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CONTRACTING ORGANIZATION: Emory University

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14. ABSTRACT

The goal of this project is to investigate the role of circular RNA of *utrophin* genes, which likely explains the difference of extraocular muscle phenotypes between two *dystrophin/utrophin* dKO mice and as the sparing mechanism of extraocular muscles in Duchenne Muscular Dystrophy (DMD). We identified 3 species of circular *utrophin* RNAs in extraocular muscles. We examined the role of RNA-binding proteins, such as TIA-1 and TIAR, to stabilize the circular *utrophin* RNA containing Exon 7 (Exon 4-8 junction) using si-RNA transfection. We established the protocol of extraocular myofiber isolation to test membrane fragility and response to calcium overload, which explains the spared muscle membrane property. To mimic the increased expression of circular-utrophin RNAs in extraocular muscles, we developed a plasmid to overexpress circular-*utrophin* Ex 7. Lastly, we confirmed the overexpression of circular-*utrophin* Ex7 in myoblasts *in vitro* and in FDB muscles *in vivo* using electroporation.

15. SUBJECT TERMS
Duchenne Muscular Dystrophy, DMD, Extraocular muscles, sparing from DMD, circular RNA, utrophin

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1. INTRODUCTION:

Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disease with muscle degeneration due to lack of muscle membrane scaffolding protein, dystrophin. While proximal limb muscles are mainly affected by DMD, extraocular muscles are spare from DMD. Although mechanism is unclear, mostly accepted hypothesis is the compensation of dystrophin by utrophin, which is highly expressed in extraocular muscles. We found the different susceptibility of EOM from two different dystrophin/utrophin double knockout (dKO) mice. We investigate the role of circular RNA of utrophin genes, which likely explains the difference of extraocular muscle phenotypes between two dystrophin/utrophin dKO mice and as the sparing mechanism of extraocular muscles in DMD.

2. KEYWORDS:

Duchenne Muscular Dystrophy, Sparing Mechanism, extraocular muscles, circular RNA, Utrophin

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: To define the role of circ-*utrophin (Utrn)* Exon 7 (Ex7) in extraocular muscle (EOM) sparing. (30% completed)

Major Task 1: Profile miRNA and RBPs binding with circ-*Utrn* Ex7. (2/6 subtasks completed)

- Subtask 1: Submit documents for Emory IACUC and ACURO approval (Completed)
- Subtask 2: Profiling of differentially expressed circular-Utrophin RNA in extraocular muscle (EOM) compared to gastrocnemius (GA) muscles by RNA-seq. (Completed)
- Subtask 3: Identification of network between microRNAs (miRNAs) and RNA-binding proteins (RBPs) including AGO2 with circ-*Utrn* Ex7 RNAs. (30% completed)
- Subtask 4: Generating EOM and GA myogenic progenitor cells (MPCs) and circ-*Utrn* Ex7 stability (half-life) assay (20% completed)

Specific Aim 2: To verify the role of circ-*Utrophin* RNAs for DMD-spared muscles. (30%)

Major Task 2: Confirmation of membrane integrity and calcium injury resistance of *mdx/utrn* dKO EOM (20% completed)

- Subtask 1: Isolation of EOM fiber and perform laser injury/calcium imaging (20% completed)
- Subtask 2: Perform immunostaining of dystroglycan proteins, sarcospan, desmin, annexin A6 (20% completed)

Major Task 3: The role of circ-*Utrn* Ex7 in membrane stability and calcium overload (30% completed)

- Subtask 1: Generate circ-*Utrn* Ex7 plasmid and confirm their expression *in vivo* transfection to FDB muscle fibers (80% progress)

What was accomplished under these goals?

Major task 1- Subtask 2: We completed RNA-seq analysis of gastrocnemius (GA) and extraocular muscle (EOM) tissues to identify differential expression of circ-*Utrn* species in both muscles. We identified two species of circ- *Utrn* RNAs containing Ex 59 (Exon 59-62 junction and Exon 59-63 junction (**Figure 1**). Although we detected circ- *Utrn* containing Exon 7 (Exon 4-8 junction) using regular PCR and quantitative PCR (qPCR), Ex 4-8 junction containing Exon 7 has not been detected in bulk RNA-seq, may be due to ***possible over fragmentation of input RNAs during library preparation and limited abundance compared to other junction species.*** By qPCR, Ct value for circ- *Utrn* Exon 59-62 is around 27 in EOM but 31 in FDB muscles (foot muscles). Ct value for circ- *Utrn* Ex 4-8 is around 34 in EOM and not detected in FDB. Considering the higher FPKM value and lower Ct values of circ- *Utrn* Exon 59-62, circ- *Utrn* Exon 4-8 may be under the detection threshold of bulk RNA-seq.

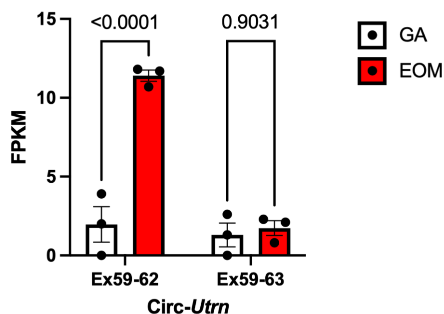

Figure 1. Comparison of Circ-*Utrn* Ex 59-62 and 59-63 junction levels in EOM and GA muscles. C57BL/6 male mice used for muscle sampling. N=3 for each group. Analyzed by two-way ANOVA. P-values are placed on top of graph.

Major task 1- Subtask 3: To elucidate the underlying mechanism of EOM-specific expression of cric-*Utrn* Ex7, we depleted candidate RBPs possibly interacting with circ-*Utrn* Ex4-8 RNAs. We confirmed the reduction of three RBPs (TIA-1, TIAR, HuR) after transfection of small interfering RNAs (si-RNAs) (**Figure 2A**) and change of circ- *Utrn* Ex4-8 levels. Thus, we conclude that TIA-1 and TIAR may affect the stability or production of circ- *Utrn* Ex4-8 (Ex7).

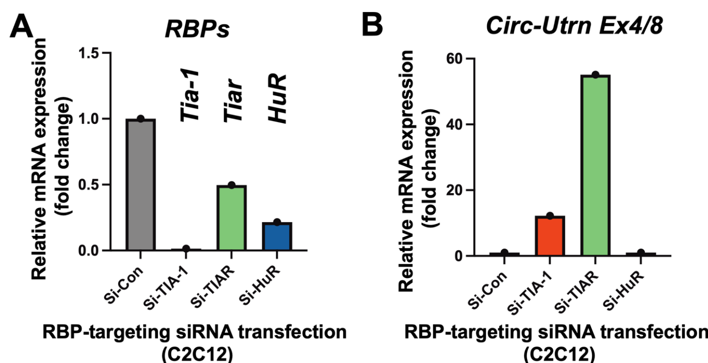

Figure 2. RNA-binding proteins influence abundance of circ-utrn RNAs. C2C12 myoblasts were transfected with the indicated si-RNAs (100nM) by electroporation (Neon). Two days after electroporation, cells were harvested in Trizol for RNA isolation. Quantitative PCR was performed to measure mRNA levels of target genes of each si-RNAs (*A*), Circ-*Utrn* Ex4/8 (*B*). *Hprt* mRNA was used as internal normalization control to calculate delta Ct values. Relative expression of each transcript was compared with Control siRNA- transfected cells.

Major task 1 - Subtask 4: We are isolating and proliferating myogenic progenitor cells from GAs and EOMs for circ-*Utrn* Ex4-8 RNA biogenesis and stability assays.

Major task 2 – Subtask 1: To investigate membrane fragility and calcium signaling of EOM fiber between two *mdx/utrn* double knockout mice, we isolated single EOM fibers as shown in **Figure 3**. While we had technical challenges due to the small size of EOM and heavy tendinous muscle attachments, we established a successful protocol for EOM isolation, which will provide samples for membrane laser injury and calcium imaging.

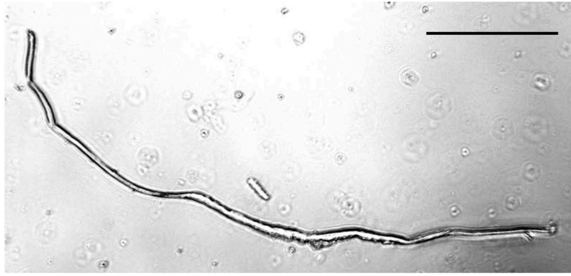

Figure 3. Isolated EOM fibers. We incubated EOM for 3 hours with collagenase type I and isolated single fibers. EOM produces muscle fibers with various lengths. Bar indicated 330μm.

Major task 2- Subtask 2: We are collecting EOM and FDB muscles for immunostaining. We expect that we will have enough samples (n=4 for each group) within 6 months.

Major task 2- Subtask 3: To investigate the role of cric-*Utrn* Ex4-8 in muscle membrane fragility and calcium signaling, we generated a plasmid overexpressing circ-*Utrn* Ex4-8. We confirmed the overexpression of circ- *Utrn* Ex4-8 in C2C12 myoblasts and FDB muscles by electroporation (**Figure 4A-B**). To isolate the circ- *Utrn* -overexpressing FDB for imaging, we co-transfected circ-*Utrn* Ex4-8 with GFP expression plasmid (**Figure 4C**). We are currently generating circ-*Utrn* Ex4-8 -overexpressing plasmid with co-expression of a fluorescent protein (mCherry protein) by using pTRE-Dual2 plasmid from Clonetech.

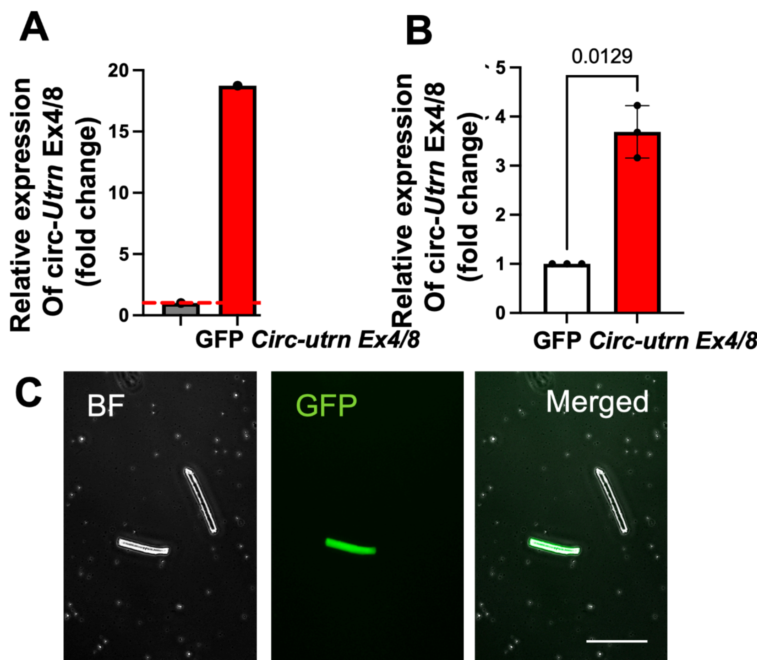

Figure 4. Overexpression of *circ-Utrn Ex4/8*. (A-B) Increased expression level of *circ-Utrn Ex4/8* measured by qPCR in C2C12 myoblasts (*A*) or FDB myofibers (*B*) transfected with *circ-Utrn Ex4/8* overexpressing plasmid (or GFP plasmid as control) using electroporation. (C) Isolated FDB myofibers after 4 days of electroporation of *circ-Utrn Ex4/8* with GFP. Transfection efficiency is about 30%. Scale bar indicated 330μm.

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

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

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

Task 1: Dr. Yoon's lab moved from MUSC to OU in the middle of the first reporting year, which significantly impacted the progress of task 1. In year 2, Yoon lab will focus on subtasks 3-6 to identify the mechanism of circ-*Utrn* Ex7/Ex 59 biogenesis and binding candidates. Choo lab already sent muscle tissues to Yoon Lab and is preparing MPCs to send soon.
Task 2: Dr. Choo's lab will perform subtasks 1-2 with isolated EOM/FDB fibers and muscle tissues.
Task 3: Choo lab will perform subtasks 2-3 with isolated FDB fibers after circ-*Utrn* plasmids transfection.

4. IMPACT:

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

Nothing to Report.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

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

Choo lab: 1. Technical difficulty of EOM isolation delayed the progress of task 2. After several trials, we established a successful protocol to isolate EOM.
2. Temporal poor breeding of Mdx/utrn colonies delayed the production of samples for RNA sequencing, qPCR, and immunostaining. We set up multiple breeders so the current breeders are feasible to generate enough number of samples for the above experiments.
Yoon lab (subcontract): Institution change in year 1 caused a delay in the progress of Major task 1. Although Yoon lab will produce diligently, we may request no-cost extension to complete the proposed research.

Changes that had a significant impact on expenditures

Yoon lab (subcontract): Institution change in year 1 caused a delay in the progress of Major task 1. Due to the change of institution and process of administration, the settlement of subaward was delayed. The remaining fund in year 1 will be used in year 2 to complete the proposed research.

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

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to Report.

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

Nothing to Report.

Other publications, conference papers and presentations.

Nothing to Report.

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

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

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

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

.

Name:	*Hyojung Choo – no change*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	
Funding Support:	
Name:	*Je-Hyun Yoon – no change*
Project Role:	*Co-investigator (subaward PI)*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	
Contribution to Project:	
Funding Support:	
Name:	*Xiaoxing Xu*
Project Role:	*Postdoctoral Researcher (Choo lab)*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-1561-1715*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Xu managed mdx/utrn mouse colonies and collected circ-Utrn muscle samples performed RNA isolation and qPCR of circ-Utrn RNAs in GA and EOM muscles.*
Funding Support:	

Name:	*Yufei Du*
Project Role:	*Undergraduate Student (Choo lab)*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Ms. Du performed RNA isolation and qPCR of circ-Utrn RNAs in GA and EOM muscles.*
Funding Support:	*NIH*

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

Choo lab: NIH R01 AR071397 is closed.

Nothing to Report

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: *N/A*

9. APPENDICES: *N/A*