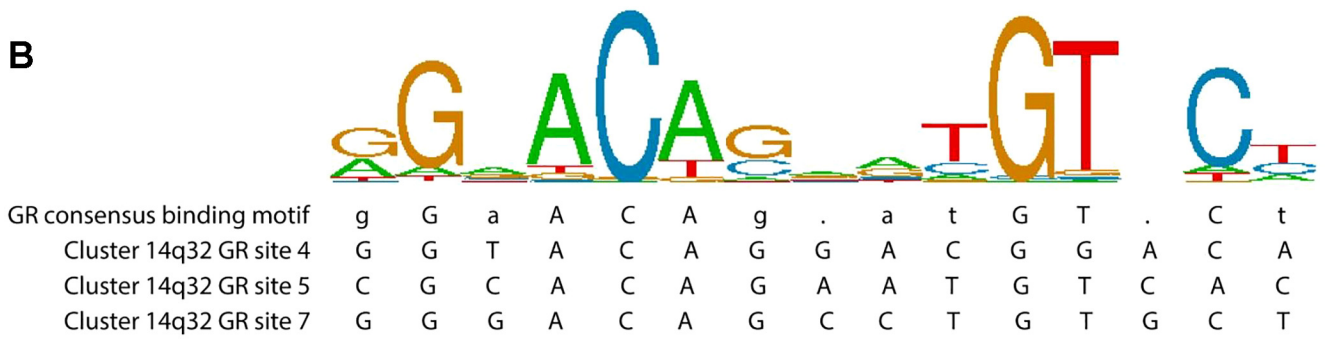
involved in proinflammatory signaling. By comparing prednisone and vamorolone, we see that these two effective but distinctly different GR ligands share efficacy as anti-inflammatory drugs that downregulate this network of inflammatory miRNAs. In contrast, vamorolone, unlike prednisolone, avoids off-target activation of miRNA transcription associated with negative steroid side effects, such as insulin resistance, adrenal suppression, hypertension, and behavior issues. This is consistent with both preclinical *mdx* mouse and recently completed human Phase I trials, which show vamorolone avoids or has substantially reduced steroidal side effects in comparison to prednisone (23, 26). Together, our work identifies a network of miRNAs associated with chronic inflammation and validates NF-κB signaling as an in vivo target of efficacious dissociative steroids (see Fig. 5 for model).

miRNAs fine tune gene expression in a multitude of signaling pathways and cellular processes. Similarly, miRNAs play a key regulatory role in the core inflammatory signaling pathway driven by NF-κB. This pathway is inappropriately upregulated in many inflammatory disorders and drives chronic muscle inflammation in DMD (7, 47). Of the miRNAs we describe here, eight are directly regulated by NF-κB (6, 13, 35, 48, 51, 74). Some, in turn, also regulate the initiation and resolution of the inflammatory response by targeting other key factors in the NF-κB signaling pathway. Thus, understanding the mechanisms governing miRNA dysregulation in relation to NF-κB signaling is particularly relevant to anti-inflammatory drug development in DMD. We will discuss some of the miRNAs of note below.

miR-142 is dysregulated across multiple diseases and may provide a novel therapeutic target. Here, we find both mature miR-142 family members (miR-142-3p, miR-142-5p) are elevated with dystrophic disease and their expression is quelled by both prednisolone and vamorolone treatment. miR-142-3p is highly expressed in monocytes (18) and lymphocytes (30), suggesting it is a marker of inflammatory infiltration. Corroborating our data here, another report observes elevated miR-142-3p levels in the gastrocnemius of three distinct muscular dystrophy model mice: *Dysferlin-null*, *α−sarcoglycan* (*Sgca*)*-null*, and *mdx* (28). This same report finds when *Sgca* is reexpressed in *Sgca-null* mice, miR-142-3p levels move toward their wild-type counterparts (28). Not surprisingly, studies focusing on miR-142-5p show similar findings. One report shows miR-142-5p is increased in single muscle fibers isolated from *mdx* and *Sgca-null* muscles and is induced in response to acute injury in *mdx* (40). Of particular interest, a recent study in mouse models of experimental colitis finds that inhibition of miR-142-5p is effective at improving disease outcomes (14). In this colitis study, the most important disease feature that improves with miR-142-5p inhibition is muscle wasting, which is known to be regulated by NF-κB (2). Together, these findings suggest miR-142 family members can serve as biomarkers of drug efficacy across different muscular dystrophies. Furthermore, the ubiquitous nature of miR-142 dysregulation across a multitude of inflammatory disorders makes these miRNAs attractive as potential therapeutic targets in states of chronic inflammation.

miR-146a is one of the most prevalent miRNAs that appears in the literature in instances of chronic inflammatory disorders (9, 15, 34, 36, 39, 49, 51, 71). miR-146a is highly associated with inflammation, is induced by NF-κB in immune cells (68), and is also expressed directly in muscle (17). Acute miR-146a activation dampens NF-κB-mediated inflammation (68); however, prolonged induction of miR-146a exacerbates inflammation (19, 37). In diseases where chronic inflammation is pres-





C

miRNA	Regulated by	Sources	Upregulation of miRNA is associated with:
miR-134	GR †	(22, 43, 73, 74)	Insulin resistance, stress, heart failure, fibrosis
miR-370	GR †	(74, 81)	Type II Diabetes and insulin resistance
miR-409-3p	GR †	(45, 74)	Fibrosis
miR-433	GR †	(69, 74)	Heart fibrosis
miR-493	GR †	(74, 84)	Stress, depression and mood disturbances
miR-543	GR †	(11, 74)	Insulin resistance, hypertension, osteosarcoma

Fig. 4. Prednisolone increases miRNAs associated with GR regulation and the 14q32 mega cluster. For the conserved miRNAs that were specifically elevated in prednisolone-treated mouse muscle, we analyzed the genomic loci, transcription factor binding sites (GR and NF-κB), and histone (H3) modifications using ChIP-seq data from ENCODE. DNA motifs bound by the GR were identified through Factorbook. *A*: all six of the miRNAs are transcribed from a well-conserved noncoding RNA cluster (mouse 12F1) which is extensively characterized in humans at the 14q32 locus; that human cluster is depicted here along with corresponding GR-binding sites, as well as histone modifications that correspond to active gene promoters (H3K4me3) and poised or active gene enhancer elements (H3K4me1, H3K27Ac). No NF-κB-binding sites are found at this locus. *B*: sequence logo pictogram of base frequency at GR binding sites, with the consensus GR motif sequence provided immediately below. Also provided are three representative GR-binding site sequences from this locus numbered from the 5′ to 3′ direction. *C*: summary of bioinformatic analyses for each miRNA, with a list of conditions that are associated with increased levels of each miRNA. †encoded by the 14q32 cluster of miRNAs with GR-bound enhancers. ChIP-seq, chromatin immunoprecipitation sequencing; ENCODE, Encyclopedia of DNA Elements; GR, glucocorticoid receptor; lncRNA, long noncoding RNA; MEG, maternally expressed gene; snoRNA, small nucleolar RNA.

ent, miR-146a levels are highly elevated both in the serum as well as in tissues affected by disease (15, 34, 36, 39, 49, 51, 71). Previously, we reported that miR-146a serum levels predict patient response to anti-inflammatory (prednisone, Remicade) treatment in inflammatory bowel disease (IBD; 24). In a separate report, we showed that miR-146a participates in a muscular dystrophy feedback loop wherein it specifically inhibits the production of dystrophin in Becker muscular dystrophy and in a mouse model of DMD exon skipping (17). Our current findings strengthen claims that miR-146a is both a promising therapeutic target and a pharmacodynamic biomarker.

Previous reports describe a role for miR-455 family members in immune signaling and muscle wasting. The transcription of miR-455-3p is controlled by NF-κB in macrophages (48). A report by Eisenberg et al. (15) shows increased miR-455-5p in muscle biopsies from patients with facioscapulohumeral muscular dystrophy, limb girdle muscular dystrophy 2A and nemaline myopathy. miR-455-3p is encoded within the intron of *COL27A1*, a gene that encodes a cartilage collagen (67). Expression of miR-455 is induced by TNF-like weak inducer of apoptosis, which plays a key role in skeletal muscle wasting (51), and miR-455-5p is implicated in skeletal muscle atrophy (72). Interestingly, miR-455-3p is also implicated in

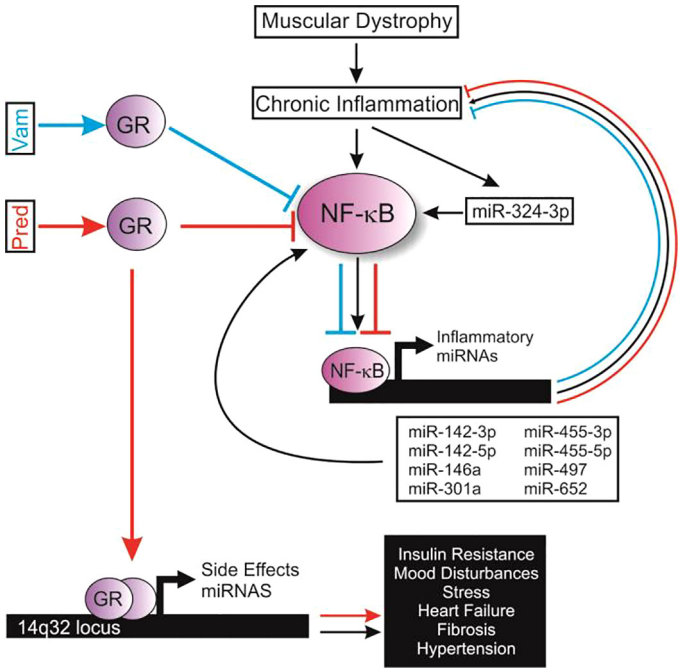


Fig. 5. Proposed model of NF-κB and GR-regulated miRNAs in the treatment of muscular dystrophy. In DMD, inflammatory signaling promotes the chronic activation of NF-κB. This, in turn, activates NF-κB gene targets, including miRNAs that regulate the expression of proteins in the NF-κB signaling pathway, creating a chronic inflammatory feedback loop. Here we show that the NF-κB-regulated miRNAs miR-142-3p, miR-142-5p, miR-146a, miR-301a, miR-455-3p, miR-455-5p, miR-497, and miR-652 are all elevated in dystrophic muscle. These NF-κB-regulated miRNAs are all effectively decreased by both vamorolone (Vam) and prednisone (Pred) treatment, via the GR. miR-324-3p, a miRNA that activates NF-κB in a positive feedback loop, is also decreased by both drugs. Acting through a separate pathway which can be selectively avoided by dissociative steroid chemistries, prednisone also directly causes GR-mediated transactivation of gene transcription. This results in elevated levels of a miRNA cluster located on chromosome 14q32. These microRNAs are associated with steroid side effects such as insulin resistance, hypertension, stress, and mood disturbances. Blue lines, pathways affected by vamorolone; red lines, pathways affected by prednisolone. DMD, Duchenne muscular dystrophy; GR, glucocorticoid receptor.

Alzheimer's disease (32). Together, these data suggest miR-455 family members may function as a potential marker of inflammation, atrophy, and drug efficacy.

Three miRNAs found in our study specifically regulate the duration and extent of NF-κB signaling via feedback mechanisms. miR-301a was reported to be the most potent activator of NF-κB out of hundreds of miRNAs, and it exerts its actions via downregulation of the NF-κB repressing factor (35). The miR-301a promoter contains an NF-κB DNA consensus element, allowing a positive feedback mechanism of NF-κB signaling: miR-301a represses NF-κB repressing factor, in turn promoting NF-κB activation, which activates miR-301a transcription. Another identified miRNA, miR-324-3p, functions in a transcription factor-like manner to trigger NF-κB transcription via sequence-specific promoter binding (13). Elevated miR-324-3p is also observed in IBD (52) and is rapidly induced after focal cerebral ischemia (12), further implicating this miRNA in driving inflammatory processes. miR-497 transcription is driven by NF-κB (42). In cases of acute inflammation, miR-497 participates in a feedback mechanism by targeting IKKβ, a kinase required for NF-κB activation (42). miR-497 is associated with regenerative capacity of muscle stem cells (62) suggesting it is transcribed both in immune cells and skeletal muscle. The above reports describing miRNA involvement in NF-κB -mediated feedback focus specifically on acute inflammation; the consequences of chronic miR-301a/miR-324-3p/miR-497 overexpression on NF-κB signaling have not been documented. Our data, however, suggest that persistent expression of these miRNAs drives a feed-forward loop of prolonged NF-κB activation and inflammation; this can be effectively attenuated by prednisolone and vamorolone treatment.

For the first time, we find that expression of a noncoding RNA cluster in the genome is increased by prednisone treatment of dystrophic muscle. Specifically, we identify six miRNAs within this cluster that appear to be coregulated and located by GR-bound enhancer elements. This cluster is well conserved across mammalian species and is among the largest polycistronic clusters. It is best characterized in humans where this cluster is on chromosome 14q32 and encodes 54 miRNAs (63). All six of the miRNAs increased from this locus are also known to be elevated in conditions that are consistent with prednisone side effects, including insulin resistance (27, 76, 81), mood disturbances (43, 81), stress (43, 84), and hypertension (11). Because prednisolone increases *mdx* heart fibrosis (9) and heart failure is a leading cause of death in DMD, increases here in miR-433 and miR-134 are interesting as they are a regulator of heart fibrosis and a serum biomarker indicative of increased risk of heart failure, respectively (22, 69). In addition to miRNAs, the 14q32 locus encodes two long noncoding RNAs, which also may have functions that are relevant to DMD and its treatment with steroids. These two long noncoding RNAs are maternally expressed gene (MEG) 8 and MEG3. MEG8 is preferentially expressed in skeletal muscle and is increased in muscle hypertrophy as observed in callipyge (or "beautiful buttocks") sheep (4). MEG3 expression is enriched in cardiac fibroblasts and intriguingly, the inhibition of MEG3 prevents heart fibrosis and diastolic dysfunction via regulation of matrix metalloproteinase 2 in a mouse model of heart damage (53). Moving forward, it will be interesting to determine the full extent of upregulation at the 14q32 locus upon prednisone treatment and the health impact that this may have on muscular dystrophy patients.

It is interesting to note that there were many similarities between dysregulated miRNAs reported here in muscular dystrophy and what has been reported in other inflammatory diseases, in particular for IBD. Specifically, in IBD six of the nine miRNAs reported here (miR-142-3p, miR-142-5p, miR-146a, miR-342-3p, miR-301a, miR-455-3p) are elevated in the serum and intestinal mucosa of patients with IBD and mouse models of IBD (3, 16, 21, 50). This suggests that the identified set of miRNAs reported here reflect a general signature of chronic inflammation and disease. Given that many of these miRNAs have been identified in inflammatory cells and processes, it is possible that these shared miRNA signatures are representative of inappropriate crosstalk between immune cells and the diseased tissue microenvironment. Moving forward, it will be important to identify and understand the mechanisms that drive inappropriate immune cell-tissue crosstalk and identify targets that could dampen these signals.

One of the miRNAs we identified in our discovery set of samples, miR-301a, did increase with disease but did not show

a response to either drug in the validation set of samples. This may be due to a switch in its transcriptional control as the *mdx* disease transitions from a younger and more inflammatory stage, to an older and more fibrotic stage. In addition to NF-κB, the transcriptional promoter of miR-301a is affected by pathways that increase with fibrosis and with age, via transcription factors within transforming growth factor-β (TGF-β) (7, 55, 56, 74, 83) and β-catenin (78, 79) pathways, respectively. Because fibrosis increases with *mdx* age, particularly in the diaphragm, a shift to combinatorial control of miR-301a expression by TGF-β and/or β-catenin pathway transcription factors could circumvent the effects of GR ligands on NF-κB. Because miR-301a increases NF-κB signaling and in some instances appears to avoid inhibition by GR ligands, it will be interesting to study this miRNA further, as inhibitors of it could provide a mechanism to enhance anti-inflammatory efficacy further through a cotherapy strategy.

There is growing enthusiasm with regards to targeting miRNAs as therapeutic agents via antisense technology. There are currently nine miRNA therapeutics that are in preclinical or clinical development, reviewed in (8). We suggest the miRNAs described here may be a defining signature of chronic inflammation and inappropriate immune cell-muscle cross talk. These findings provide a rationale for the development of miRNA inhibition agents as a potential strategy to treat diseases of chronic inflammation.

In broader terms, this work highlights muscular dystrophy as a good scientific system to provide insights into chronic inflammation pathways relevant to a much larger group of disorders. Elevated NF-κB signaling is present in dystrophic muscle even in infants with DMD, years before the onset of symptoms (7). We find that prednisone, the DMD standard of care and one of the most widely prescribed drugs in the world, shares efficacy with a more selective steroid by inhibiting this chronically elevated NF-κB signaling in *mdx* mice. Here we identify nine miRNAs that appear to largely behave as a set, all increasing with dystrophy and responding to treatment with drugs that share NF-κB inhibition as a mechanism of action. Interestingly, the majority of these miRNAs appear to be conserved across diseases, as others have observed them to be elevated in IBD and other diseases with chronic inflammation (14, 16, 35, 38, 52, 67). We find that at least one of these miRNAs, miR-146a, shows conserved behavior across species with muscular dystrophy (mouse, dog, and human) (17). We also find that miR-146a shows conserved drug responses between tissue and serum (47), providing a noninvasive serum biomarker that responds to both disease and treatment. Moving forward, it will be important to determine if the other miRNAs here can provide serum biomarkers as well, and if these miRNAs can be targeted as a next-generation approach to treat diseases of chronic inflammation.

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DISCLOSURES

A. Fiorillo, C. Tully, and C. Heier have no conflict of interest relevant to this study. J. Damsker is employed by and has stock options in ReveraGen BioPharma, Inc., which owns intellectual property relating to vamorolone. K. Nagaraju and E. Hoffman have founder shares and a board membership with ReveraGen BioPharma, Inc.

AUTHOR CONTRIBUTIONS

A.A.F., J.M.D., K.N., E.P.H., and C.R.H. conceived and designed research; A.A.F., C.B.T., and C.R.H. interpreted results of experiments; A.A.F., C.B.T., and C.R.H. analyzed data; C.B.T. and C.R.H. performed experiments; A.A.F. and C.R.H. prepared figures; A.A.F. and C.R.H. drafted manuscript; A.A.F., K.N., E.P.H., and C.R.H. edited and revised manuscript; A.A.F., E.P.H., and C.R.H. approved final version of manuscript.

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