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14. ABSTRACT

Previously, we discovered that a small, non-coding RNA, called microRNA 146a (miR-146a), is significantly higher in DMD muscle than in healthy muscle. miR-146a increases as DMD disease worsens and its expression is tightly linked with inflammation. We also found that miR-146a directly blocks dystrophin protein production and its high expression in DMD muscle prevents successful exon skipping dystrophin rescue. Worked proposed in this application test the *hypothesis* that miR-146a is detrimental to DMD disease course and that blocking miR-146a in combination with exon skipping will improve the amount of rescued dystrophin. We found that inhibiting miR-146a in combination with exon skipping increases dystrophin rescue in the muscle of mdx mice. Further, we found that mdx mice with genetic deletion of miR-146a show significant improvement in muscle function and pathology as compared with mdx mice. This suggest that miR-146a inhibition alone may improve patient outcomes and miR-146a inhibition in combination with exon skipping can improve overall dystrophin rescue.

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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	Complete,
Subtask 2: Meet monthly with Co-mentors	1-24	Complete
Subtask 3: Meet every 6 months with mentoring committee	1-24	Complete
Milestone(s) Achieved: Bi-directional evaluation of career development plan and mentorship	24	Complete
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 their website.
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 2018.
Milestone (s) Achieved: Comprehensive training in new fields	24	Completed

(immunology and comprehensive mouse disease models) and leadership skills achieved.		
Major Task 3: Interdisciplinary Seminars	**Months**	**Completion date and/or percentage completion**
Subtask 1: Attend weekly and monthly seminars at CNMC	1-24	Completed
Subtask 2: Attend weekly Partridge Laboratory meetings	1-24	Completed
Milestone (s) Achieved: Present 2x per year at both departmental seminars and laboratory meetings	24	Completed
Major Task 4: Grant Writing and Career Skills	**Months**	**Completion date and/or percentage completion**
Subtask 1: Attend CTSI Grants Enhancement Seminars	1-24	Completed
Subtask 2: Work with CTSI on putting together R01 Submission	12-24	Completed
Milestone (s) Achieved: 3 grant submissions at the end of the award period; R01 in preparation by end of award period.	24	Completed - Will submit R01 grant in June 2020
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; April 2019 attended Muscular Dystrophy Association Conference 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	We redirected our focus to phenotyping Mdx/146a-/- double KO mice (aka 146aX mice) as we were able to generate these crosses and saw phenotypic improvements in Mdx mice with deletion of miR-146a.
Milestone(s) Achieved: Determination of how overexpression or loss of miR-146a affects DMD disease pathogenesis	12	Completed this in miR-Mdx/miR-146a-/- mice
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 completed in order to focus on Mdx/miR-146a-/- phenotyping
Subtask 2: Perform AAV9-miR-146a delivery in mdx and WT mice 2 groups x 12 mice/group	6-12	Not completed in order to focus on Mdx/miR-146a-/- phenotyping
Milestone(s) Achieved: Determination of how miR-146a overexpression affects inflammation and functional outcomes in mdx mice; manuscript submission	12	Not completed in order to focus on Mdx/miR-146a-/- phenotyping
Major Task 3: Create mdx52/miR-146aKO mice	**Months**	**Completion date and/or percentage**

		completion
Subtask 1: Breed mice and genotype	12-24	Completed
Subtask 2: Perform phenotyping, functional assays	12-24	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 preliminary studies and determined that the best course of action moving forward is to perform exon skipping on the Mdx/miR-146a-/- mice. We have designed the assays to measure skipping and ordered the morpholino and are waiting on getting enough males to perform injections.
Subtask 2: Perform Phenotyping and end-point assays	12-18	Dec 2017 Completed, Trial on Mdx/miR-146a-/- mice forthcoming
Subtask 3: Perform dystrophin quantification and post-sacrifice analysis	18-24	Dec 2017 Completed, Trial on Mdx/miR-146a-/- mice forthcoming
Milestone(s) Achieved: Determine outcomes of miR-146a inhibition in exon skipping; manuscript submission	24	Dec 2017 Completed, Trial on Mdx/miR-146a-/- mice forthcoming

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. Additionally I have hired a lab manager and post-doctoral fellow.

Interdisciplinary Seminars

- I have presented once at a Departmental seminar, at Partridge Lab Meetings and have additionally conducted my own weekly lab meetings with my lab manager, post-doctoral fellow, and faculty member Christopher Heier along with his medical fellow.

Grant Writing and Career Skills

- I have attended grants enhancement seminars and Departmental Seminars with senior faculty for grant writing

- I was awarded a foundation grant (Jan 2019), have submitted an MDA grant as a co-investigator (Dec 2019) and am in the process of putting together an NIH R01 grant (June 2020).

Conferences

- I have attended the Ottawa Neuromuscular Diseases Conference (Sept 2017, poster presentation), the Ascona Molecular Mechanisms of Muscle Wasting and Disease Conference (Sept 2018) and the Muscular Dystrophy Association Clinical and Scientific Meeting (March 2019).

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 5x miR-146a binding sites to check for the efficiency of miR-146a inhibition in vitro.

- We have crossed mdx52 mice to miR-146a$^{-/-}$ mice to create double knockout mice that we are referring to as miR-146aX mice.

    - With the generation of these mice we have decided to focus our attention on phenotyping these as our main measure of the role of miR-146a in dystrophic disease etiology.
    - We have performed phenotyping assays on these mice to assess muscle strength at different timepoints, harvested mice to look at muscle weights and are beginning to perform molecular assays using these mice (data below).

In year 1 we generated and validated *mdx*: miR-146a-/- mice (Figure 1). We performed functional assays on these mice and found that deletion of miR-146a:

- Improves forelimb and hindlimb grip strength as compared to age-matched *mdx* mice (Figure 2).
- Reduces weight compared to age-matched *mdx* mice (Figure 2).
- Improves muscle strength as measured by two and four limb wire hang as compared to age-matched *mdx* mice (Figure 3).
- Reduces muscle hypertrophy and spleen enlargement that is typically seen in mdx mice. (Figure 4).

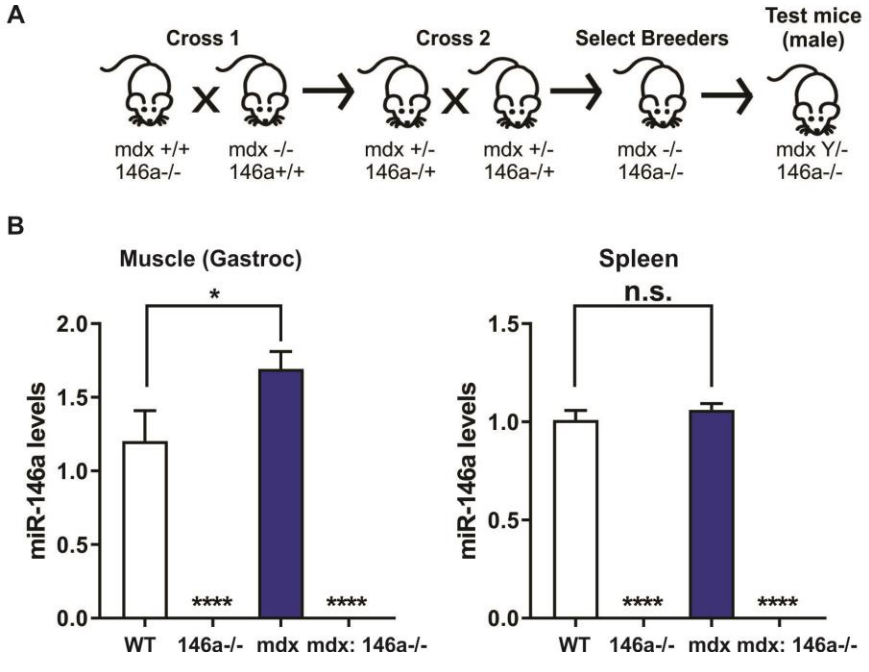

Figure 1 Confirmation of miR-146a deletion in mdx: miR-146a-/-mice. (A) Mdx52 mice were crossed with miR-146a-/- mice for two generations before mdx: 146a-/- were generated. Breeders were selected and then male test mice were used for phenotyping and molecular experiments. (B) WT, miR-146a-/-, mdx and mdx:146a-/- were sacrificed at 8 week and RNA was extracted from the gastrocnemius muscle and the spleen. qRT-PCR was performed to confirm deletion of miR-146a. Graphs show miR-146a levels in both tissues, and elevated miR-146a in the gastroc of mdx mice, as previously reported.

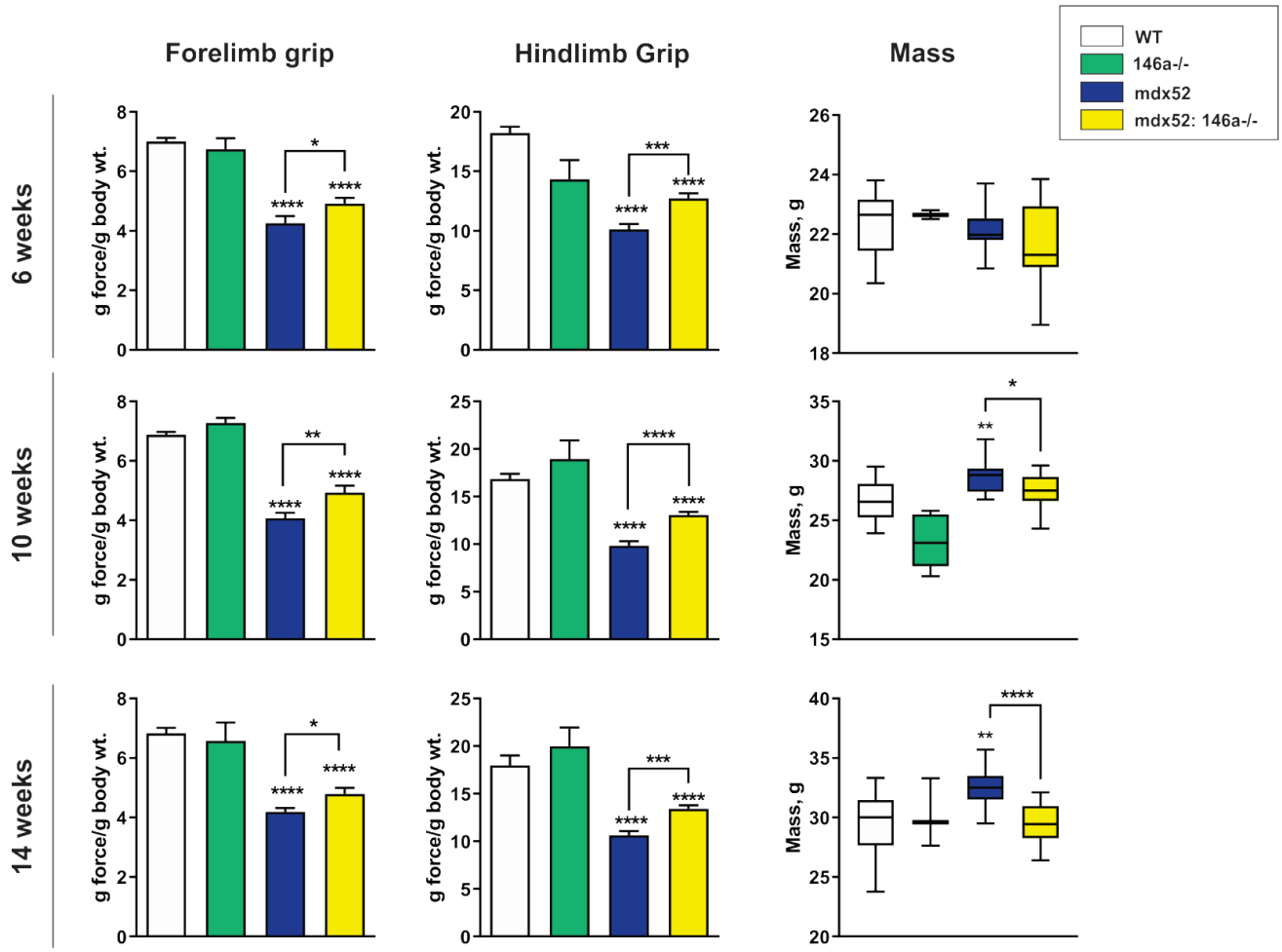

Figure 2. mdx: miR-146a-/- mice show improved forelimb and hindlimb grip strength and have reduced mass as compared to age-matched mdx mice. Indicated mice were weighed and their fore -and hindlimb grip strength was measured using a Columbus Instruments grip strength meter. Graphs show mean values for each measurement and error bars represent SEM. For each group at least 12 mice were used (except miR-146a-/- mice where n=4 due to less males in our litters. More data is being collected for this group. A one-way ANOVA was performed to determine which groups were significantly different from WT mice. Additionally a two-tailed student's t-test was performed to compare mdx vs. mdx: 146a-/- mice.

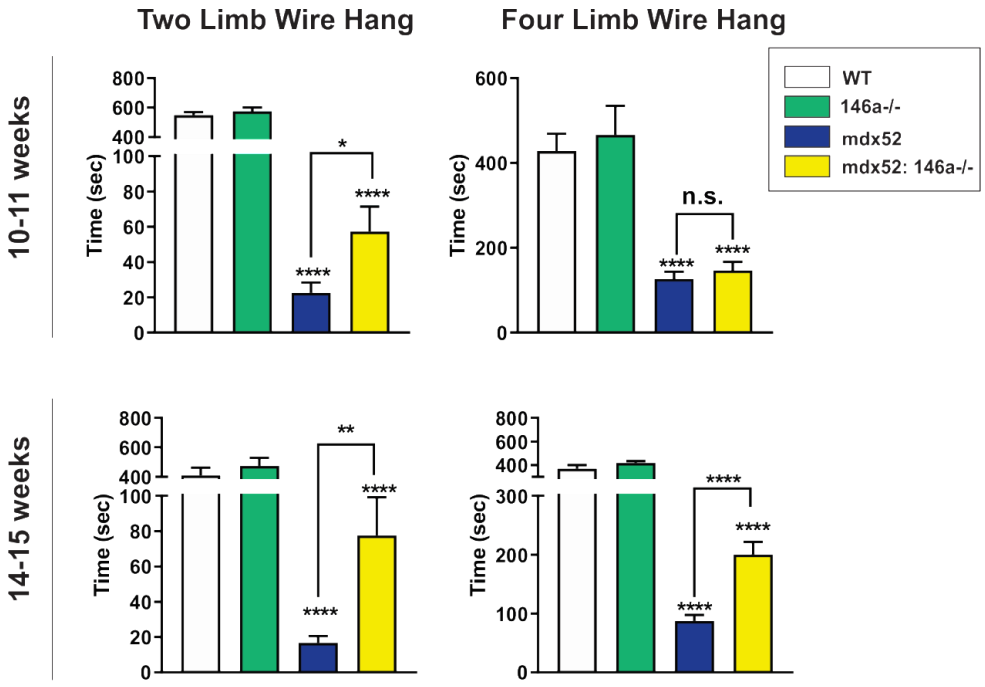

Figure 3. mdx: miR-146a-/- mice show increased hang time on two and four limb wire hang tests. Indicated mice were tested for muscle strength using two or four-limb wire hang tests. Graphs show mean values for each measurement and error bars represent SEM. For each group at least 12 mice were used (except miR-146a-/- mice where n=4 due to less males in our litters). More data is being collected for this group. A one-way ANOVA was performed to determine which groups were significantly different from WT mice. Additionally a two-tailed student's t-test was performed to compare mdx vs. mdx: 146a-/- mice.

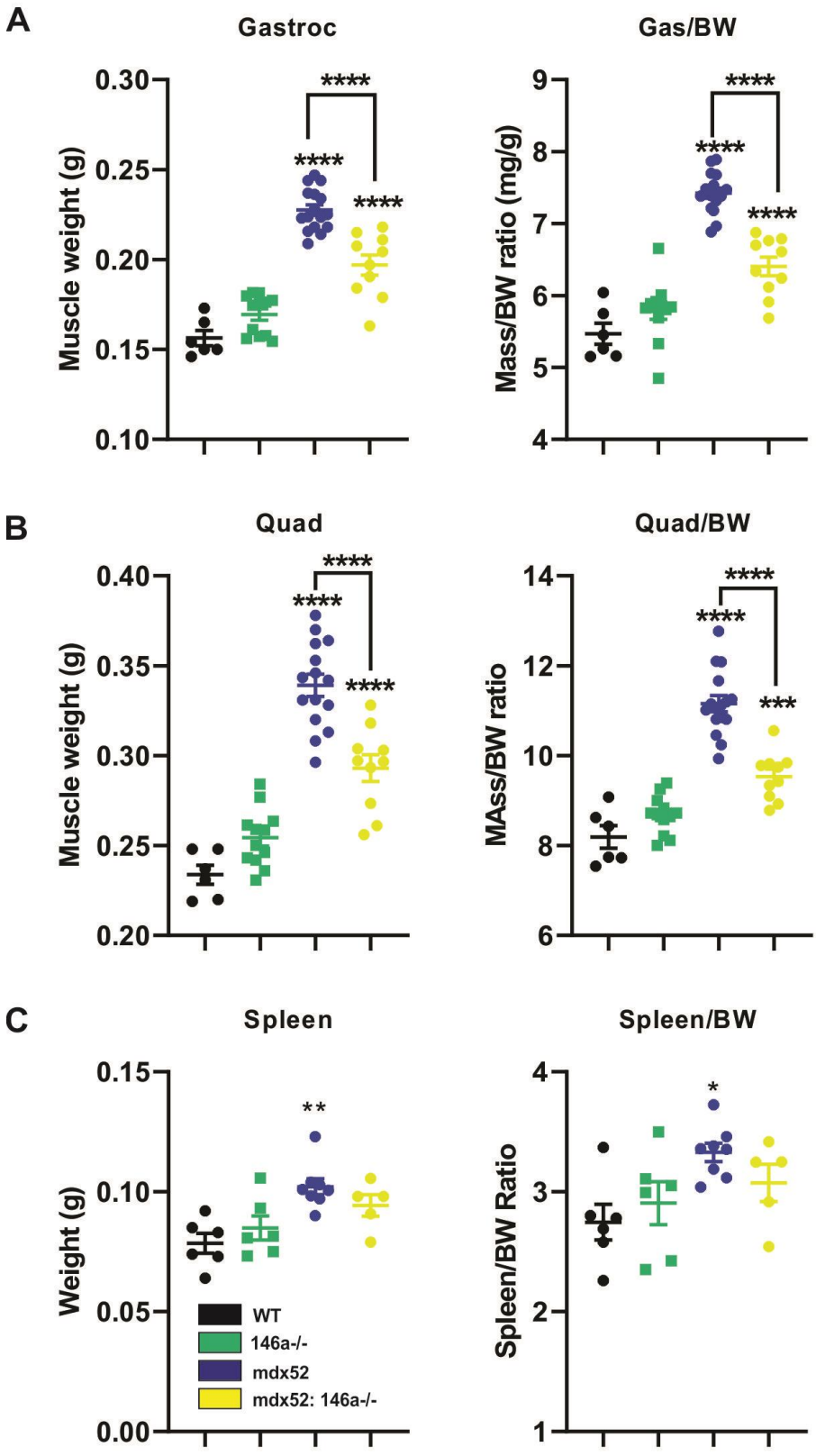

Figure 4. mdx: miR-146a-/- mice show reduced muscle and spleen weights. Indicated mice were sacrificed and their tissues harvested and weighed. A one-way ANOVA was performed to determine which groups were significantly different from WT mice. Additionally a two-tailed student's t-test was performed to compare mdx vs. mdx: 146a-/- mice.

The highlighted data suggests that the presence of miR-146a in mdx mice exacerbates disease course. We are following up on these studies by performing histology, immunofluorescence and qPCR to look at inflammation in *mdx*: miR-146a-/- mice vs. *mdx* mice. Further, as we also postulate miR-146a inhibition/deletion will improve dystrophin restoration, we will also inject *mdx* or *mdx*: miR-146a-/- with 800 mg/kg exon-skipping morpholino and compare levels of dystrophin in skeletal muscles. We have optimized dystrophin quantification using capillary electrophoresis and have been able to detect dystrophin levels as low as 0.5%, and are therefore confident that if there is an improvement in dystrophin restoration with miR-146a deletion, we will be able to accurately detect it.
Using data generated in Aims 1 and 2, we will submit an R01 in June 2020.

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)

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 5.

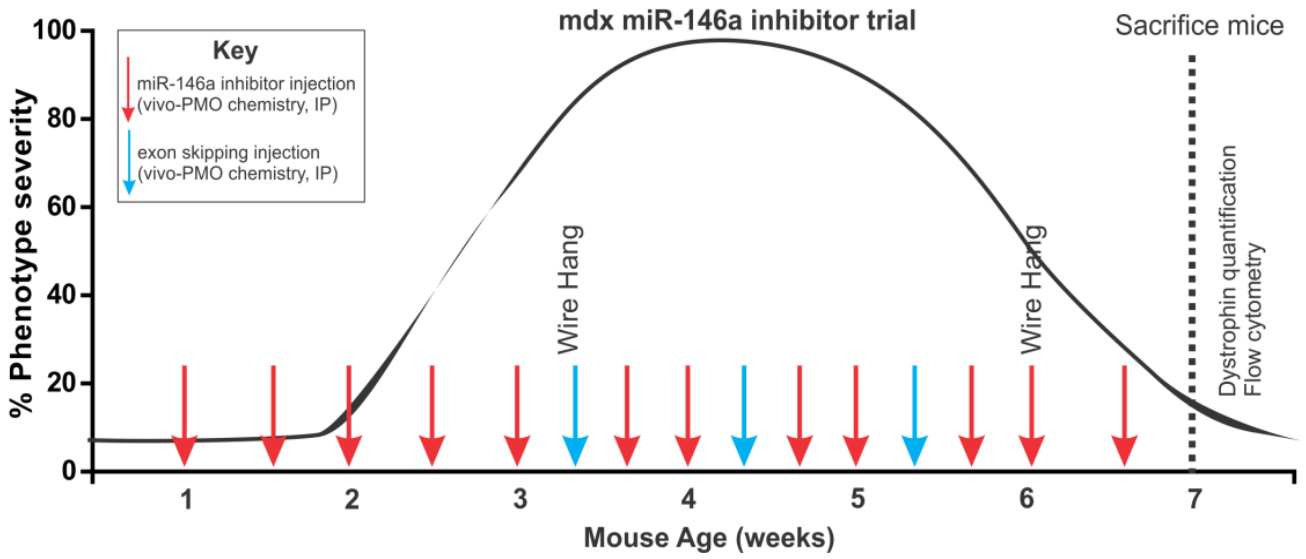

Figure 5. Experimental design of exon skipping + miR-146a inhibitor study.

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 6). 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 7a,b), but miR-146aI-injected mice show modest dystrophin rescue in the heart while CTRLI-injected mice did not (Figure 7c,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).

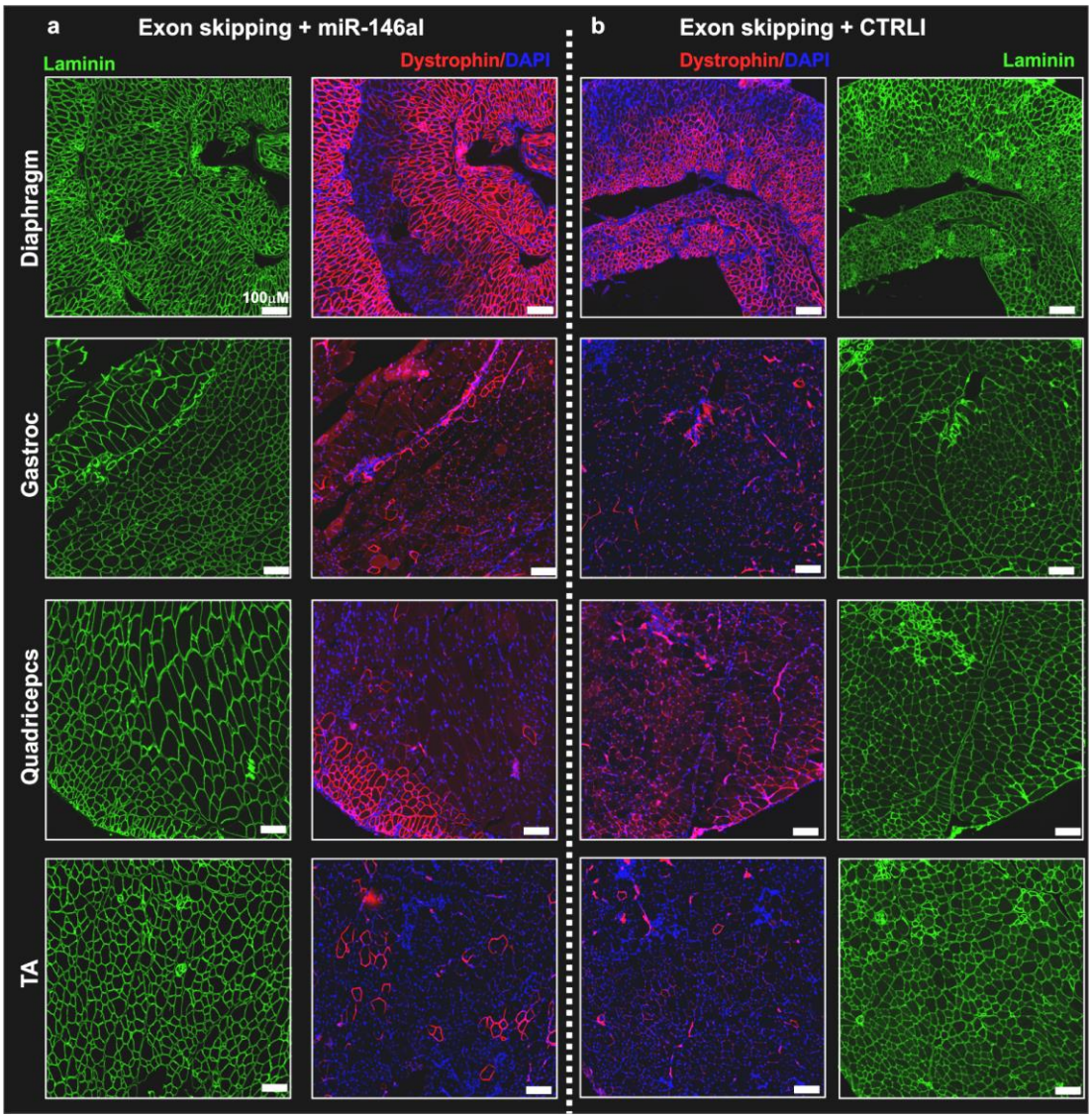

Figure 6. Exon skipping + miR-16aI administration increases dystrophin rescue in skeletal muscles. *Mdx* mouse trial was performed as described in the text. Images of entire muscle cryosections (8μM) were taken using the Olympus VS-120 Virtual Scanning Microscope at 20x magnification. A) Muscles from 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 100μM).

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.

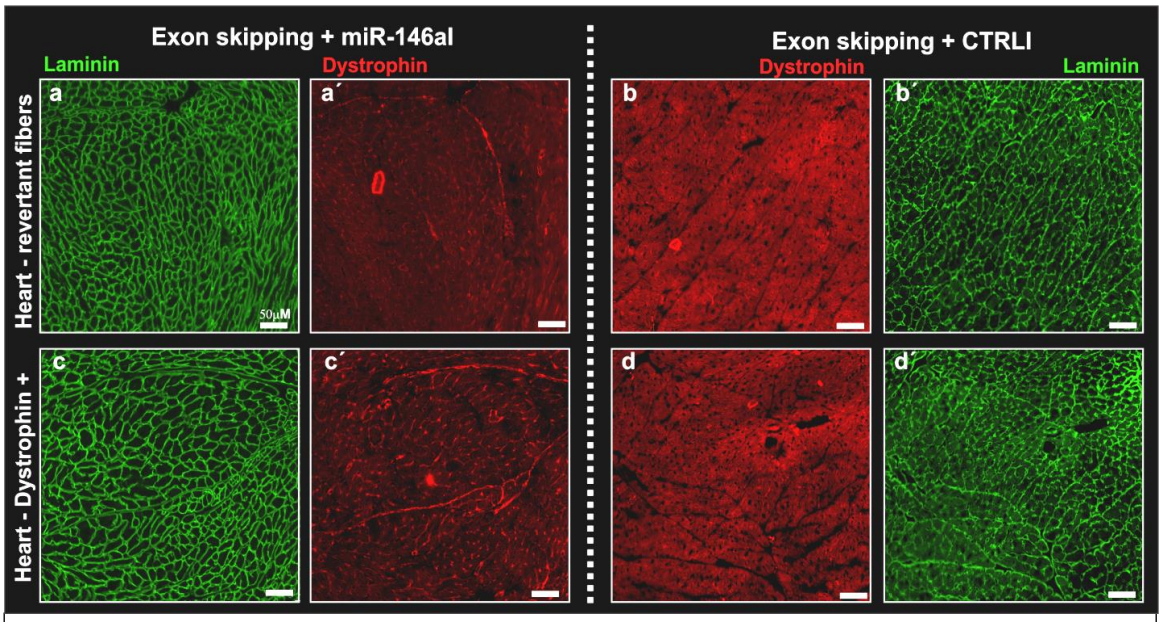

Figure 3. Exon skipping + miR-16aI administration increases dystrophin rescue in heart. Images of entire heart cryosections (8μM) were taken using the Olympus VS-120 Virtual Scanning Microscope at 20x magnification Laminin staining is shown in green and dystrophin staining in red. A & A') Heart sections 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).

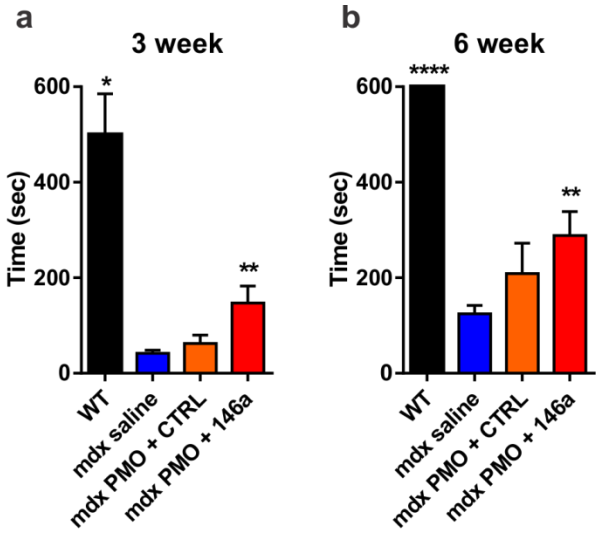

Figure 4. miR-146aI administration improves muscle strength. A wire hang test was performed in all 4 treatment groups at 3 (A) weeks (before exon skipping administration) and at 6 weeks (B). In both cases miR-146aI administration significantly increased hang time in mdx mice.

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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, Ascona, Switzerland and Orlando, FL 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, September 2019), A Night of Irish Treasures (March 2018).

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 patent that describes the use of microRNAs inhibition as a means to decrease muscle inflammation in dystrophic muscle:

Heier CR and **Fiorillo AA**. Compositions and Methods for Treating and Preventing Muscular Disorders and Dystrophies, Steroid Side Effects, and Inflammation. Application No. PCT/US2019/035264.

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 - Heier CR and **Fiorillo AA**. Compositions and Methods for Treating and Preventing Muscular Disorders and Dystrophies, Steroid Side Effects, and Inflammation. Application No. PCT/US2019/035264.

CHANGES/PROBLEMS

a. Changes in approach and reasons for change.

In aim 1 we refocused our effort on phenotyping the novel mdx: miR-146a-/- mouse. We will perform a second trial of exon skipping once we have sufficient mice in each genotype to inject.

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.

Kinder T, Heier CR, Tully, CB, Vandermeulen J, Hoffman EP, Nagaraju K, **Fiorillo AA**. Muscle weakness in myositis: microRNAs mediate dystrophin reduction in MHC class I transgenic mouse model and human muscle biopsies. Arthritis Rheumatol. 2020 Feb 3. doi: 10.1002/art.41215. PMID: 32009304 [Epub ahead of print].
Acknowledgement of federal support: Yes.

Fiorillo AA, Heier CR, Huang Y, Tully CB, Punga T, Punga AR. Estrogen Receptor, Inflammatory and FOXO transcription factors regulate expression of myasthenia gravis-associated circulating microRNAs. **Front. Immunol.** 21 February 2020 https://doi.org/10.3389/fimmu.2020.00151.
Acknowledgement of federal support: Yes.

Books:

Fiorillo A.A., Heier C.R. (2019) Approaches to Studying the microRNAome in Skeletal Muscle. In: Burniston J., Chen YW. (eds) Omics Approaches to Understanding Muscle Biology. Methods in Physiology. Springer, New York, NY. First Online 06 November 2019. DOI https://doi.org/10.1007/978-1-4939-9802-9_6

Presentations:

Fiorillo AA, Hogarth MW, Boehler, JF, Nagaraju K, Partridge TA, Hoffman EP. "miR-146a inhibits dystrophin production and promotes inflammation in multiple muscle disorders." 4th Ottawa International Conference on Neuromuscular Disease and Biology. October 2017. *Poster Presentation*.

Fiorillo AA, Tully, CB, Kinder, TB, Nagaraju, K, Hoffman, EP, Heier CR. miR-146a inhibits dystrophin production and directly activates innate immune signaling to promote inflammation in multiple muscle disorders. Molecular Mechanisms of Muscle Wasting and Disease. Ascona, Switzerland. September 2018. ***Oral Presentation***.

Tully CB, Heier CR, **Fiorillo AA**. Digital PCR and capillary protein analysis: An updated approach to dystrophin transcript and protein quantification as a biomarker of dystrophin restoration therapies. Muscular Dystrophy Clinical and Scientific Conference. April 2019. ***Poster Presentation***.

Fiorillo AA, Tully, CB, Kinder, TB, Nagaraju, K, Hoffman, EP, Heier CR. miR-146a inhibits dystrophin production and directly activates innate immune signaling to promote inflammation in multiple muscle disorders. Muscular Dystrophy Clinical and Scientific Conference. April 2019. ***Oral Presentation***

b. Inventions, patent applications and/or licenses

Heier CR and **Fiorillo AA**. Compositions and Methods for Treating and Preventing Muscular Disorders and Dystrophies, Steroid Side Effects, and Inflammation. Application No. PCT/US2019/035264.

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	24
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 received a foundation grant from the Foundation to Eradicate Duchenne.

c. What other organizations were involved as partner

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

d. Partner's contribution to the project

Financial

APPENDICIES

Accepted Article

DR. ALYSON FIORILLO (Orcid ID : 0000-0003-3458-8036)

Article type : Full Length

Title: Muscle weakness in myositis: microRNAs mediate dystrophin reduction in MHC class I transgenic mouse model and human muscle biopsies.

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Subtitle: MicroRNAs mediate dystrophin reduction in myositis

Funding:
This work was supported by grants from the Foundation to Eradicate Duchenne, the Clark Charitable Foundation, the Department of Defense and the NIH. TB Kinder was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (F31AR065362). AA Fiorillo is supported by the Department of Defense (W81XWH-17-1-047), the (NIH L40AR070539), the Foundation to Eradicate Duchenne and the Clark Charitable Foundation. CR Heier and this project were funded by the NIH (K99HL130035, R00HL130035, and L40AR068727).CR Heier and AA Fiorillo, were supported by the NIH (T32AR056993). Microscopic analysis for this study was conducted at the Children's Research Institute (CRI) Light Microscopy and Image Analysis Core, which is supported by CRI and the Intellectual and Developmental Disabilities Research Center Award (U54HD090257) through the National Institutes of Health and the National Institute of Child Health and Human Development (NICHD).

Abstract
Objective: Muscle inflammation is a feature in myositis and Duchenne Muscular Dystrophy (DMD). Autoimmune mechanisms are thought to contribute to myositis muscle weakness. However, the lack of correlation between inflammatory infiltrates and muscle weakness indicates a role for non-immune pathological mechanisms. We previously identified two microRNA sets elevated in DMD muscle. One is an "inflammatory" set that is dampened with glucocorticoids; the other is a "dystrophin-targeting" set that inhibits dystrophin translation. Here we test the hypothesis that these miRNAs are similarly dysregulated in myositis muscle and contribute to muscle weakness and disease severity.

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Methods: We utilized the MHC class I transgenic myositis mouse model and validated findings in 6 myositis patient muscle biopsies. Mice were classified as mild or severe based on transgene expression, weight, histological severity, and muscle strength/weakness. *Results:* Severe myositis mice show mononuclear cell infiltration along with elevated expression of Type 1 IFN and NF-κB-regulated genes including *Tlr7* (3.8-fold, $p<0.05$). Severe mice show elevated inflammatory miRNAs (miR-146a/miR-142-3p/miR-142-5p/miR-455-3p/miR-455-5p, ~3-40-fold increase, $p<0.05$) and dystrophin-targeting miRNAs (miR-146a/miR-146b/miR-31/miR-223, ~3-38-fold increase, $p<0.05$). ChIP-seq bioinformatics identify at least one NF-κB consensus element within the promoter/enhancer regions of these miRNAs. Western blot and immunofluorescence demonstrate reduced dystrophin levels in severe myositis muscle. We also observe elevated NF-κB-regulated genes, TLR7, and miRNAs along with reduced dystrophin in histologically severe human myositis muscle biopsies. These data demonstrate an acquired dystrophin deficiency may occur through NF-κB-regulated miRNAs in myositis and suggest a "unifying theme" for perpetuation of muscle injury, inflammation and weakness in both myositis and DMD.

Introduction

The Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of systemic connective tissue diseases characterized by chronic muscle inflammation and weakness. This group of diseases, which includes dermatomyositis, polymyositis, and inclusion body myositis (collectively referred to as myositis) affects both adults and children. Although rare, there is evidence that disease incidence is increasing (1). Histological features of myositis include 1) endomysial, perimysial or perivascular infiltration by inflammatory cells consisting predominantly of macrophages and T-cells (2, 3), 2) myofiber-specific expression of major histocompatibility complex (MHC) class I molecules, and 3) bouts of muscle degeneration/regeneration, fibrosis, and fat deposition (4). Of these, the most striking feature is overexpression of MHC class I in myositis patient muscle (5, 6).

The role of the innate and adaptive immune systems in myositis is well known studied, but recent reports suggest non-immune mechanisms also contribute to disease [Reviewed in (7, 8)] as there is not a strong correlation between inflammatory infiltrates and extent of myositis patient

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weakness (9). Interestingly, myositis muscle seems primed for inflammatory signaling events as it inappropriately expresses Toll-like receptors (TLRs) (10, 11) and MHC Class I (5, 6); receptors that are normally confined to immune cells. Stimulation of TLR7 and MHC Class I activates the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB). In addition, TLR7 is a potent inducer of Type I Interferon (IFN) genes (12); this gene expression signature has been shown to correlate to disease severity in dermatomyositis (13-16) and juvenile dermatomyositis (15, 17).

NF-κB is a key regulator of inflammation in immune cells; however, it is also turned on in skeletal muscle under inflammatory conditions. Not surprisingly, in myositis muscle NF-κB is highly activated and its activation corresponds to disease severity (18). Classical NF-κB activation is triggered via inflammatory cytokines such as TNF-α which is found at high levels in myositis patient muscle (19). Non-classical NF-κB activation occurs through ER-stress and autophagy; both of these processes/pathways are increased in myositis (20, 21). It has been previously shown that muscle-specific MHC Class I expression in muscle activates the NF-κB pathway and turns on classical NF-κB target genes such as TNF and TLR7, exacerbating inflammatory signaling (22). Further confirming its importance in disease pathogenesis, downstream gene targets of NF-κB are highly upregulated in myositis muscle (19, 23).

At the molecular and histological levels, there is significant overlap between inflammatory myopathies and other muscle disorders, in particular, Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD) and manifesting DMD female carriers (24, 25). Similar to myositis, DMD muscles show increased TLR7 expression (26), elevated NF-κB activation (18, 27), and overexpression of muscle-specific MHC class I (28). Previously, we described two distinct sets of miRNAs that are elevated in DMD muscle. The first is an "inflammatory set" which is increased in *mdx* mice and reduced by administration of two anti-inflammatory drugs (29). The second is a "dystrophin-targeting" set (termed dystrophin-targeting miRNAs or DTMs). DTMs are elevated both in DMD and Becker Muscular Dystrophy (BMD) and are prohibitory to dystrophin protein production in BMD and in exon-skipping mediated dystrophin rescue in DMD (30). Some of these miRNAs are increased in inflammatory connective tissue disorders that overlap with myositis including scleroderma, Rheumatoid arthritis, and Sjogren's syndrome, suggesting that inflammatory miRNAs and DTMs play a broader role in regulating chronic inflammation.

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In mice, skeletal muscle-specific conditional overexpression of MHC class I induces myositis (3). Following MHC class I upregulation, female mice develop early histological and functional changes mirroring human myositis, while male mice develop a more mild disease (3). In the work presented here, we test the hypothesis that inflammatory miRNAs and DTMs contribute to myositis disease by reducing dystrophin in muscle fibers.

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Materials and Methods

Mice

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 within the guidelines and approval of the Institutional Animal Care and Use Committee of Children's National Medical Center. Animals were housed at room temperature with a 12–12-h light-dark cycle. Genotyping was carried out at 3–4 weeks as previously described (21). The mouse model of myositis used in this study is known as the HT ((TRE)-H-2Kb (H) and mCK-tTA (T)) mouse model has been described previously (3). For this study, transgene expression was induced by withdrawal of doxycycline from the drinking water at 5 weeks old and analyses were performed at 9-13 weeks old. Female mice were used for all studies as the disease manifestation is more severe.

Muscle biopsies.

Muscle biopsies were provided by the Biobank of skeletal muscle, peripheral nerve, DNA and cell lines, member of the Telethon Network of Genetic Biobanks (project no. GTB12001). Further details can be found in Supplemental Methods.

qRT-PCR of miRNAs and mRNAs

qRT-PCR of miRNAs and mRNAs was performed as previously reported (29, 30). Details can be found in Supplemental Methods.

Western blot analysis

Protein from muscle was collected using a lysis buffer containing 75 mM Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate, 10 mM EDTA, and 5% 2-mercaptoethanol. Protein samples (25µg) were separated using a 3-8% Tris-Acetate gel (Bio-Rad) and then transferred onto a nitrocellulose membrane overnight (4^{o}C; 20V), followed by a 1hr pulse (4^{o}C; 80V). Antibodies used included DYS-1, a mouse monoclonal antibody against dystrophin (1:100, Novacastra), and a rabbit polyclonal antibody against vinculin (1:1000, GenTex clone N3C1). Horseradish peroxidase–conjugated anti–mouse or anti–rabbit goat immunoglobulin (1:5000, Millipore) were used as secondary antibodies.

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Enzyme chemiluminescence (Pierce) was used for detection. The blots were scanned digitally with ChemiDoc Touch Imager.

Hematoxylin and Eosin Staining and Histological Grading of muscle sections

8 μM sections were cut from frozen mouse quadricep muscles or from frozen human muscle biopsies. Sections were air-dried for 1 hour on SuperFrost Plus slides and then stained with Hematoxylin and eosin. Stained slides were imaged on a VS120 Virtual Slide Microscope (Olympus) at 20x magnification. Details on pathological grading can be found in Supplemental Methods.

Immunofluorescence

Frozen muscle sections (8 μM) were dried for 1 hour on SuperFrost Plus slides and then stored at -80^{o}C. For staining, slides were thawed for 1 hour at room temperature and then fixed in either ice cold acetone (4^{o}C) for 10 minutes or 4% paraformaldehyde for 10 minutes at RT. Further details on primary and secondary antibody dilutions and incubation times can be found in Supplemental Materials.

Force Contractions on Isolated Skeletal Muscle

At time of sacrifice, EDL muscle was isolated from live anaesthetized mice and contractile properties were measured *ex vivo*. Details can be found in Supplemental Materials.

Bioinformatics

We examined each miRNA gene promoter to gain insight into the mechanisms of response to treatment as we previously reported (29). Details for identifying NF-κB/RELA binding sites can be found in Supplemental Materials.

Results

Variable disease severity in myositis model mice

In the present study, we utilized female mice that overexpressed MHC Class I after doxycycline withdrawal at 5 weeks of age. After doxycycline withdrawal for 4-8 weeks, we observed variable disease severity, allowing us to investigate molecular factors driving disease progression. We initially observed that mice weighing less at time of sacrifice appeared to have a more aggressive disease. To determine if body weight was related to transgene expression, we plotted the weight of mice at time of sacrifice against the extent of transgenic MHC class I expression in the most severely affected muscle (quadriceps). As shown in **Figure 1A**, body weight and transgene expression showed a strong

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inverse correlation (r_s= -0.9146, Spearman's correlation) corroborating our observations that mice weighing less show a more severe disease, likely due to increased MHC class I expression. The scatter plot revealed two distinct phenotypes: HT mild (HT-M) and HT severe (HT-S). HT-S myositis mice weighed 12-16 grams at time of sacrifice, expressed muscle-specific MHC class I at a level of 300-fold or greater vs. WT. HT-M myositis mice weighed 17-20 grams, showed transgene expression less than 300-fold. To determine if differences in transgene expression were reflected in muscle pathology, we used a grading system on Hematoxylin and eosin (H&E)-stained muscles from HT-M and HT-S mice. HT-S myositis muscles showed 1) higher endomysial inflammation with invasion into the myofibers, 2) more highly variable muscle fiber size and 3) increased necrosis and degeneration, showing a mean pathology score of 2.3 out of 3. In contrast, HT-M mice showed 1) less endomysial inflammation with no invasion into fibers, 2) less fiber size variation and 3) little to no necrosis and degeneration a mean histological grade of 1.3 (**Figure 1B, B′**, $p<0.0001$).

To determine if molecular and histological differences in HT-S and HT-M mice resulted in functional differences, we assessed the muscle strength of isolated EDL muscles *ex vivo*. Specific force in HT-S mice was reduced by 62% ($p<0.0001$) as compared to WT mice while specific force in HT-M mice was reduced by 41% ($p<0.0001$, **Figure 1C**). Collectively, these data show HT-S myositis mice indeed display a more severe disease as compared to HT-M mice.

Type I IFN signature and NF-κB activation in mice with severe myositis

Recent studies suggest a mechanistic role for molecules induced by Type I interferons (IFNs) in the pathogenesis of myositis (13-16); this is most commonly observed in adult and juvenile dermatomyositis (15, 17). To determine if Type I IFN gene expression was related to disease severity in our study, we performed qRT-PCR on three Type I IFN signature genes, interferon-induced protein with tetratricopeptide repeats 1 (*Ifit*), interferon regulatory factor 7 (*Irf7*) and MX dynamin like GTPase 2 (*Mx2*). HT-S myositis muscles showed significant increases (~10-80-fold) in all three genes vs. WT ($p<0.0001$, $p<0.0001$, $p<0.01$, respectively, **Figure 1D**). In comparison, HT-M muscle showed modest increases in *Ifit1*, *Irf7* and *Mx2* with only *Ifit1* reaching statistical significance ($p<0.05$).

We have previously shown NF-κB is highly activated in human inflammatory myopathies and mouse models of myositis (22). To determine if NF-κB activation was associated with disease

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severity, we assessed NF-κB -activated genes including tumor necrosis factor (*Tnf)* and C-C motif chemokine ligand 5 (*Ccl5*). Both *Tnf* and *Ccl5* followed a similar pattern to Type I IFN signature genes: in HT-S muscles, both *Tnf* and *Ccl5* were highly increased over WT levels ($p<0.001$, **Figure 1E**). In HT-M muscles, NF-κB -induced gene expression showed a lower, but significant elevation vs. WT ($p<0.01$).

In response to their cognate ligands, Toll-like receptors (TLRs) activate NF-κB inflammatory signaling and, in turn, induce both Type I IFN and inflammatory (NF-κB) gene expression (31). Recent reports have observed myofiber-specific expression of TLR7 in several muscle disorders (10, 26, 32). Because we observed elevated expression of both gene expression signatures in HT-S mice, we next assessed TLR7 expression in HT-M and HT-S myositis muscles. Using immunofluorescence, we observed an almost complete absence of TLR7 from WT muscle (**Figure 2A**). In contrast, HT-M muscles showed modest TLR7 staining, while HT-S muscles showed strong staining (**Figure 3A**). Using confocal microscopy, we observed co-localization of TLR7 and LAMP1, a marker of late endosome/early lysosomes, indicating TLR7 was in an activated state (33) (**Figure 2B,** positive controls shown in **Figure S1**). qRT-PCR corroborated immunofluorescence data showing elevated *Tlr7* in HT-S myositis (**Figure S2A**). qPCR also indicated increased *Lamp1* gene expression in HT-S myositis, however, it did not reach statistical significance (**Figure S2B**).

Signaling through TLR7 promotes macrophage recruitment and invasion into muscle fibers. Immunofluorescence of mouse macrophage marker F4/80 showed increased macrophage infiltration in HT-S muscles as compared to both HT-M and WT muscles (**Figure 2C**). This observation was confirmed by qRT-PCR to measure the macrophage-specific marker, EGF-like module-containing mucin-like hormone receptor-like 1 (*Emr1*). qRT-PCR showed HT-S muscles expressed elevated *Emr1* ($p<0.05$, **Figure S2C**). Together these data suggest myositis disease progression involves distinct changes in the molecular signature of myofibers, which begin to take on an innate immune-like state via expression of Type I IFN signature genes, NF-κB -regulated genes, and myofiber-specific expression of TLR7, all of which may result in increased macrophage recruitment.

Inflammatory and dystrophin-targeting miRNAs correspond to disease severity

To further characterize molecular changes that drive myositis disease progression, we analyzed two defined sets of miRNAs in muscle. The first set encompassed nine miRNAs that we

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previously found are elevated in dystrophic mice, regulated by NF-κB, and decreased in response to anti-inflammatory treatment (termed inflammatory miRNAs) (29). Of these, five (miR-146a, miR-142-3p, miR-142-5p, miR-455-3p, miR-455-5p) were significantly elevated in HT-S mice vs. WT mice (~4-6-fold increase, $p<0.05$, **Figure 3A**). miR-142-3p and miR-146a were also significantly elevated in HT-M myositis muscles, but to a lesser extent.

We next assayed a second defined set of miRNAs (termed DTMs) which we have previously reported are upregulated in DMD and BMD, and bind to the dystrophin 3′UTR to inhibit its translation. qRT-PCR showed four of seven DTMs (miR-146a, miR-223 and miR-31, miR-146b) were highly elevated (~3-40-fold increase, $p<0.05$) in HT-S quadriceps (**Figure 3B).** We also observed significantly elevated miR-146a and miR-31 in HT-M muscles, but to a lesser extent,

Because we observed elevated TLR7 in myositis muscles, we also assessed expression of three miRNAs known to activate TLR7; miR-21 (34), miR-29a (34) and let-7(35). miR-21 was undetectable in all samples assessed, while miR-29a and let-7 showed no significant differences between WT, HT-M and HT-S muscles (**Figure S3**).

DTMs (miR-146a, miR-223) are induced by TNF-α in dystrophic myotubes, suggesting regulation by NF-κB (30). To gain insight into DTM regulation in myositis, we performed bioinformatics on ChIP-seq data at the sites of each miRNA promoter/enhancer region. Bioinformatics analysis showed all four elevated DTMs possess sites that are directly bound by NF-κB at consensus elements within their promoter/enhancer regions (**Figure 3C**) (36). These elements overlapped with corresponding ChIP-seq data that detects histone modifications which correspond to active regions of transcriptional regulation (H3K4me3, H3K27Ac, H3K4me1). This is supported by previous reports demonstrating NF-κB -specific regulation of miR-146a (37), miR-146b (37), miR-223 (38) and miR-31 (39). Together, these data suggest these defined sets of miRNAs play a key role in myositis disease progression and NF-κB regulates their expression.

Reduction of dystrophin is linked to myositis disease severity

Our lab has demonstrated that DTMs inhibit dystrophin translation, and their level of expression inversely corresponds to dystrophin levels in BMD muscle and in exon-skipping treated DMD muscle (30). To determine if elevated DTMs reduce dystrophin in myositis muscle we performed dystrophin immunostaining in WT, HT-M, and HT-S quadriceps. Dystrophin was

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markedly reduced in HT-S myositis mice as compared to HT-M mice, most notably in areas of inflammation as shown by macrophage (F4/80) staining (**Figure 4A**). We next performed both dystrophin and laminin immunostaining to better visualize muscle fibers lacking dystrophin. HT-S muscle fibers lacking dystrophin stained positively for laminin, indicating the presence of intact muscle fibers (**Figure 4B**). To corroborate immunofluorescence we also performed Western blot on muscle extracts from HT-M and HT-S mice which similarly showed dystrophin is reduced in HT-S myositis muscles (**Figure 4C**).

To determine the relevance to human disease, we obtained human muscle biopsies from patients with dermatomyositis (DM) and inclusion body myositis (IBM) as well as muscle biopsies from healthy control subjects. Analysis by H&E revealed different histological severities based on the extent of inflammation and fiber type variability. Based on histology, we partitioned biopsies into "mild" or "severe" cohorts. Two dermatomyositis biopsies and one inclusion body myositis biopsy were grouped as "histologically mild" and the remaining three inclusion body myositis biopsies were grouped as "histologically severe." Representative images are shown in **Figure 5A**.

We next assessed MHC Class I gene expression (HLA-B) in human muscle biopsies. Histologically severe biopsies showed a ~6-fold elevation ($p<0.05$) in MHC Class I whereas the mild biopsies showed no significant elevation above healthy control levels. Histologically severe biopsies showed elevated levels of NF-κB-driven gene expression, showing elevated TNF and CCL5 ($p<0.05$) while histologically mild biopsies show no appreciable upregulation (**Figure 5B**). We also observed elevated TLR7 gene expression in histologically severe biopsies, consistent with our observations in HT-S myositis muscle ($p<0.05$, **Figure 5B**). We assessed Type I IFN gene expression and found no significant differences between healthy, mild and severe groups. We did, however, observe elevated Type 1 IFN gene expression in 1 of 2 DM muscle biopsies, consistent with previous reports showing this gene expression signature is a hallmark of DM, particularly in muscles that have perifascicular atrophy (15, 17) (data not shown).

We next assessed levels of NF-κB-regulated DTMs found to be elevated in HT-S muscles. Here we observed increased expression of both miR-146a ($p<0.10$) and miR-146b ($p<0.001$) in histologically severe biopsies, while histologically mild biopsies showed no differences in miR-146a expression, but did show slightly higher miR-146b levels in comparison to healthy muscles ($p<0.10$,

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Figure 5B). We then performed dystrophin immunofluorescence on muscle biopsies, using sarcolenmal-localized laminin as a control for integrity of muscle fibers. While dystrophin staining in control and histologically mild biopsies was uniform, we found areas of reduced and discontinuous dystrophin staining in histologically severe biopsies (**Figure 5C**). Collectively, our data suggests a mechanism where increased TLR7 signaling activates inflammation and NF-κB -driven miRNAs to promote muscle weakness through dystrophin reduction (refer to model in **Figure 6**). Further, elevated levels of DTMs may be responsible for reduced and discontinuous dystrophin observed in myositis muscles.

Discussion

Here, we utilize HT myositis model mice with variable disease severity to better understand molecular and cellular pathways that contribute to myositis. Previous studies with these mice describe a chronic disease progression where reduced locomotor activity is seen as early as 1 month off doxycycline, and clinical and histopathological signs are observable at about 2 months, which become profound and severe by 4-6 months (3). The analyses we perform here at 1-2 months off doxycycline enabled us to identify early molecular aberrations and additionally measure weakness in isolated EDL muscles *ex vivo* due to the sensitivity of the assay. The cohort of HT mice in our study possessed variable rates of disease progression, and due to the progressive nature of the model, we predict that the mild HT mice would become more severe through time. We classify mice in two distinct cohorts: HT-M mice with low transgenic expression of muscle-specific MHC class I, and HT-S mice characterized by high MHC class I transgene expression. Comparing these two groups and age-matched WT littermates, we find HT-S mice develop a more severe disease as shown by weight, histological severity, muscle strength/weakness as well as elevated Type I IFN and NF-κB gene

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signatures. HT-S mice show inappropriate expression of endolysosomal-localized TLR7 within myofibers and increased macrophage infiltration as visualized by F4/80 staining. Correspondingly, HT-S muscles display markedly increased levels of miRNAs from two defined sets: an inflammatory set previously described in DMD model mice (29) and a dystrophin-targeting set previously described in both BMD and in exon-skipping treated DMD model mice (30). All miRNAs elevated in severe myositis muscle from these sets possess at least one NF-κB regulatory element within their promoter or enhancer regions. Using both immunofluorescence and Western blot we find dystrophin is reduced in HT-S muscles. To corroborate our data in HT model mice, we additionally analyze human muscle biopsies from myositis patients and find histologically severe muscles have elevated MHC Class I, elevated NF-κB-driven gene expression and high levels of miR-146a and miR-146b. Histologically severe human myositis muscles also showed patchy/reduced levels of dystrophin immunostaining. Importantly, the samples classified as severe were from IBM patients, whereas the DM biopsies were classified as mild. This is consistent with previous studies where MHC expression was shown to be more prominent in IBM muscle (40). Collectively, our data suggest a model where myositis disease progression is driven by myofibers adopting a more immune-like state, as evidenced by inappropriate expression of TLR7, and of MHC Class I. TLR7 activates NF-κB-driven gene expression, eliciting release of inflammatory cytokines and leading to macrophage recruitment. NF-κB-driven gene expression also triggers an NF-κB feed-forward loop where production of inflammatory cytokines, including TNF-α, further turns on NF-κB. TLR7 activation also leads to production of Type I IFN, which in turn, triggers expression of NF-κB and Type I Interferon-stimulated genes. NF-κB additionally activates dystrophin-targeting miRNAs which downregulate dystrophin protein levels. Together, these events contribute to a self-amplifying inflammatory feedback loop of muscle damage and inflammation. As dystrophin is required for mechanical stability in muscle, our observations of reduced dystrophin in myositis muscle may contribute to muscle weakness (**Figure 6**).

Many signaling pathways we describe here are similarly overexpressed or overactive in DMD. For instance, in DMD there is strong activation of multiple components of the innate immune system before the onset of clinical symptoms, including elevated signaling from TLRs (TLR4, TLR7), NF-κB activation, and expression of MHC class I molecules on muscle cells. It is recognized that there is substantial overlap between the histopathological presentation of myositis and muscular dystrophies,

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specifically DMD, BMD and manifesting DMD female carriers. Transcriptional profiling also shows shared pathways between these diseases (41). A few case studies describe DMD female carriers that were initially misdiagnosed with myositis, further illustrating the multitude of confounding factors that blur the boundaries between the two diseases (25, 42). In the work presented here, we describe miRNAs that are elevated in both diseases. These similarities give rationale for development of therapeutics that could target shared pathways in DMD and myositis.

Recent studies describe inappropriate expression of TLR7 detected in myofibers from myositis patients (10, 11) and this expression exacerbates disease (43). Similarly, TLR7 is elevated in DMD myofibers at an early age (<1 year of age) (27). Because of the high TLR7 levels observed in DMD muscle, it is postulated that myofiber-derived RNA molecules may be the most potent of all of the contributing damage-associated molecular patterns (or DAMPs) as they serve as the natural ligand for TLR7 (44). In support of this, we have previously found that a more common feature of DMD and BMD muscle is miRNA upregulation as opposed to miRNA downregulation (30). Further, recent studies suggest that the overabundance of specific miRNAs may trigger activation of TLR7 (34, 35). Collectively, high levels of miRNAs in muscle disease and mis-expression of TLR7 in myofibers seem to be alluding to a mechanism where diseased muscle itself is primed to activate a strong inflammatory response.

TLR7 is trafficked to the endolysosomal compartment when activated (45). Here we observe elevated endosomally-localized TLR7 in severe myositis muscle. We also observed elevated LAMP1 expression which may be indicative of impaired autophagy (21, 46). It has been reported that autophagy is required for activation of NF-κB, (47) and that TLR7 and its ligands regulate autophagy (48, 49). Thus, these seemingly intertwined pathways make attractive targets for myositis and perhaps for other inflammation-associated muscle disorders.

miRNAs are responsible for fine-tuning gene expression in all cell and tissue types, including muscle and immune cells. When the balance of miRNAs is shifted in a specific tissue type, gene expression programs are altered driving tissue from a healthy state to a diseased state. In HT-S mice, we found significant upregulation of 5/9 miRNAs from a pre-defined "inflammatory set" (miR-146a, miR-142-3p, miR-142-5p, miR-455-3p, miR-455-5p) and 4/7 from a pre-defined dystrophin-targeting set (miR-146a, miR-223, miR-31, miR-146b). We additionally found elevated miR-146a and miR-

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146b in histologically severe human muscle biopsies. All of these miRNAs are regulated by the inflammatory transcription factor NF-κB, (36) and some, in turn, regulate key components of the NF-κB signaling pathway. This highlights the importance of NF-κB signaling in myositis disease pathogenesis. We will discuss the significance of these miRNAs below.

miR-146a is highly upregulated in severe myositis mice, is induced by NF-κB in immune cells (37), and is expressed directly in muscle (30). In diseases where chronic inflammation is present, miR-146a levels are highly elevated both in serum and in tissues affected by disease (50-57). miR-146b is similarly overexpressed in severe myositis, is induced by TNF-α/NF-κB (37, 58) and is elevated in the serum of patients with inflammatory disorders such as inflammatory bowel disease (57). Both miR-146a and miR-146b are defined as DTMs, and we have shown that they downregulate dystrophin by binding to its 3′UTR (30). The miR-142 family is highly expressed in monocytes (59) and lymphocytes (60). We and others have reported increased miR-142 in DMD as well as other genetic muscular dystrophies (29, 61). The miR-142 locus possesses 13 NF-κB binding sites within its promoter/enhancer region, illustrating the interdependence of its expression and inflammatory signaling. Increased expression of miR-31 has been reported in DMD patients, BMD patients and *mdx* mice (30, 62-64). miR-31 also regulates dystrophin expression by targeting its 3′UTR (30, 65) and its expression is induced by NF-κB (39). Collectively the miRNAs that we describe here seem to be uniquely primed to shift the balance towards a pro-inflammatory phenotype as they become increasingly expressed in diseased muscle.

A few early studies noted reduced and discontinuous dystrophin staining in human myositis biopsies (66, 67). Here, we investigated dystrophin levels in myositis mice and human muscle biopsies after we observed elevated levels of DTMs in severe myositis (HT-S) mice. Immunofluorescence revealed that severe myositis muscles possess a reduced and mosaic pattern of dystrophin staining that partly resembles what is observed in muscles of BMD patients or DMD female carriers. Reduced dystrophin was in part associated with the extent of inflammation as shown by macrophage (F4/80) staining. Given that DTMs are regulated by inflammation, it is plausible that DTMs within in the muscle microenvironment could dictate the local expression of dystrophin. As disease progresses, both inflammation and dystrophin reduction could "spread" through the muscle, as patches of damaged muscle communicate through DAMPs to healthy areas, in turn activating

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inflammatory signaling, reducing dystrophin, and causing muscle weakness. Interestingly, reduced dystrophin has been reported in other muscle disorders where dysregulated NF-κB signaling and chronic inflammation is present (67-69). Loss of dystrophin could provide a possible mechanism for muscle weakness in myositis which, to date, is not fully understood. It will be particularly important to study dystrophin deficiency in other muscle disorders, especially those with high inflammation, to determine if a secondary dystrophin deficiency could contribute to disease.

In this report, we have provided novel insight into mechanisms driving muscle weakness and disease progression in myositis model mice. Future work will focus on determining 1) if the microRNAs we describe here also play roles in other muscle/inflammatory disorders and 2) if these miRNAs can serve as therapeutic targets in muscle disease.

Figure Legends

Figure 1. Variable disease severity in HT myositis mice. (**A**) Classification system used for severity of HT myositis mice. Body weights were plotted against the extent of Major Histocompatibility Complex Class I (MHC class I) transgene expression. Body weight and MHC class I showed a strong inverse correlation (Spearman's correlation r_s=-0.9147, p<0.0001). Groups were classified as either mild, denoted as "HT-M" (blue dots, body weight >16g, MHC class I expression < 300-fold) or severe, denoted as "HT-S" (red dots, body weight <16, MHC class I expression >300-fold). (**B**) H&E staining from WT, HT-M and HT-S quadriceps (n=3, 6 and 8 mice respectively, ANOVA). Bars= 50uM. (**B′**) Graph of histological scoring. Data are mean + SEM (n> 4 mice/group, ANOVA, *p<0.05, ***p<0.001; ****p<0.0001). (**C**) Isolated EDL muscle specific force measurements ($n \geq 10$ mice/group, ANOVA; ****p<0.0001). **(D)** Type I IFN gene expression levels

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(*Ifit1*, *Irf7* and *Mx2*) in WT, HT-M and HT-S quadriceps. Data were normalized to 18S rRNA. (**E)** NF-κB-induced gene expression (*Tnf*, *Ccl5)* in WT, HT-M and HT-S mice. Data were normalized to 18S rRNA (n= 5, 7 and 10 for WT, HT-M, and HT-S, respectively, ANOVA) and presented as the mean + SEM. *P<0.05, **P<0.01; ***P<0.001, ****P<0.0001.

Figure 2. Increased TLR7 staining and macrophage infiltration in severe myositis muscles. (**A-B**) Representative images of WT, Mild (HT-M), and Severe (HT-S) quadriceps immunolabeled with an antibody against TLR7 (green) and LAMP1 (red). DAPI counterstain was used to visualize nuclei and is shown in blue. (**A**) Images were taken using a VS-120 scanning microscope at 20x magnification, Bar = 50 μM. (**B**) Images from a second set of stained sections (stained as in A) were taken using confocal microscopy Bar = 20 μM. In both (A) and (B) co-localization of TLR7 and LAMP1, a marker of late endosome/early lysosomes is an indication that TLR7 is localized to the endosomes and is in an activated state. (C) Quadriceps muscle from HT-M and HT-S quadriceps stained with an antibody against F4/80, which recognizes a glycoprotein expressed by murine macrophages (green), an antibody against laminin (red) and DAPI (blue). Bar = 50 μM.

Figure 3. Dystrophin-targeting and inflammatory miRNAs are elevated in myositis muscle and correspond with disease severity. (**A**) Inflammatory miRNAs were analyzed by qRT-PCR using quadriceps of Mild (HT-M), Severe (HT-S) or WT mice. Data were normalized to sno202 (n= 5, 6 and 8, respectively, ANOVA; + SEM. *P<0.05, **P<0.01; ***P<0.001, ****P<0.0001.) (**B**) qRT-PCR analysis of dystrophin-targeting miRNAs (DTMs) as in (A). (**C**) Transcription factor (NF-κB) binding sites and histone (H3) modifications that mark regulatory regions were examined using ChIP-seq data from ENCODE. Binding motifs for each transcription factor were identified through the Factorbook repository. *Left top*; Schematic of the gene locus for miR-146b, illustrating the binding sites of 3 neighboring DNA loci bound directly by NF-κB. Epigenetic modification maps are provided showing the location of histone modifications associated with active promoters (H3K4me3) and poised/active enhancers (H3K4me1/H3K27Ac. *Left bottom;* Sequence logo pictogram of base frequency at NF-κB binding sites with the consensus NF-κB motif. Two representative NF-κB binding site sequences near miR-146b are also provided. *Right*; Summary of promoter analysis and

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literature data for all DTMs indicating each miRNA and known factors associated with its transcriptional regulation. (DMD, Duchenne Muscular Dystrophy, UC, ulcerative colitis, HPA, hypothalamic–pituitary–adrenal axis)

Figure 4. Dystrophin is reduced in severe myositis. (A) Representative Mild (HT-M) and Severe (HT-S) quadriceps immunolabeled with an antibody against Dystrophin (red) and macrophage staining (F4/80, green). Bar = 50μM. (**B**) Representative WT, HT-M and HT-S quadriceps immunolabeled with an antibody against Dystrophin (red). Anti-Laminin (green) was used as a control to show sarcolemnal integrity. DAPI counterstain to visualize nuclei is shown in blue. Bar = 100μM (**C**) Western blot of Dystrophin using protein extracts of tibialis anterior from HT-M and HT-S myositis mice (n=4). Vinculin was used as a loading control and was run on the same Western blot.

Figure 5. Elevated MHC Class I, NF-κB-driven gene expression, Dystrophin-targeting miRNAs and reduced dystrophin in histologically severe human muscle biopsies. (**A**) Representative H&E staining of human muscle biopsies (n=3 Healthy control, 2 dermatomyositis (DM), 4 inclusion body myositis (IBM)). Bar = 100μM. (**B**) Human muscle biopsies were analyzed for expression of the indicated genes. Data were normalized to Hprt for mRNAs and RNU48 for miRNAs. (n=3 healthy control, 3 histologically mild, 3 histologically severe; #$p<0.10$, *$p<0.05$, ***$p<0.001$, Student's t-test). (**C**) Representative healthy, histologically mild and histologically severe human muscle biopsies immunolabeled with an antibody against Dystrophin (red). Anti-Laminin (green) was used as a control to show sarcolemnal integrity. In Histologically severe muscle, stars highlight muscle fibers where laminin staining is uniform, but dystrophin is reduced, discontinuous or absent. DAPI counterstain to visualize nuclei is shown in blue. Bar = 100μM and 20μM for inset.

Figure 6. Model of self-amplifying feedback loop between macrophages and myofibers in myositis. Myositis disease progression is driven by myofibers adopting a more immune-like state, as evidenced by inappropriate expression of TLR7 and MHC Class I. TLR7 activates NF-κB-driven gene expression, eliciting the release of inflammatory cytokines and promoting macrophage

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recruitment. Activation of NF-κB-driven genes such as TNF-α and IFN-α/β, leads to a feed-forward loop where these cytokines activate their receptors (TNFR and IFNAR, respectively) leading to enhanced NF-κB activation. TLR7 additionally activates IRF7, driving production of IFN-1, which in turn activates IFNAR and expression of Type I interferon-stimulated genes (ISGs) which includes IRF7. NF-κB also triggers expression of inflammatory and dystrophin-targeting miRNAs (infla-miRNA and DT-miRNA), leading to dystrophin reduction and muscle weakness, in turn causing increased release of pro-inflammatory signals. All these components contribute to a positive, self-amplifying inflammatory feedback loop. (Type I IFN, Type 1 Interferon; IFNAR, Interferon Receptor; ssRNA – single stranded RNA).

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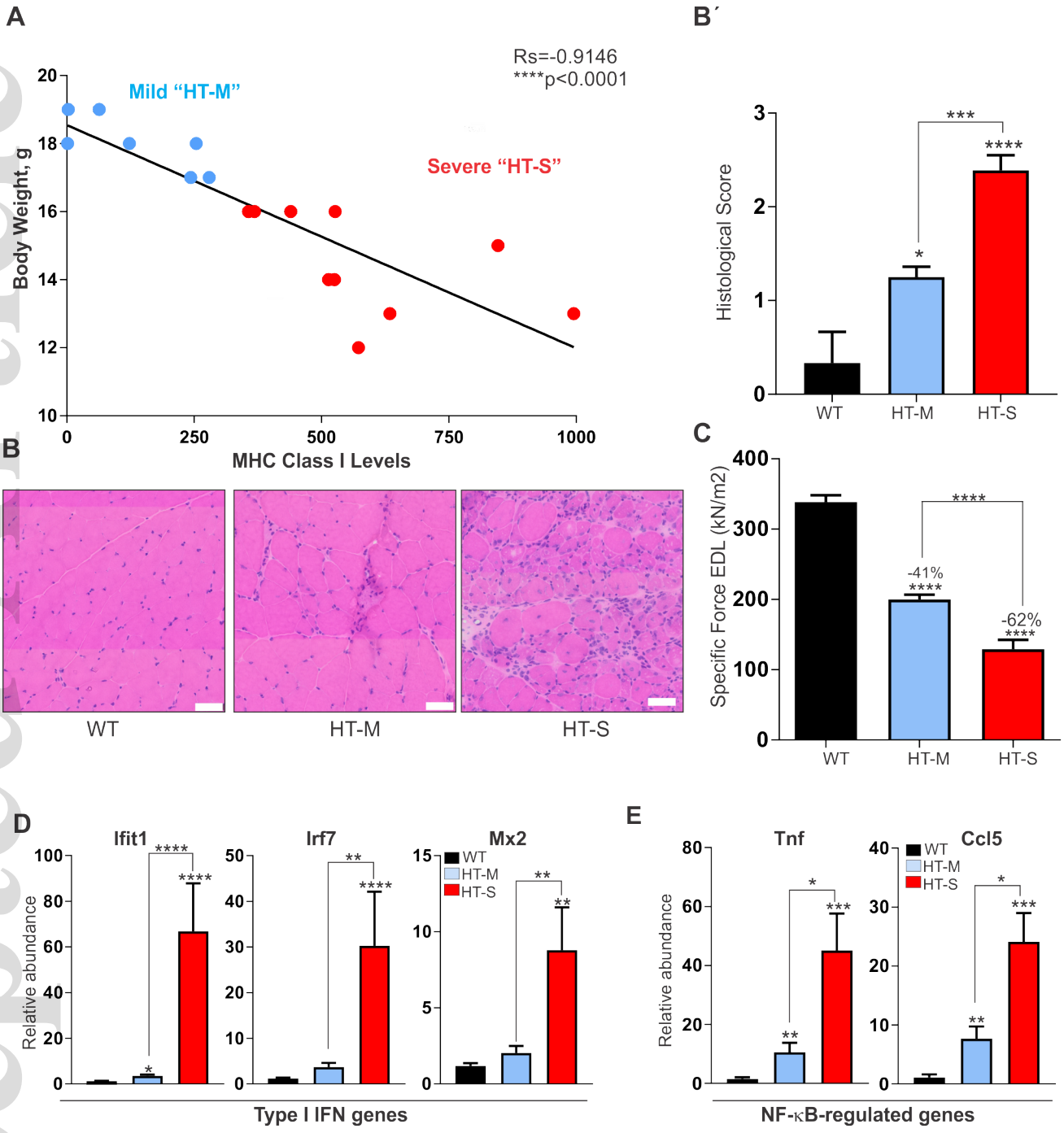

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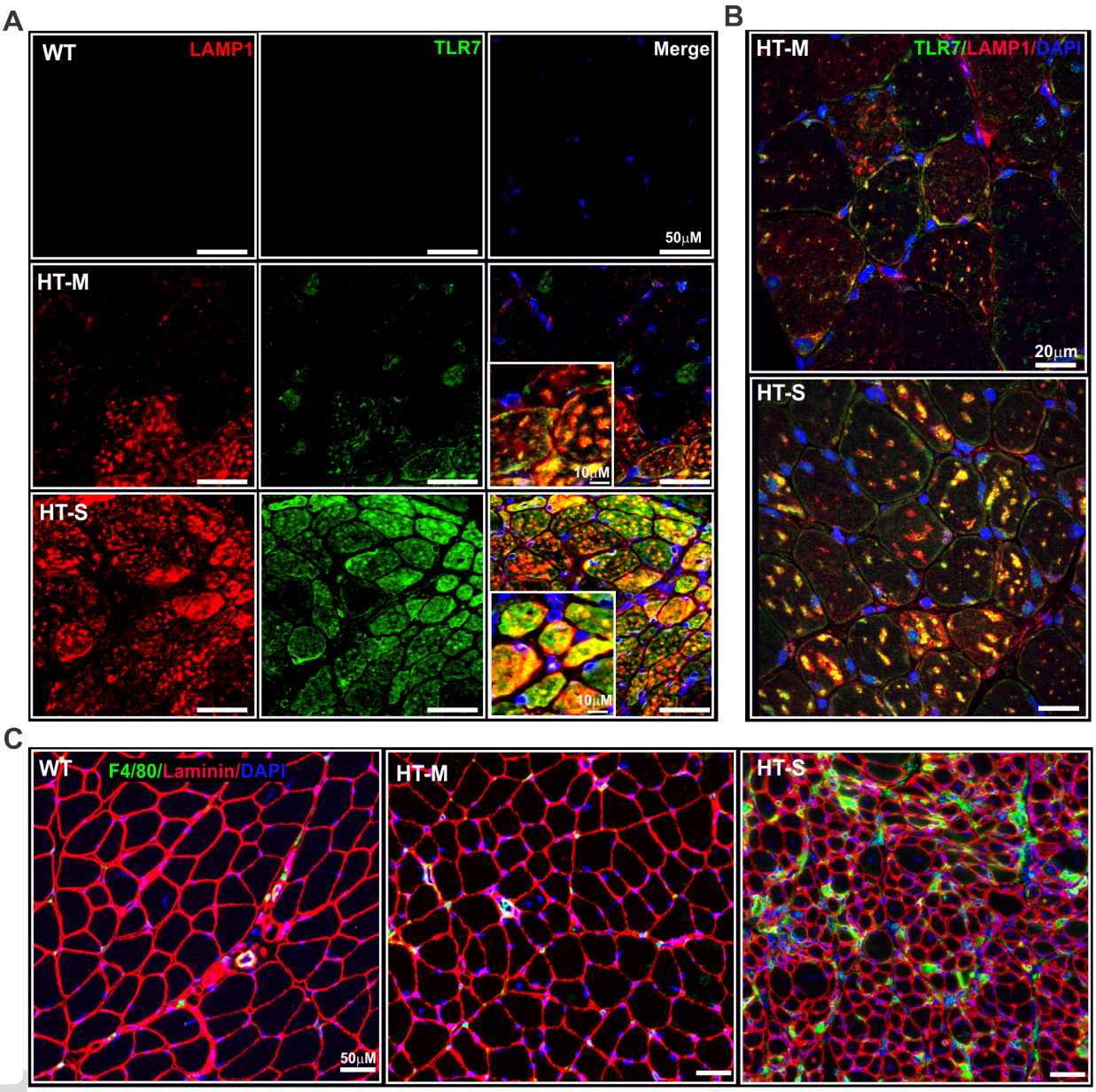

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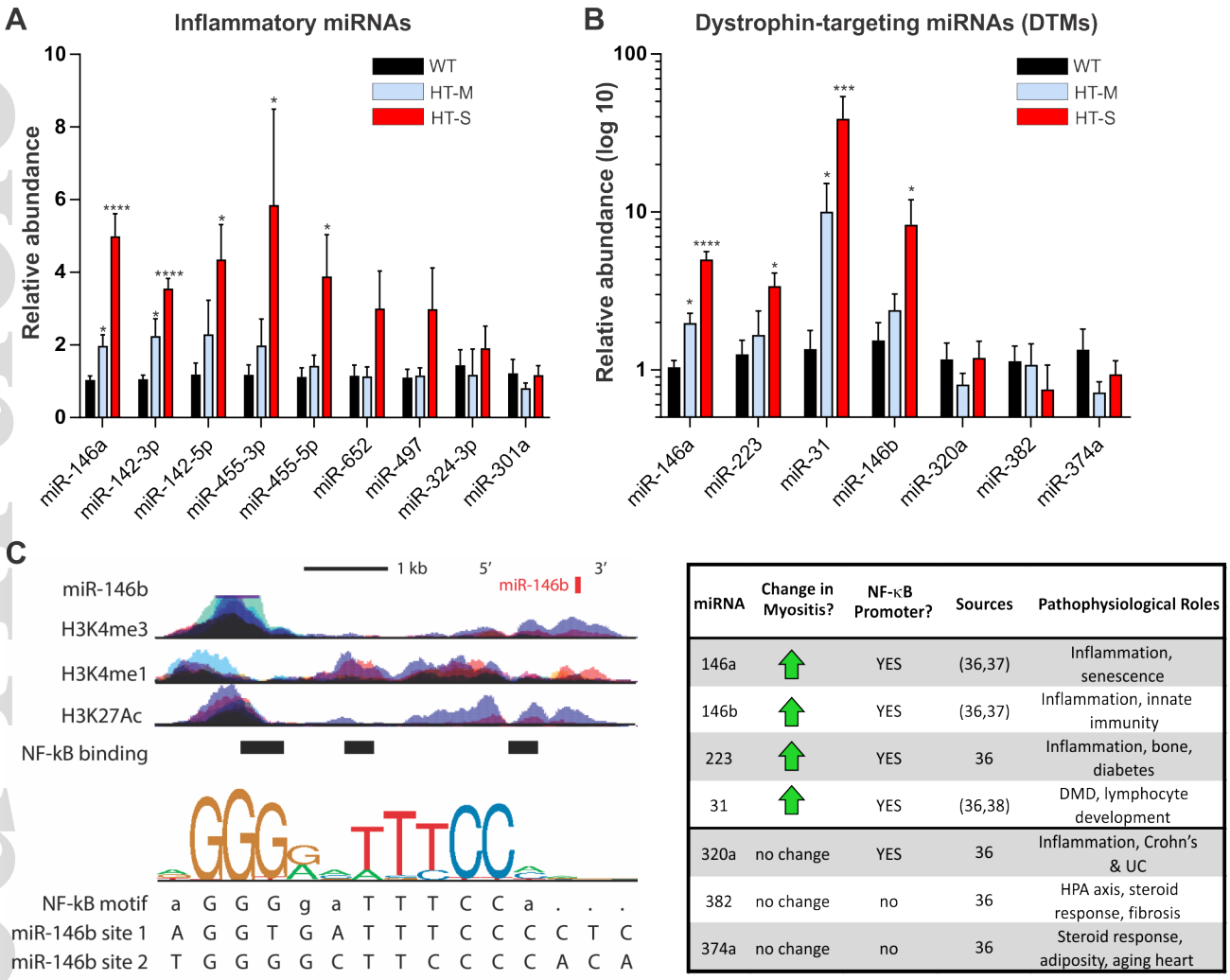

miRNA	Change in Myositis?	NF-κB Promoter?	Sources	Pathophysiological Roles
146a	↑	YES	(36,37)	Inflammation, senescence
146b	↑	YES	(36,37)	Inflammation, innate immunity
223	↑	YES	36	Inflammation, bone, diabetes
31	↑	YES	(36,38)	DMD, lymphocyte development
320a	no change	YES	36	Inflammation, Crohn's & UC
382	no change	no	36	HPA axis, steroid response, fibrosis
374a	no change	no	36	Steroid response, adiposity, aging heart

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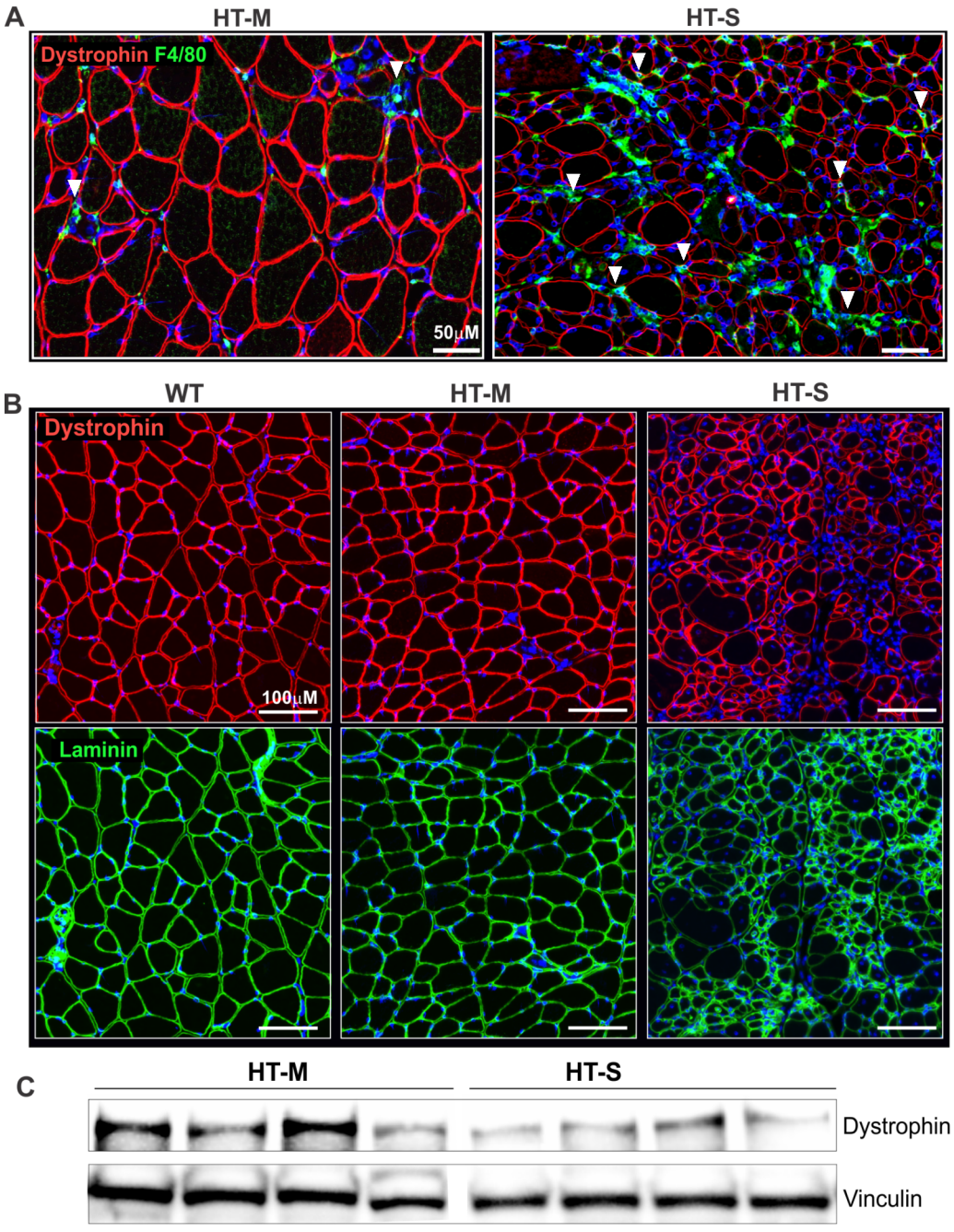

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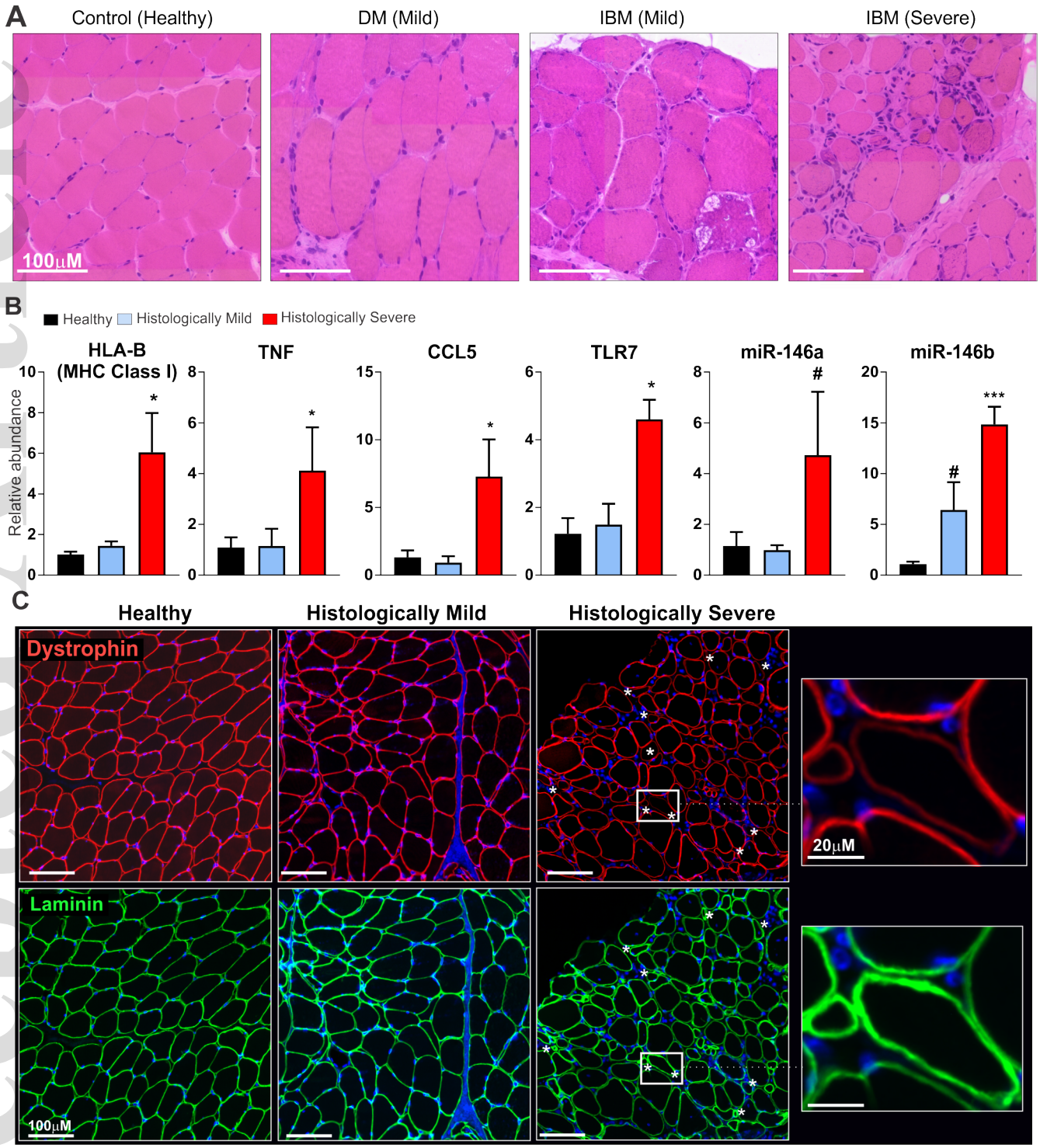

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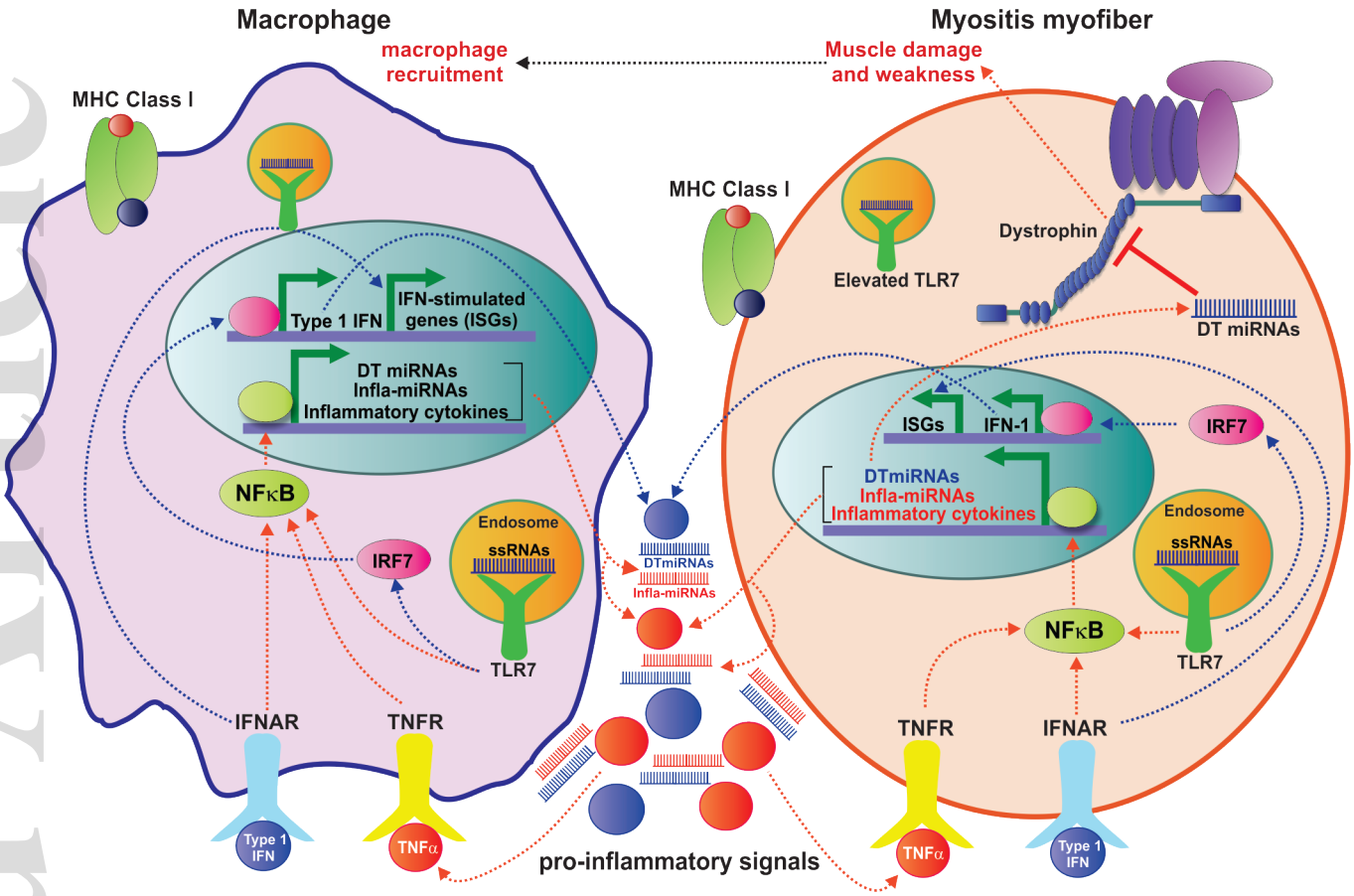

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Estrogen Receptor, Inflammatory, and FOXO Transcription Factors Regulate Expression of Myasthenia Gravis-Associated Circulating microRNAs

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MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate important intracellular biological processes. In myasthenia gravis (MG), a disease-specific pattern of elevated circulating miRNAs has been found, and proposed as potential biomarkers. These elevated miRNAs include miR-150-5p, miR-21-5p, and miR-30e-5p in acetylcholine receptor antibody seropositive (AChR+) MG and miR-151a-3p, miR-423-5p, let-7a-5p, and let-7f-5p in muscle-specific tyrosine kinase antibody seropositive (MuSK+) MG. In this study, we examined the regulation of each of these miRNAs using chromatin immunoprecipitation sequencing (ChIP-seq) data from the Encyclopedia of DNA Elements (ENCODE) to gain insight into the transcription factor pathways that drive their expression in MG. Our aim was to look at the transcription factors that regulate miRNAs and then validate some of those *in vivo* with cell lines that have sufficient expression of these transcription factors This analysis revealed several transcription factor families that regulate MG-specific miRNAs including the Forkhead box or the FOXO proteins (FoxA1, FoxA2, FoxM1, FoxP2), AP-1, interferon regulatory factors (IRF1, IRF3, IRF4), and signal transducer and activator of transcription proteins (Stat1, Stat3, Stat5a). We also found binding sites for nuclear factor of activated T-cells (NFATC1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), early growth response factor (EGR1), and the estrogen receptor 1 (ESR1). AChR+ MG miRNAs showed a stronger overall regulation by the FOXO transcription factors, and of this group, miR-21-5p, let-7a, and let 7f were found to possess ESR1 binding sites. Using a murine macrophage cell line, we found activation of NF-κB -mediated inflammation by LPS induced expression of miR-21-5p, miR-30e-5p, miR-423-5p, let-7a, and let-7f. Pre-treatment of cells with the anti-inflammatory drugs prednisone or deflazacort attenuated induction of inflammation-induced miRNAs. Interestingly, the activation of inflammation induced packaging of the AChR+-specific miRNAs miR-21-5p and miR-30e-5p into exosomes, suggesting a possible mechanism for the elevation of these miRNAs in MG

patient serum. In conclusion, our study summarizes the regulatory transcription factors that drive expression of AChR+ and MuSK+ MG-associated miRNAs. Our findings of elevated miR-21-5p and miR-30e-5p expression in immune cells upon inflammatory stimulation and the suppressive effect of corticosteroids strengthens the putative role of these miRNAs in the MG autoimmune response.

Keywords: microRNA, myasthenia gravis, estradiol, miR-21-5p, NF-κB, FOXO

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate important intracellular biological processes. The field of circulating miRNAs in autoimmune disorders, especially as potential biomarkers have expanded in the recent years, including myasthenia gravis (MG) (1). In MG there is a shortage of biomarkers to predict the clinical course and this is particularly troublesome due to the fluctuating nature of skeletal muscle fatigue with periods of unpredictable worsening (2). MG is a heterogeneous disease that still can be subdivided into two main subgroups based on antibody target: patients with antibodies against the acetylcholine receptors (AChR+ MG; ∼85%) and those with antibodies against the muscle-specific tyrosine kinase (MuSK+ MG; ∼7%) (3). These two antibody subtypes differ, for example, in the involvement of the thymus which is considered to play a major role in AChR+ MG, especially in terms of thymus inflammation (hyperplasia) but also in regards to treatment response (4).

Recent reports have defined different signatures of elevated serum miRNAs in AChR+ and MuSK+ MG (1). In AChR+ MG, the miRNAs miR-150-5p, and miR-21-5p were upregulated compared to healthy controls and these levels were also lower in patients treated with immunosuppression as compared to immunosuppression naïve patients (5, 6). In addition to miR-150-5p and miR-21-5p, levels of miR-30e-5p were found to be upregulated in late onset AChR+ MG patients and expression of these miRNAs correlated with disease course (7). Interestingly, miR-30e-5p levels were found to be higher in patients with ocular AChR+ MG who later develop generalized MG as compared to those that remain ocular. Given this, expression of miR-30e-5p has the potential to predict clinical disease course (8). On the other hand, the miRNA profile in sera from MuSK+ MG patients revealed elevated miR-151a-3p, let-7a-5p, let-7f-5p, and miR-423-5p levels (9).

miRNAs modulate gene expression by targeting multiple mRNAs encoding proteins involved in a variety of signaling pathways and cellular processes (10). In a similar manner, miRNAs could influence and alter the autoimmunity signaling pathway involved in autoimmune disorders such as MG. Several pathways including the Nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) pathway are inappropriately regulated in several inflammatory disorders including chronic muscle inflammation (11). Toll-like receptor signaling activates NF-κB-driven inflammation. Elevated levels of Toll-like receptors (TLRs) 2, 3, 4, 5, 8, and 9 have been detected in MG peripheral blood mononuclear cells (PBMCs) (12). Moreover, TLR4, TLR7, and TLR9 have been implicated in amplifying autoimmunity in the MG thymus (13, 14).

In MG, T regulatory cells (Tregs) from the thymus of MG patients are profoundly defective in their suppressive activity (15) and there is also an imbalance in peripheral blood Tregs (16). Therefore, genes involved in these two pathways, Treg differentiation and NF-κB signaling, are anticipated to be associated with MG predisposition. Nevertheless, it is not clear which transcription factors regulate the MG-associated miRNA expression in AChR+ vs. MuSK+ MG. In this study, we examined the regulatory transcription factors that drive expression of extracellular miRNAs found in the serum of patients with AChR+ and MuSK+ MG. Our overall objective in this analysis was to outline transcription factors that regulate these miRNAs and then validate a subset of these (i.e., NF-κB and Estrogen Receptor, ESR1) *in vivo* with cell lines that have sufficient expression of these transcription factors [RAW macrophage cells for NF-κB (17) and T-cells for ESR (18)].

MATERIALS AND METHODS

Bioinformatics

We examined the surrounding regulatory region of each miRNA gene to gain insight into the mechanisms of response to treatment as previously reported (19). Briefly, we examined the binding of transcription factors that are most relevant in MG (20–22) using chromatin immunoprecipitation sequencing (ChIP-seq) data. ChIP-seq data from the Encyclopedia of DNA Elements (ENCODE) was queried for physical binding to DNA loci encoding the human miRNA target of interest (23, 24). Both the independent promoter/enhancer of the miRNA was queried, and for miRNAs that were encoded within introns of a gene, the promoter and enhancer of that gene was additionally queried. The proximal promoter was considered the region directly upstream of the miRNA or gene, within 2 kb (25) while the enhancer was considered the region within 10 kb of the miRNA or gene (26). 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 (UCSC) Genome Browser Release 4 (https://genome.ucsc.edu/index.html) with alignment to the GRCh37/hg19 genome build. Each ChIP-seq dataset was analyzed using the ENCODE Regulation Super-Track listed under the Regulation menu. Transcription factors were 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 H3K27Ac 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 of transcription factors was queried in ChIP-seq datasets produced using all 9 cell line tracks to identify all possible transcription factor binding. These include: GM12878, a lymphoblast cell line; H1-hESC, an embryonic stem cell line; HeLa-S3, a cervical cancer cell line; HepG2, a liver cancer cell line; HSMM, a human skeletal muscle myoblast cell line; HUVEC, a human umbilical vein endothelial cell line; K562, a human immortalized myelogenous leukemia cell line; NHEK, Human Epidermal Keratinocytes; and NHLF; a human lung fibroblast cell line. Histone modifications were also queried in ChIP-seq datasets using all nine available cell line tracks.

Circulating miR-21-5p Analysis in Patients and Healthy Controls

Detailed patient characteristics have been described previously (5). In brief, stored serum samples were collected in tubes without additives from 54 healthy blood donors (27 females, mean age: 45 ± 16 years; 27 male, mean age: 47 ± 15 years) without any medications and from 73 MG patients (42 females; mean age: 57 ± 15 years), at Uppsala University Hospital. The MG group consisted of 27 female MG patients without any immunosuppression (mean age: 55 ± 13 years), 10 male MG patient without any immunosuppression (mean age: 62 ± 18 years), 15 female MG patients with immunosuppression (mean age: 53 ± 15 years), and 21 male MG patients with immunosuppression (mean age: 62 ± 14 years). In the MG patient cohort without immunosuppression, 76% were AChR+ and 24% were AChR/MuSK antibody seronegative (AChR/MuSK-) and among those with immunosuppression 70% were AChR+ and 30% were AChR-/MuSK-. Mean MGC score was 6.1 (range: 0–34) in the group without immunosuppression and 4.6 (range: 0–18) in the group with immunosuppressive treatment (5). The study was approved by the local Ethical Review Board in Uppsala (Dnr 2010/446) and all patients signed informed consent. RNA isolation, cDNA synthesis and qPCR were performed as previously described (5, 6). In brief, total RNA was isolated from 200 µl serum by using miRCURY™ RNA Isolation Kit—Biofluids (Exiqon#300112) and 2 µl of isolated RNA sample was applied for cDNA synthesis in a 10 µl reaction mix by using Universal cDNA Synthesis Kit II (Exiqon #20330). The RT-qPCR analysis was performed with ExiLENT SYBR® Green master mix (Exiqon #203421) and cDNA reactions were diluted 100X. The following quality controls were included on Pick-&-Mix microRNA PCR panel plates: the interpolate calibration (UniSp3), RNA extraction control (UniSp2 and UniSp4), cDNA synthesis control (UniSp6), and hemolysis test (miR- 23a-3p–miR-451a) (Exiqon). The ΔCT value of hemolysis markers [ΔCT (hemolysis) = CT(miR-23a-3p) – CT(miR-451a)], was used to detect hemolysis and samples with a ΔCT >7 were excluded. Reference miRNAs were miR-93-5p, miR-191-5p, miR-423-4p, and miR-103a and quantification of relative miRNA expression was performed with the comparative CT method.

ELISA

Serum samples from 20 female AChR+ MG patients (mean age: 54 ± 12 years) without immunosuppressive medication that were also analyzed for miR-21-5p levels, and 20 age matched female healthy controls (mean age: 51 ± 14 years) were analyzed. Enzyme-linked immunosorbent assay (ELISA) was performed for 17-β-estradiol using Human Estradiol kit (KAQ0621, Invitrogen), following the manufacturer's instructions. All samples were assayed in duplicate at an absorbance of 450 nm, and the detection range was 13–935 pg/ml. Inter-assay CV was 6.1%. Calculating and analysis of data was conducted by Spark® Multimode Microplate Reader (Tecan Trading AG).

miR-21-5p Expression in T Cells Treated With β-Estradiol and 4-Hydroxytamoxifen

Human T cell line KE-37 (DSMZ, ACC 46) was used for β-estradiol (E2758, Sigma) and 4-hydroxytamoxifen (H7904, Sigma) treatments, since human T cells express estrogen receptor (18).

Cells were plated in 35 mm dishes with RPMI1640 media (R7509, Sigma) with 10% charcoal stripped FBS (TA3382101, Thermo Fisher Scientific) at a density of 1,000,000 cells/dish. Each condition was performed in triplicate.

For β-estradiol and 4-hydroxytamoxifen co-treated group, cells were treated with 0.1 nM β-estradiol and 10^{-}µM 4-hydroxytamoxifen for 24 h. For dose-response and time-course group, cells were treated for 24, 48, and 72 h, respectively, with ß-estradiol at 0, 5, and 50 nM. The expression of miR-21-5p was examined and analyzed by qRT-PCR, using reference genes miR-103a and miR-191-4p for normalization. miRNA isolation was performed using miRNeasy Mini Kit (217004, Qiagen) and cDNA was synthesized. PCR amplification system included cDNA templates (1:50 dilution), ExiLENT SYBR® Green Master Mix (201420-01, Exiqon) and miRNA-specific primers (Qiagen). PCR amplification was conducted in presence of Rox Reference Dye by Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies). Log conversion of the miRNA expression data was done in order to obtain data more similar to a normal distribution for the statistical tests.

miRNA-Induction in Macrophage Cells

The murine macrophage cell line RAW 264.7, a standard model system for assessment of LPS-induced inflammatory response (17), was cultured in DMEM with 10% FBS. Cells were seeded in a 6-well plate at a density of 500,000 cells/well. Each condition was performed in triplicate. Cells were pretreated with 10 µM of the indicated drug or a DMSO vehicle-only control. Inflammatory signaling was induced using LPS (Thermo Fisher Scientific) or a PBS-only vehicle control at a dilution of 1:1,000 as previously reported (27). After 48 h exosomes were collected from the media

using ExoQuick (SBI), and RNA was subsequently extracted using TRIzol. Cells were also lysed for RNA using TRIzol at 24 or 48 h post-LPS induction. Expression of exosome-specific miRNAs was quantified by TaqMan Assay and results were normalized to the Geometric Mean of miR-17 and miR-93 as these miRNAs are reported to remain unchanged in exosomes (28). Expression of cell-specific miRNAs was also quantified by TaqMan Assay and values were normalized to sno202. The Taqman IDs for all miRNAs queried is as follows: snoRNA202, 001090; miR-93, 001090; miR-17, 002308; miR-151-3p, 001190; miR-21-5p, 000397; let-7a, 000377; let-7f, 000382, miR-150−5p, 000473; miR-30e-5p, 002223; miR-423-5p, 002340.

Statistical Analysis

D Ágostino & Pearson normality test revealed that qPCR data were normally distributed (p>0.05) whereas ELISA data were not ($p < 0.05$). Comparison of miR-21-5p serum data between male and female controls and MG patients was done using one-way ANOVA, followed by unpaired *T*-test comparing female controls and MG patients as well as male controls and MG patients. Comparison of 17-β-estradiol levels between female MG patients and healthy controls was done by Wilcoxon matched-pairs signed rank test. A $p < 0.05$ was considered significant. Spearman Rank correlation was used to determine any correlation between estradiol and miR-21-5p levels as well as between miR-21-5p and age.

RESULTS

Transcription Factors Regulating AChR+ vs. MuSK+ MG-Associated miRNAs

The previously identified MG-associated miRNAs in serum included miR-150-5p, miR-21-5p and miR-30e-5p (AChR+ MG) as well as miR-151, miR-423, let-7f, and let-7a (MuSK+ MG) (1). Our bioinformatics analysis of the ChIP-seq data from ENCODE revealed several transcription factor binding sites on the aforementioned miRNA genes (**Figure 1**). For this analysis, we utilized data from all available cell lines deposited into ENCODE, to get an overview of the regulation of each miRNA. We used an arbitrary cutoff of 2 kb for the proximal promoter region and 10 kb for the proximal enhancer region. To increase confidence that the regions analyzed were bona fide regulatory elements we also overlayed ChIP-seq data showing verified histone modifications indicative of active promoters (H3K4 trimethylation) and enhancers/regulatory regions (H3K4 monomethylation, H3K27 acetylation (**Figure 1**).

At least nine transcription factors were found to have binding sites in the defined regulatory regions of 7 MG-associated miRNAs. The identified transcription factors included the Forkhead box proteins (FoxA1, FoxP2), the interferon regulatory factors (IRF1, IRF4), signal transducer and activator of transcription proteins (STAT1), AP-1 transcription factors (FOS, Jun, JunB) and NF-κB (denoted as RELA in ENCODE) (**Figure 1**). When we looked at the totality of transcription factor binding sites on the 7 miRNA regulator regions queried, we found that FOS, JunD, FoxA1, and RELA had the highest occupancy on the MG-associated miRNAs ($N = 30$, 28, 27, and 23, respectively). Intriguingly, only miR-21-5p, let-7a, and let-7f were found to possess estrogen receptor 1 (ESR1) binding sites. We also found nuclear factor of activated T-cells (NFATC1) and early growth response factor (EGR1) binding sites on the majority of the miRNA genes queried (**Figure 1**).

Due to the different signature of circulating miRNAs in AChR+ MG vs. MuSK+ MG, we next in silico determined the set of transcription factors that more specifically regulate the expression of these subtype specific miRNAs. AChR+ MG miRNAs showed a stronger overall regulation by the FOXO transcription factors, considering that miR-21-5p, miR-150-5p, and miR-30e-5p together possessed 52 binding sites for FOXO transcription factors (**Figure 2A**). On the contrary, MuSK+ miRNAs possessed less binding sites for the FOXO family (nine binding sites, **Figure 2A**) and instead had the most binding sites for the FOS, Jun, JunD, and Stat3 proteins. Further, in-depth analysis of each miRNA regulatory region revealed binding sites for the FOXO transcription factors within the miR-21 and the miR-30e loci (**Figures 2B–D**).

Higher miR-21-5p Levels in Female Immunosuppression Naïve MG Patients

We next focused on the relationship of estrogen and miR-21-5p, as our bioinformatics analysis demonstrated heavy regulation of this miRNA by ESR1, showing six binding sites within its promoter/enhancer region (**Figures 1**, **3A,B**). Based on this, we tested the hypothesis that miR-21-5p levels would be markedly different between male and female MG patients. To do this, we re-analyzed miR-21-5p levels from archival data of AChR+ MG patients and healthy controls, this time grouping them by gender (5). There was no difference between serum miR-21-5p levels of healthy female controls ($N = 27$) and healthy male controls ($N = 27$; $p = 0.33$) or between female ($N = 27$) or male ($N = 10$) MG patients with immunosuppression ($p = 0.90$). However, female MG patients without immunosuppression had significantly higher values than male MG patients without immunosuppression ($p = 0.015$; **Figure 4A**). miR-21-5p levels were also higher in female MG patients without immunosuppression compared to healthy female controls ($p = 0.0001$; **Figure 4B**) as well as female MG patients with immunosuppression ($p = 0.019$, **Figure 4B**). One-way ANOVA did not reveal any difference in miR-21-5p levels between the groups of AChR+ and AChR/MuSK- patients (without and with immunosuppression, respectively; data not shown; $p = 0.21$).

We next explored whether age could play a role for miR-21-5p levels. However, correlation analysis did not reveal any relationship between age and miR-21-5p levels in healthy female controls ($R = 0.25$; $p = 0.21$) nor in male healthy controls ($R = -0.04$; $p = 0.85$). Neither was there any correlation between miR-21-5p levels and age in female MG patients without immunosuppression ($R = 0.17$; $p = 0.39$). A significant age correlation was seen however for miR-21-5p in male MG patients without immunosuppression ($R = 0.75$; $p = 0.017$), i.e., increasing miR-21-5p levels with increasing age.

A

Transcription factor	miRNAS					
	miR-21-5p	miR-150-5p	miR-30e-5p	miR-151-3p	miR-423-5p	let7a-1-5p, let-7f-1-5p
ESR1	6					1
EGR1	5	3	8	3		4
FOS	10		4	10	1	5
JUN	10	1	1	6	1	2
JUNB	4					
JUND	13	4	4	4	2	1
FOXA1	18	1	5	1	2	
FOXA2	8	1	3			
FOXM1	9	1	2	1	1	
FOXP2	1		3	2	1	1
IRF1	5	2	1	1	2	2
IRF3						2
IRF4	5	1	2	1	2	1
NFATC1	5	2	1	1	1	
RELA (NF-kappaB)	13	3	4	1	1	1
STAT1	7	1	2	2	2	1
STAT2	3		1			1
STAT3	14	1		4	3	2
STAT5A	5	1	2	1	1	

B

Group	Transcription factors	Disease relevance
Forhead box proteins	FoxA1, FoxA2, FoxM1, FoxP2	Cancer, Aging , Autoimmunity
AP-1 transcription factors	FOS, Jun, JunB, JunD	Elevated in Rheumatoid arthritis and cancer
Interferon regulatory factor	IRF1, IRF3, IRF4	Autoimmune disorders; aging & autoimmunity
Signal transducer & activator of transcription (STAT)	Stat1, Stat3, Stat5a	Autoimmune disease, Activated by cytokines
NF-kappaB	NF-kappaB	Inflammatory diseases, Stimulated by TNF-α and IL-1
NFAT (Nuclear factor of activated T-cells)	NFATC1	Autoimmune disease/ Cyclosporin A blocks activity
Early Growth Response Factor	EGR1	Regulates genes involved in genes synaptic homeostasis and plasticity
Estrogen Receptor	ESR1	Elevated in female MG patients; regulated by estrogen

FIGURE 1 | Overview transcription factors that regulate MG-associated miRNAs. **(A)** Using the UCSC Genome browser, each of the seven miRNAs identified from our previous reports (1, 5) were queried for transcription factor regulation. **(A)** Shows an overview of all transcription factor binding sites within the proximal promoter (∼2 kb) of the miRNA, the proximal promoter of the gene surrounding the miRNA locus (if applicable), the enhancer region (within ∼10 kb) of the miRNA, and the enhancer region of the gene surrounding the miRNA locus (if applicable). **(B)** Overview of the disease relevance and function for each major transcription factor group listed in **(A)**.

Based on the link between ESR1 and miR-21-5p, we assumed that serum estradiol levels could be correlated to miR-21-5p levels. Therefore, we next assessed estrogen levels in female controls vs. female MG patients. While there was no significant difference between 17-β-estradiol levels in female MG patients compared to their age-and sex matched controls, there was a trend toward increased serum estradiol in female MG patients ($p = 0.07$; **Figure 4C**). Nevertheless, there was no clear correlation between miR-21-5p and 17-β -estradiol levels when taking the entire female cohort, i.e., immunosuppression naïve MG patients and healthy controls, into consideration ($p = 0.75$; Spearman $R = -0.05$; **Figure 4D**). In summary, the observed higher miR-21-5p levels in female MG patients without immunosuppression compared to male patients does not appear to be mediated by estradiol levels alone.

Finally, in order to determine whether estradiol would induce expression of miR-21-5p in a human T cell line, these cells were treated with 17-β-estradiol at different concentrations and duration. Treatment with 17-β-estradiol for 24, 48, and 72 h did not result in a significant increase in miR-21-5p levels (data not shown). Further, treatment with the estrogen receptor inhibitor tamoxifen did not induce significant reduction of miR-21-5p levels (**Supplementary Figure 1**).

NF-κB Pathway Up-Regulates Expression of MG-Associated miRNAs

Based on the finding that AChR+ MG-associated miRNAs have a large number of putative NF-κB binding sites within the regulatory domains (**Figure 5A**), the effect on the expression of these miRNAs was assayed through induction of inflammatory signaling in a murine macrophage cell line. Upon inflammatory induction by LPS, which activates NF-κB through TLRs, such as TLR4, levels of miR-21-5p, miR-30e-5p, miR-151a-3p, miR-423-5p, let-7a-5p,

A

miRNA	Number of putative FOXO binding sites
miRNAs dysregulated in AchR+ MG	
miR-21-5p	36
miR-150-5p	3
miR-30e-5p	13
miRNAs elevated in MuSK+ MG	
miR-151-3p	4
miR-423-5p	4
let7a-1-5p, let-7f-1-5p	1

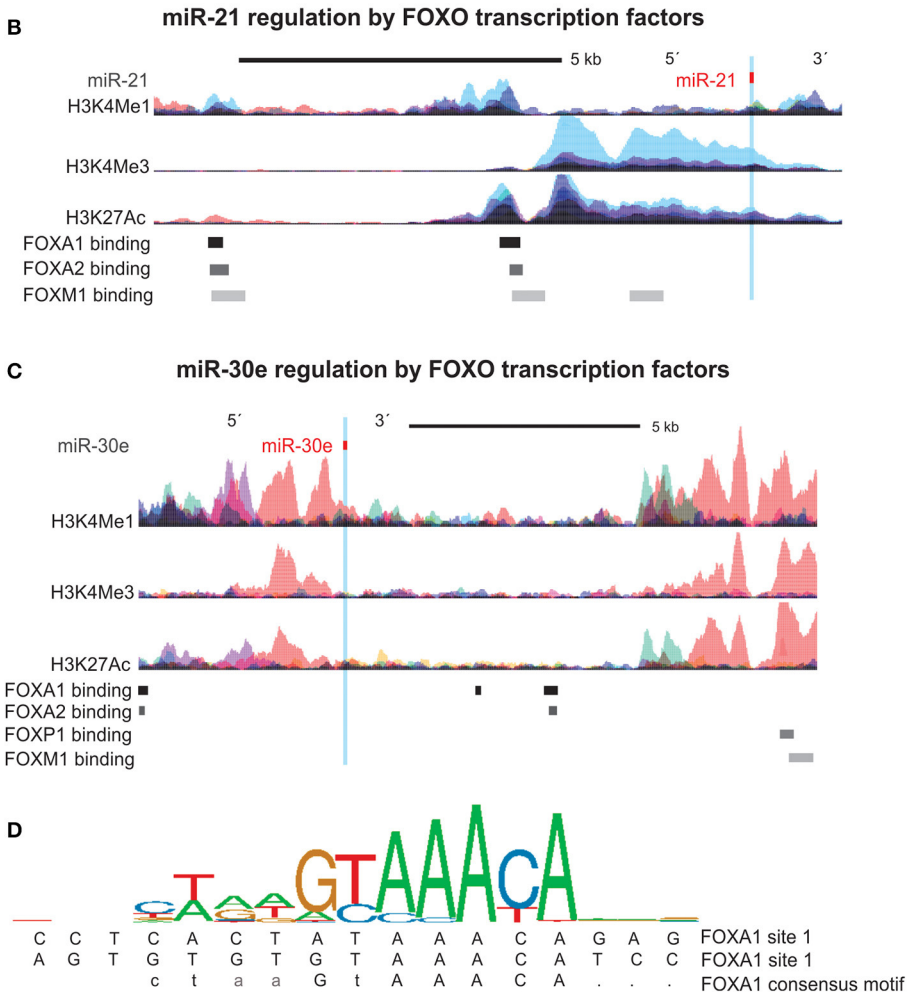

FIGURE 2 | Promoter analysis of miRNAs indicates regulation by FOXO transcription factors. FOXO Transcription factor binding sites and histone (H3) modifications that mark regulatory regions were examined using ChIP-seq data from ENCODE. DNA binding motifs for each transcription factor were identified through the Factorbook repository. **(A)** Table of all FOXO binding site found in regulatory regions of miRNAs dysregulated in both AchR+ and MuSK+ MG, demonstrating a high number of binding sites within miRNAs specific to the AchR+ subtype. **(B,C)** Schematic illustrating binding sites for FOXO transcription factors within the **(B)** miR-21 locus (seven of 26 binding sites shown) and **(C)** miR-30e locus (seven of 13 binding sites shown). 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 both loci. **(D)** Sequence logo pictogram of base frequency at FOXA1 binding sites, with the consensus FOXA1 motif provided immediately below. Also provided are two representative FOXA1 binding site sequences near miR-30e, listed in order from the 5′ to 3′ direction.

and let-7f-5p were significantly elevated (**Figure 5B**). In addition, packaging of miR-21-5p and miR-30e-5p into exosomes was induced by inflammation (**Figure 5C**). Cell-specific expression of miRNAs was then assessed after anti-inflammatory treatment. Macrophages pretreated with the immunosuppressive treatment prednisolone and deflazacort attenuated the induction of the same miRNAs, miR-21 and miR-30e (**Figures 5D,E**).

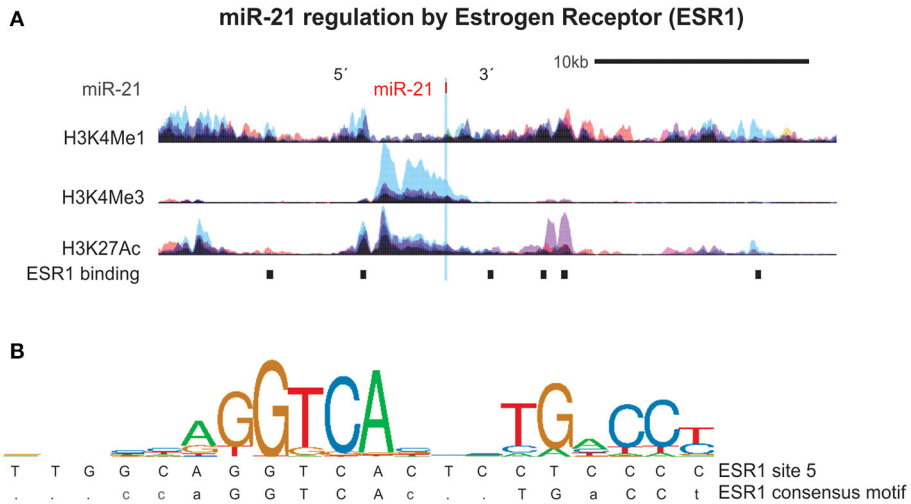

FIGURE 3 | miR-21 is regulated by the Estrogen Receptor 1 (ESR1). **(A)** Estrogen receptor 1 (ESR1) binding sites and histone (H3) modifications that mark regulatory regions were examined using ChIP-seq data from ENCODE as detailed in **Figure 2**. Shown are the six ESR1 binding sites in the region surrounding the miR-21 DNA locus. **(B)** Sequence logo pictogram of base frequency at ESR1 binding sites, with the consensus ESR1 motif provided immediately below. Also provided is a representative ESR1 binding site sequences near miR-30e, listed in order from the 5′ to 3′ direction.

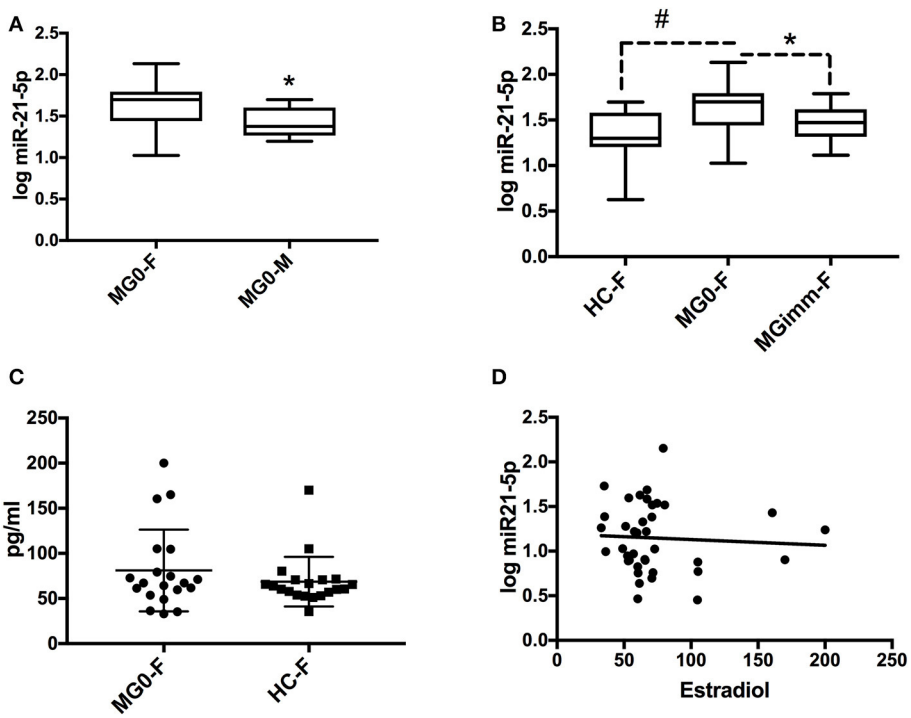

FIGURE 4 | miR-21-5p levels are highest in female MG patients without immunosuppression. **(A)** miR-21-5p levels in female (MG0-F; $N = 27$) and male MG (MG0-M; $N = 10$) patients without immunosuppression. **(B)** miR-21-5p levels in female healthy controls (HC-F; $N = 27$), MG0-F and MG patients with immunosuppression (MGimm-F; $N = 15$). Data in panels **(A,B)** are shown in logarithmic (log) scale and the boxplot refers to mean ± SD and whiskers state min to max values. **(C)** Serum 17-ß-estradiol in female MG patients ($N = 20$) vs. female age matched healthy controls ($N = 20$). **(D)** Correlation between 17-ß-estradiol and miR-21-5p among female healthy controls ($N = 20$) and MG patients without immunosuppression ($N = 20$). Spearman $R = -0.053$; $p = 0.75$. $^{*}p < 0.05$; $^{\#}p < 0.0001$.

A

miRNA	Number of putative NF-kappaB binding sites
miRNAs dysregulated in AchR+ MG	
miR-21-5p	13
miR-150-5p	3
miR-30e-5p	4
miRNAs elevated in MuSK+ MG	
miR-151-3p	1
miR-423-5p	1
let7a-1-5p, let-7f-1-5p	1

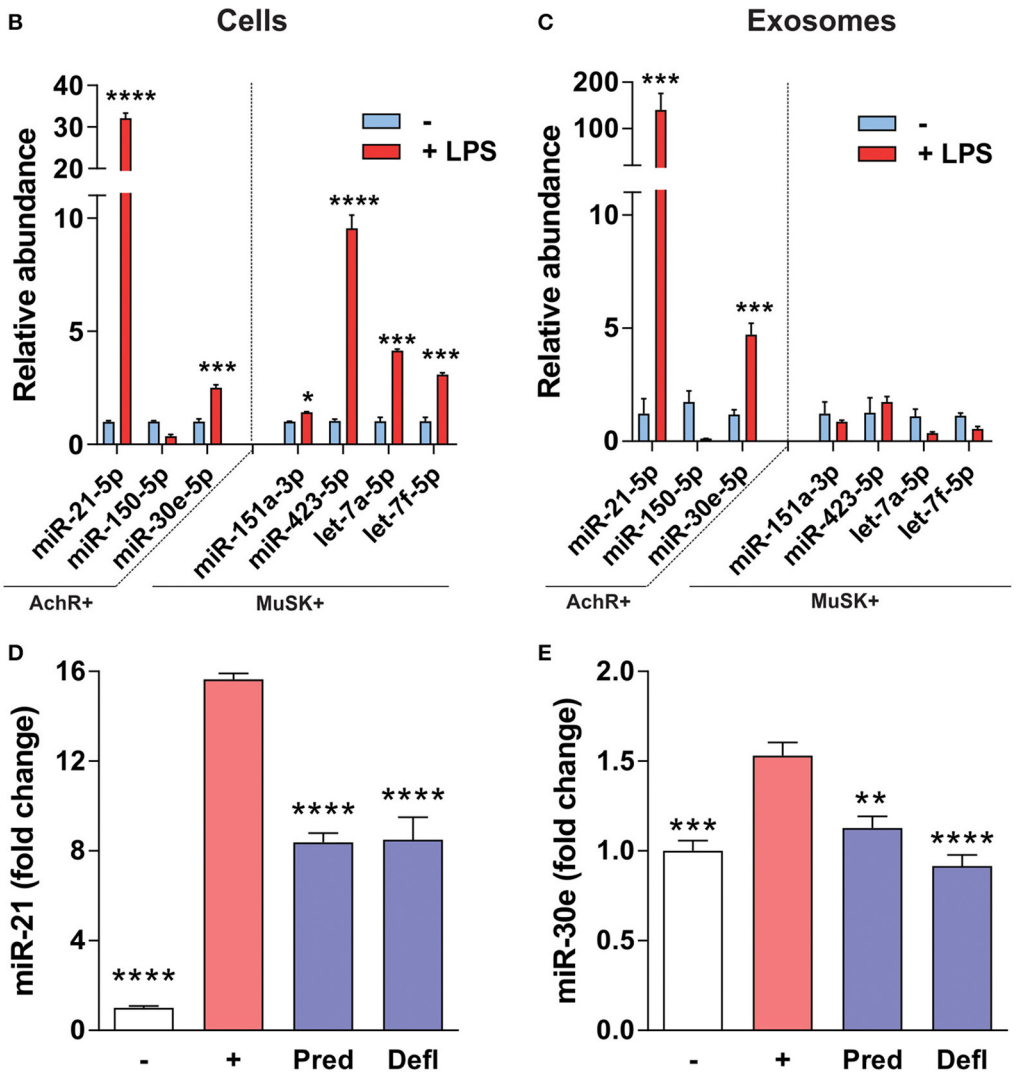

FIGURE 5 | NF-κB-mediated regulation of MG miRNAs. **(A)** Table shows the number of putative NF-κB binding sites within the regulatory regions of each miRNA listed. **(B,C)** Inflammatory signaling was induced in RAW 264.7 for 48 h using LPS. Expression of miRNAs was assayed by qRT-PCR. For each figure LPS treatment was compared to the respective no treatment control. **(B)** In a murine macrophage cell line, LPS induces miR-21-5p, miR-30e-5p, miR-151a-3p, miR-423-5p, let-7a-5p, and let-7f-5p **(C)** LPS induces packaging of miR-21-5p and miR-30e, but not other MG-associated miRNAs, into exosomes ($n = 4$, student's two-tailed *t*-test, $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{*}p < 0.05$). **(D,E)** RAW 264.7 macrophages were pretreated with the indicated drug at 10 μM and inflammatory signaling was induced for 24 h using LPS. Expression of miRNAs within cells was assayed by qRT-PCR for **(D)** miR-21-5p and **(E)** miR-30e-5p, respectively ($n = 4$ $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$, ANOVA with *post hoc* vs.; [–] = no LPS control, [+] = LPS plus vehicle, Defl, deflazacort; Pred, prednisolone.

DISCUSSION

Research on circulating miRNAs in autoimmune disorders, including MG, has developed drastically in recent years. In this study, we examined the transcription factors that regulate MG-associated miRNAs in order to understand what drives the dysregulated serum miRNA profile observed in MG patients. Moving forward, understanding these mechanisms will have important implications toward immunosuppressive and anti-inflammatory drug development in MG. Pathway-based analyses that combine information across multiple genes into a limited number of molecular networks have been found to be a powerful approach that can also be used for miRNAs. The majority of identified transcription factors for MG-associated miRNAs are involved in autoimmunity-related pathways, especially the FOXO proteins, AP-1 transcription factors, NF-κB, IRF, and STAT family and NFAT. In line with this, the AChR+ MG-associated miRNAs might undergo stronger overall regulation by the FOXO transcription factors, considering the 52 binding sites in miR-21-5p, miR-150-5p, and miR-30e-5p for FOXO transcription factors. In AChR+ MG, T regulatory cells (Tregs) have reduced suppressive activity and one of the potential causes of this is decreased expression of FOXP3 in the MG thymus and peripheral blood (15, 29). The Treg specific transcription factor, Fork head/winged-helix transcription factor (FOXP3), has been shown to regulate both the development and the function of Tregs (30). FOXP3 expression is partly controlled by the phosphorylation of signal transducer and activator of transcription 5 (STAT5) induced by the IL-2 transduction pathway and low FOXP3 levels in Tregs may result from decreased STAT5 phosphorylation after IL2-signaling (31). The subtype of MuSK+ MG and AChR+ MG differ in several ways, mainly in that MuSK antibodies are of IgG4 subclass (32, 33), whereas the AChR antibodies are of IgG1 subclass. Further in MuSK+ MG, thymus pathology is normal compared to AChR+ MG (34), where thymus hyperplasia is considered to play a major role. In line with this, the circulating miRNA profile differs between these antibody subtypes of MG and thus it could be anticipated that the transcription factor profile is also different, as encountered here.

The proinflammatory NF-κB signaling pathway is related to inflammatory diseases and stimulated by TNF-α and IL-1. NF-κB had 23 binding sites across all MG-associated miRNAs, the majority being located in the AChR+ MG-associated miRNA genes. This is in support of a large study on almost 1,200 European MG patients with early onset AChR+ MG, where several risk genes were found related to the NF-κB signaling pathway (35). Here, the genetic associations to MG outside the HLA complex indicate VAV1, a key signal transducer crucial for T and B cell activation and BAFF, a cytokine important in the proliferation and differentiation of B cells. Combined VAV1 and BAFF haplotypes conferred a greater risk in combination and in addition to CD86, and these share the same signaling pathway, NF-κB (35). It was therefore not entirely surprising to find that NF-κB regulates transcription of the miRNAs found to be upregulated in AChR+ MG. Together, these data implicate that patients with early onset AChR+ MG may very well be predisposed to dysregulated proinflammatory signaling. miR-21-5p is also regulated by NF-κB, which would, in part, explain why MG patients on immunosuppressive drug therapy, i.e., mainly prednisone, have reduced levels of miR-21 compared to patients without immunosuppression (5). Nevertheless, other immunomodulatory medications such as azathioprine with a slower onset treatment effect and more long-acting properties, potentially have different effects on miR-21-5p and miR-150-5p as compared to the more rapid onset treatment effect of prednisone (36).

After querying ChIP-seq data to understand how MG-associated miRNAs are regulated, we functionally tested NF-κB-specfic miRNA regulation in a murine macrophage cell line and ESR1-specific miRNA regulation in a human T cell line. Our results have important implications about how the overexpression of MG-associated miRNAs may occur, but they also have their limitations. For instance, administration of LPS along with prednisone/deflazacort pre-treatment in murine macrophage cells serves to confirm that NF-κB can indeed induce expression of MG-associated miRNAs (miR-21-5p, miR-30e-5p, miR-151a-3p, miR-423-5p, let-7a-5p, and let-7f-5p) within cells and that blocking NF-κB can attenuate expression of a subset of these miRNAs (miR-21-5p and miR-30e-5p). However, in future studies it will be important to determine how these miRNAs respond to inflammation in individual patients that may have different levels and activation of NF-κB/NF-κB signaling. We also detected miR-21-5p and miR-30e in the exosomes of murine macrophages in response to LPS administration. This observation suggests that in MG patient serum, miR-21-5p and miR-30e may have originated from inflammatory cells that shed miRNA-containing exosomes in response to elevated inflammation. In future experiments it will be important to perform these experiments in patient-derived cell lines to determine (1) the totality of cell/tissues types that may be responsible for releasing miRNAs into the serum via exosomes and (2) other transcription factors might drive miRNA packaging into exosomes.

We analyzed miR-21-5p expression in a human T-cell line after treatment with estradiol, but did not, however, see significant induction. It is possible that estradiol was not sufficient to induce miR-21-5p expression because other transcription factors that co-regulate this miRNA (such as NF-κB) confounding the data. This is a likely scenario given that in female MG patients, we only observed significant elevation of serum miR-21-5p in when patients on immunopressive drugs were removed from analysis; this suggests heavy regulation of miR-21-5p by NF-κB. It is also possible that already high baseline ESR1 activation in this cell line prevented any additional activation of miR-21-5p upon estradiol administration.

To build our bioinformatics and cell line results, in future studies it will be important to determine how each of the identified transcription factors is expressed and how these collectively coordinate miRNA transcription in MG. While the cell culture experiments performed here were to determine if, in our hands, specific miRNAs are regulated by NF-κB and ESR1, these experiments do not yield the full picture of how these MG-associated miRNAs become dysregulated in MG.

While the data presented here allow us to postulate on which transcription factors are responsible for driving the expression of AChR+ and MuSK+ MG-associated miRNAs, what would be quite interesting in future studies is to derive macrophage and T-cells from AChR+ and MuSK+ patients and perform similar treatments, as mentioned above. Further, we could perform RNA-seq in comparison to healthy controls to look at potential upregulation of specific transcription factors. In follow up studies, such experiments will help us to determine which transcription factors would be the best therapeutic targets for AChR+ and MuSK+ MG.

There are distinct sex-associated differences in the expression of tissue-specific antigens that are controlled by the autoimmune regulator, a key factor for central tolerance in the autoimmune response. A recent study showed that in females, estrogen induces epigenetic changes in the autoimmune regulator (AIRE) gene, causing reduced AIRE expression below a threshold that increases female susceptibility to autoimmune diseases and in particular MG (37). In addition, worsening in female MG patients have been described in particular phases of the normal menstrual cycle, commonly (up to 50%) with symptoms being at their worst just before the next menstrual flow (38), when both progesterone and estrogen levels are the lowest. Previous studies indicate that 17ß-estradiol participate in the tolerization process by decreasing the expression of α-AChR and HLA-DR proteins and increases the expression of type I interferon and related molecules in thymic epithelial cells (37). With all the aforementioned effects of 17-β-estradiol on different aspects in MG in mind, the finding that ESR1 could regulate miR-21-5p, let-7a, and let-7f through binding sites is intriguing. This is in line with the higher levels of miR-21-5p in female immunosuppressive naïve MG patients compared to their male counterparts. However, we did not find a direct correlation between serum miR-21-5p and estradiol levels in female healthy controls or female MG patients without immunosuppression, indicating that other factors may influence the measurable circulating levels. Further, since 17-β-estradiol in women fluctuates during different phases of the menstrual cycle (39) and we did not sample the women during only one phase so this makes the interpretation of 17-β-estradiol levels more difficult.

In conclusion, the findings of our study highlight the connection between the MG-associated miRNAs and signaling pathways that govern inflammatory events and risk factors known to be important at least in AChR+ MG. This supports the important role of having biomarkers, such as miRNAs, that also are connected to the autoimmune events underlying the disease.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Review Board of Uppsala University (Dnr 2010/446). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF, CH, TP, and AP contributed conception and design of the study. AF, CH, Y-FH, and CT performed experiments. AF and AP performed statistical analysis. AP and AF wrote the first draft of the manuscript. CH, Y-FH, and TP wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00151/full#supplementary-material

Supplementary Figure 1 | Human T-cell line KE-37 was treated for 24, 48, and 72 h, respectively, with ß-estradiol at 0 nM (control group), 5 and 50 nM. The relative expression of miR-21-5p (%) compared to the control group is displayed on the y-axis.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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