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TITLE: Development of Orally Bioavailable Therapeutics by the Chloroplast Expression System to Counter Muscle Degeneration, Weakness, and Fibrosis in DMD

PRINCIPAL INVESTIGATOR: Elisabeth Barton

CONTRACTING ORGANIZATION: University of Florida
207 GRINTER HALL
GAINESVILLE FL 32611-0001

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Elisabeth Barton

E-Mail: erbarton@ufl.edu

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University of Florida
207 Grinter Hall, Gainesville,
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14. ABSTRACT

Patients with DMD suffer from progressive muscle weakness and damage, resulting in fibrotic replacement. The goal of this project is to evaluate the therapeutic potential of the anti-fibrotic agents, ACE2/Ang(1-7), when produced in plants using a chloroplast expression system. Lyophilized plant material was delivered by oral gavage to the mdx mouse model for DMD. Initial studies were done to ensure that the plant material and protein was orally bioavailable. Further, additional studies confirmed that ACE2 protein accumulated in the circulation over the course of the treatment. Functional assessment of mice treated for 2 weeks showed improved strength in the diaphragm and EDL muscles. However, by 2 months of treatment the benefits were reduced back to untreated controls. This lack of long-term benefit was not due to compound delivery, which accumulated in the circulation over the course of the study. Instead, we believe that the muscle tissue compensated by down regulating the receptors that are sensitive to the heightened Ang(1-7) levels. Even though this therapeutic agent did not provide benefit, we assert that this delivery strategy may provide a new way to introduce therapeutic proteins for treating neuromuscular disease.

15. SUBJECT TERMS
DMD, chloroplast expression system, renin-angiotensin pathway, fibrosis, skeletal muscle function.

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Table of Contents

Page

1. Introduction

The overarching goal of this proposal is to evaluate a novel delivery system as a strategy to treat DMD, using a promising anti-fibrotic therapy, Angiotensin Converting Enzyme 2 and its enzymatic product Angiotensin-(1-7) (ACE2/Ang-(1-7)). Specifically, we have tested chloroplast derived ACE2 and Ang-(1-7) in lettuce to evaluate each agent independently and in combination in the *mdx* mouse model for DMD. Our outcome measures include a standard battery of physiological and morphological assessments, including muscle function (force generation capacity, stiffness, and fragility), extent of fibrosis (Sirius red staining), and circulating and tissue ACE2 activity. This analysis will provide key pre-clinical data for both the use of Ace2/Ang(1-7) as a treatment for DMD, as well as the potential of this delivery strategy for additional proteins for muscle disease.

2. Keywords

Renin-angiotensin pathway
Angiotensin Converting Enzyme 2
Angiotensin-(1-7)
Fibrosis
Duchenne Muscular Dystrophy
Mdx
Skeletal muscle
Chloroplast expression
Oral bioavailability

3. Accomplishments

The major goals and accomplishments are listed below, organized by the Tasks in the Statement of Work. In some cases, representative data is shown to illustrate the results.

Task 1. Tobacco ACE2/Ang(1-7) activity validation (Daniell) (months 1-16):

1a. Plant transgenic tobacco seeds and grow (months 1-4, typical growing period; repeated 2 times).
1b. Harvest tobacco plants (month 4, 8, 12).
1c. Prepare leaf material for protein activity (months 4, 8, 12).
1d. Perform immunoblotting to protein quantification (months 1, 4, 8, 12).
1e. Perform ACE2 activity assay (months 1, 4, 8, 12).

Plant production and quantification of ACE2 and Ang(1-7) were completed by the Daniell lab. These materials were shipped to the Barton lab for evaluation in the UF mouse colony. Note that the production level of ACE2 by the plants is lower than the other proteins. In this plant line, there was no codon optimization, which impairs the efficiency of translation of mammalian proteins by plants. While additional transplastomic plants are under production in order to attempt to boost ACE2 levels, these were not evaluated in the current study.

Leaf Sample	Concentration (ug/mg)	Dry weight (g)
CTB-ACE2	0.2	5.24
CTB-Ang(1-7)	8.64	4.97
CTB-GFP	5.6	1.13

Figure 1. Quantification of therapeutic protein per mg plant material by immunoblotting.

Task 2. ACE2/Ang(1-7) Pharmacokinetics (Daniell/Barton) (months 1-2):

2a. Oral dosing of C57 mice for muscle biodistribution (N=64) (months 1-2).

2b. Protein quantification in muscles and blood (months 1-2).

Confirmation of Circulating ACE2 after feeding was performed using a fluorescent reporter for ACE2 enzymatic activity. Shown in Figure 2 is the comparison of rates between N=3 fed mice and 1 naïve control 5 hours post gavage. Fed ACE2 activity levels were more than 2-fold higher than endogenous levels with 1 oral gavage of 25 mg plant material. We next measured the changes in circulating ACE2 activity over the course of 2 months treatment in mature Cmdx mice. Blood was obtained at trough (24 hours after last dosing and immediately before subsequent dose). As shown in Figure 3, there was an accumulation of ACE2 in the circulation out to 6 weeks when using repeated measures on the same mice. This provides confidence that the oral gavage procedures work well, and that oral bioavailability of the plant derived proteins occurs. The same assays were performed on those mice tested for therapeutic efficacy detailed in Task 5.

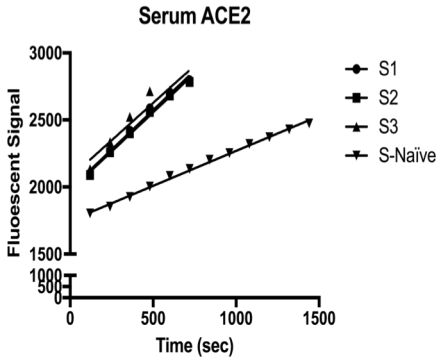

Figure 2. *Fluorescent based ACE2 activity assay. Average slope of test lines was 1.15 Fl/sec vs Naïve sample at 0.521 Fl/sec.*

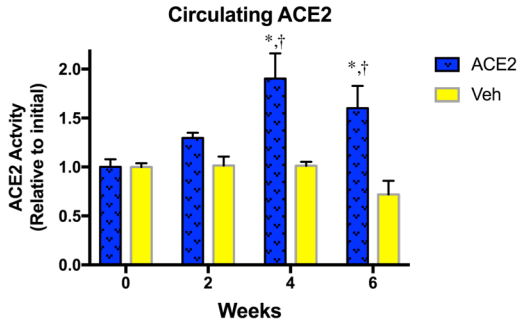

Figure 3. *Cumulative ACE2 activity relative to initial for N=6 treated and N=4 control muscle. *, P<0.05 vs initial for the same animal. †, P<0.05 vs Vehicle (Veh) for the same timepoint.*

Biodistribution studies were performed using GFP reporter plants by the Daniell lab and published (Xiao et al, 2016). While this was not the direct task, and was supported by related funds in the Daniell lab, the study demonstrated nicely that plant-based proteins could enter the circulation and also enter skeletal muscle (Figure 4). This was an important step forward in the project. However, it is clear that muscle uptake is low compared to other tissues, presumably due to the relatively low proportion of the circulation in resting muscle. Also note that the current study uses plants with the CTB extension. Interestingly PTD-GFP appears to have better exposure in muscle. The Daniell lab is working on re-designing their protein constructs to incorporate this new extension. While outside of the scope of this project, future studies are likely to incorporate the newly designed plant expression systems.

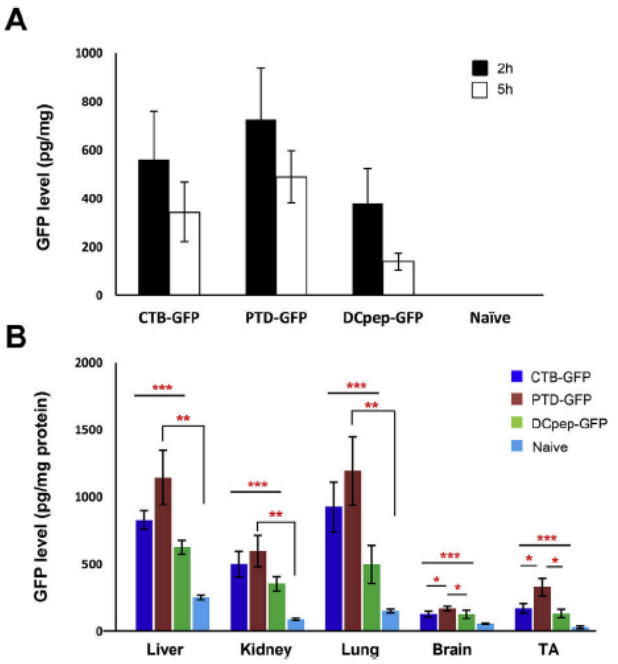

Figure 4. *Efficiency of oral delivery and biodistribution of GFP fused with different tags. Serum (A) and tissue (B) GFP levels in mice (N ¼ 6 per group) fed leaf materials expressing CTB-GFP, PTD-GFP and DCpep-GFP. Adult mice were orally fed with leaf materials from transgenic tobacco plants, with the amount adjusted to GFP expression levels, for three consecutive days. A control group (N ¼ 6) kept unfed. Blood samples were collected at 2 and 5 h after last gavage at which, mice were sacrificed and tissue samples were collected for protein isolation. GFP concentration in serum and tissues were measured with ELISA. The data was shown as average ± SEM. Statistic significance was determined by a paired Student's t test, and p value less than 0.05 were considered significant. *P < 0.05, **P < 0.01, ***P < 0.05 or P < 0.01 (CTD, PTD and DCpep versus Naïve). (From Xiao et al, Biomaterials 80 (2016) 68e79).*

Task 3. Breeding *Cmdx* and C57 mice for colony maintenance and expansion (Barton) (months 1-30)

3a. Purchase mice (month 1).
3b. Breed mice for older age group (month 2-4).
3c. Breed mice for younger age group (month 2-3; 5-6).

Mice were purchased from Jackson Labs to establish a breeding colony of Cmdx and strain matched controls. Mice were bred at UF and at Penn (the PIs former institution) to generate 6 month old mice for treatment groups. Mice were bred at UF generate 1 month old mice for treatment groups. Only male mice were used for the treatment groups. To date a total of 276 Cmdx mice and 184 C57Bl10 mice (M and F for both strains) were generated at UF. Active breeding was tapered to only maintenance in case the need arises to generate more mice for specific conditions in the final stages of the study.

Task 4. Oral gavage of ACE2/Ang(1-7) in dystrophic mice (Barton) (months 3-14)

4a. Dose Ang(1-7) into young mice (N=36) (months 3-9)
4b. Dose ACE2 into young mice (N=36) (months 4-10)
4c. Dose ACE2/Ang(1-7) into young mice (N=36) (months 5-11)
4d. Dose Ang(1-7) into mature mice (N=36) (months 6-12)
4e. Dose ACE2 into mature mice (N=36) (months 7-13)
4f. Dose ACE2/Ang(1-7) into mature mice (N=36) (months 8-14)

Oral gavage studies have been performed on 180 mice, and an additional 36 mice have been used as untreated controls. These cohorts include both the Cmdx mice and wildtype controls (C57Bl10). Control mice received oral gavage of plants containing GFP as a vehicle, and to account for any stress due to the oral gavage procedures. A total of 12 mice died or were required to be euthanized during the treatment periods. This was due to significant weight loss associated with the phenotype, which is incorporated into our humane endpoints criteria.

Task 5. Evaluation of ACE2/Ang(1-7) in dystrophic mice (Barton/Daniell) (months 4-15)

5a. Test Ang(1-7) treated and control young mice (months 4-10)
5b. Test ACE2 treated and control young mice (months 5-11)
5c. Test ACE2/Ang(1-7) treated and control young mice (months 6-12)
5d. Test Ang(1-7) treated and control mature mice (months 7-13)
5e. Test ACE2 treated and control mature mice (months 8-14)
5f. Test ACE2/Ang(1-7) treated and control mature mice (months 9-15)

In order to evaluate the efficacy of treatment, mice were subjected to isolated muscle function testing, with focus on the EDL and diaphragm. With short term treatment, we examined a treatment period of 2 weeks with young adult mice and with 6 month old mice. The primary outcome measure for these studies is muscle strength in the EDL and diaphragms.

In young mice, treatment with ACE2 resulted in an 11-15% increase in muscle strength (specific force) in the EDL muscles of both WT and Cmdx mice (Figure 5). As specific force changes can arise from either cross-sectional area and/or absolute force production, both were assessed. There were no changes in cross sectional area by ACE2 treatment, and the improved function arose from increased force production. Even with these improvements in limb muscle function, the significant deficit in diaphragm function was not improved in Cmdx diaphragms following treatment.

When the same duration treatment was performed in
in 6 month old Cmdx animals, the results differed. Mice were fed daily with ACE2 treatment regimen to determine if any acute functional benefit was achieved. While no significant improvement was observed in EDL muscles, the diaphragms exhibited a significant increase in specific force (Figure 6). Thus, there was an age-dependent effect of 2 week ACE2 delivery on muscle specific improvements.

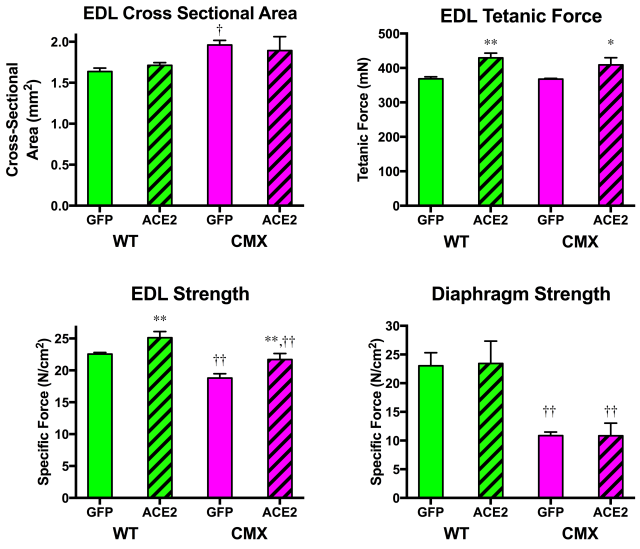

Figure 5. *2 week Treatment of 1.5 month old male WT and Cmdx mice with ACE2 plant. Compared to age matched vehicle treated Cmdx controls, there was a significant increase in EDL tetanic force and specific force. EDL cross sectional area is higher in untreated Cmdx mice, which arises from the compensatory hypertrophy from the disease phenotype. The diaphragms muscles did not exhibit any treatment dependent effects. *, p<.05,**, p<.01 vs. vehicle control; †, p<.05, ††, p<.01 vs WT within same treatment group. 2-way ANOVA followed by Sidak's comparison.*

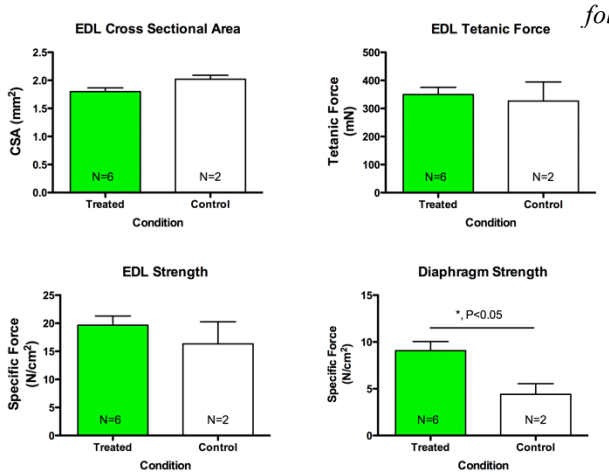

Figure 6. *2 week Treatment of 6 month old male Cmdx mice with ACE2 plant. Compared to age matched vehicle treated Cmdx controls, there was a significant increase in diaphragm specific force. The EDL muscles did not exhibit any treatment dependent effects, with no change in cross sectional area, tetanic, or specific force. *, significant difference by unpaired t-test.*

A continuation of this study design included a 2 month treatment arm for comparison. However, unlike the benefit observed after short term exposure, there was no significant difference between treated and control muscles (Figure 7). This suggests that there is a negative feedback response to ACE2 treatment over tine, even though it has built up in the circulation (Figure 3).

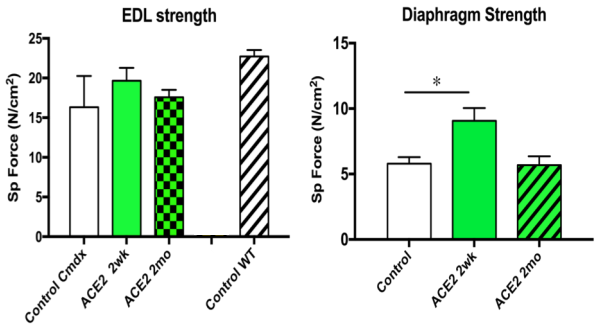

***Figure** 7. 2 month Treatment of 6 month old male Cmdx mice with ACE2 plant. No functional benefit was observed following treatment. 2 wk treatment results are shown for comparison.*

Rather than delivery the ACE2 enzymes, we delivered the desired product of the reaction, Ang(1-7). We tested young animals, starting at 4 weeks of age, using Ang(1-7) alone, or a combined treatment. Following 2 months of treatment with Ang(1-7), there was no change in force production in either the diaphragm or the EDL muscles, and strength remained significantly lower than WT (C57) muscles. In order to determine if this was a limitation of the oral bioavailability or an issue with the effects of Ang(1-7), we injected cohorts of WT and Cmdx mice with a recombinant adeno-associated virus (AAV) expressing Ang(1-7) regulated by a liver specific promoter. We have used this strategy to produce myostatin related proteins from the liver effectively (Morine et al, 2010 PLoS One. 5(2):e9176). Regardless of the delivery strategy, no benefit of increased Ang(1-7) was observed in the EDL or diaphragms from the Cmdx mice (Figure 8).

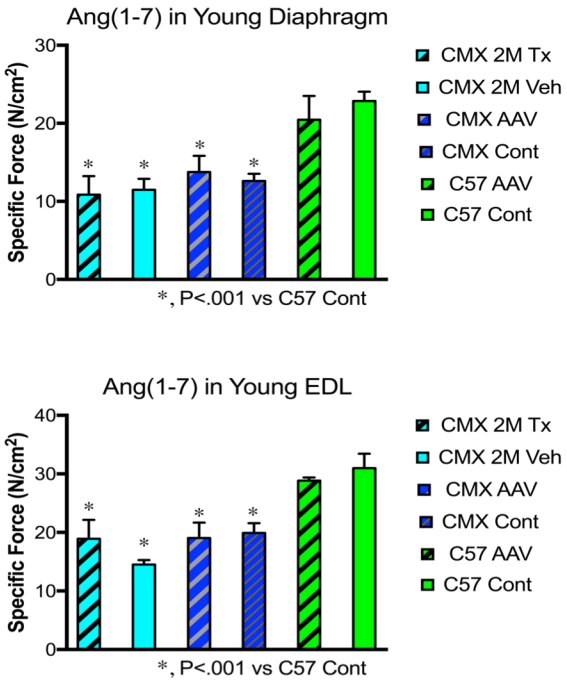

***Figure 8**. 2 month Treatment of 1 month old male Cmdx mice with Ang(1-7) delivered orally by plants or through injection of AAV. No functional benefit was observed following treatment. Statistical comparisons were performed by 1 way ANOVA followed by Tukey post-hoc testing.*

To determine if ACE2 and Ang(1-7) combined could provide functional benefit, we treated 1 month old Cmdx mice for 1 or 2 months with both plants. For the 1 month group, we delivered plant material 5 times per week. For the 2 month group, we delivered plant material 3 times per week. The rational for the lower frequency was to minimize the stress of daily oral gavaging, especially with the young animals. As shown in Figure 9, the results were similar to that of Ang(1-7) alone for this duration of treatment. In sum, there was no perceivable benefit of combined ACE2/Ang(1-7) treatment with regard to strength in EDL or diaphragm muscles.

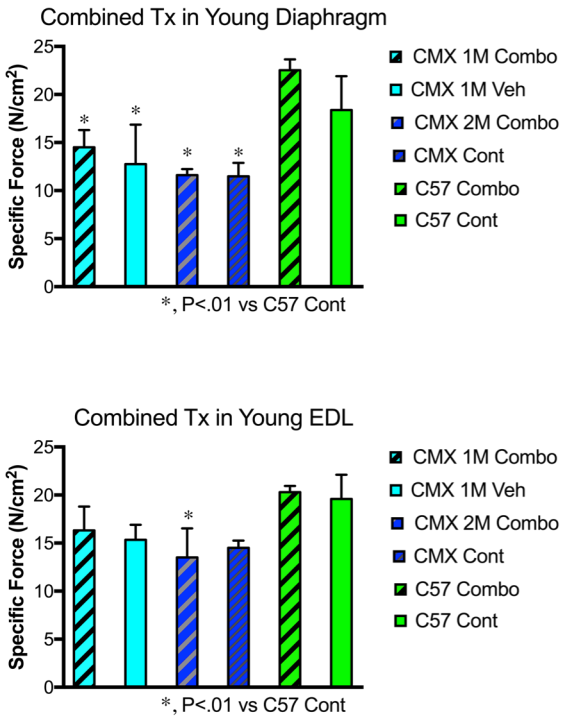

Figure 9. *1 and 2 month Treatment of 1 month old male Cmdx mice with ACE2/ Ang(1-7) delivered orally by plants. No functional benefit was observed following treatment. Statistical comparisons were performed by 1 way ANOVA followed by Tukey post-hoc testing.*

Finally, we returned to 6 month old mice, testing the efficacy of combined treatment for 2 months, using feeding 5 times per week. The rationale was to see if the delivery of both the enzyme and the product would help regain the initial observed short-term benefit at this age. As shown in Figure 10, the results were similar to all other longer term trials: there was no improvement in force production following 2 months of treatment in 6 months old mice. Thus, in summary, both in young and mature mice, functional benefit in driving Ang(1-7) production either indirectly by delivery of enzyme, or directly by delivery of the product, occurs with short term exposure. However, by 1 month, there is no longer any benefit, suggesting a negative feedback response to increased Ang(1-7) availability.

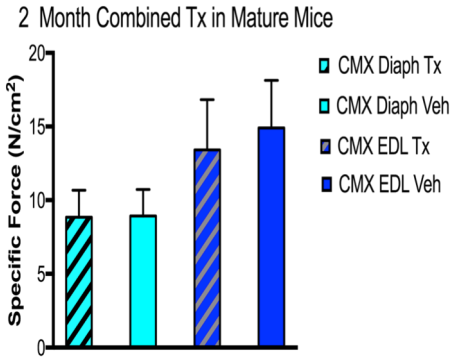

Figure 10. *2 month Treatment of 6 month old male Cmdx mice with ACE2/ Ang(1-7) delivered orally by plants. No functional benefit was observed following treatment. Statistical comparisons were performed by 2-tailed T tests.*

To determine if there was a blockade in muscle delivery of ACE2, or a compensatory decrease in its levels upon drug delivery, ACE2 activity was measured in the serum and in the muscle of control and treated mice (Figure 11). While not definitive as of yet, ACE2 activity increases in serum following feeding the chloroplast ACE2, but decreases in the muscle. It is not clear if this is a decrease in endogenous expression of Ace2 in response to the higher circulating levels, or if there is a reduction of uptake from the circulation.

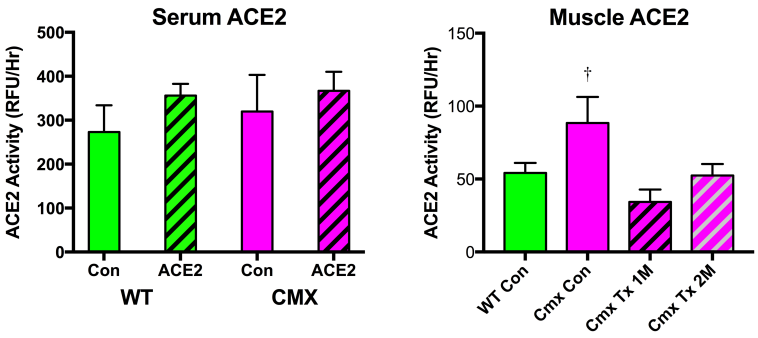

Figure 11. *Circulating ACE2 in WT animals increases with 2 month ACE2 treatment, and there is a trend for increase in the Cmdx mice. Muscle ACE2 activity is higher in control Cmndx mice than WT controls, but activity decreases at both 1 and 2 months of ACE2 treatment.*

As it stands, the study has shown that the chloroplast expression platform can effectively deliver proteins to the circulation. It has also confirmed that bolstering ACE2 levels can have short term beneficial actions on muscle function. However, in longer erm studies, the endogenous feedback systems likely react to these high levels, and shut down the efficacy of the pathway.

As there were no proposed goals for training and professional development, we have nothing to report.

Task 6. Manuscripts and proposals (Barton/Daniell) (months 12-15)

6a. Prepare manuscript on ACE2/Ang(1-7) delivery (months 12-15).

6b. Prepare proposal for continuation of original project (12-15).

Initial findings were presented by the PI at New Directions in Muscle Biology and Disease meeting in Orlando, FL June 2016 as an oral presentation, and at the European Muscle Conference in Montpellier, France in September 2016. The PI also presented the concept of chloroplast expression system and drug delivery to the US Anti-Doping Association (USADA), and the World Anti-Doping Association (WADA). A second poster presentation including the ACE2 activity findings and the comparison of short- and long-term treatments is slated for June 2018 at the New Directions in Muscle Biology and Disease meeting in New Orleans, LA. The findings are also being prepared in a manuscript for consideration of publication in calendar year 2018.

4. Impact

Development of the principal discipline(s) of the project.
We have optimized delivery of plant derived proteins to mice to ensure that there is exposure to the therapeutic proteins under evaluation.
We have recognized that chloroplast expression of human proteins requires codon optimization in order to produce the proteins efficiently. The Daniell lab is working on new algorithms for codon optimization, and this effort will be used to re-engineer the ACE2 plant lines in increase protein yields.

Impact on other disciplines.
The delivery system is currently being evaluated in models for cardio-pulmonary dysfunction, diabetes, and hemophilia. For muscle, there needs to be additional optimization in order to enhance uptake by muscle tissue, compared to other more highly vascularized tissues.

Impact on technology transfer.
All of the reagents are under patent protection through either the Daniell or Barton labs. The Daniell lab has entered negotiations with Novo Nordisk on other chloroplast expression projects.

Impact on society beyond science and technology?
The delivery system could have impact in a broader sense, as indicated by the interest form the Anti-doping commissions. If this provides a strategy to bolster performance enhancement, then their regulatory oversight will have to include guidelines for detection.

5. Changes/Problems

Two problems emerged during the funding support. First, there were significant delays in transferring funds from University of Pennsylvania to University of Florida, which caused an approximately 1 year hiatus in progressing to achieve the aims of the proposal. The second problem arises from the possibility that the therapeutic proteins under investigation may not be effective in preventing fibrosis in this animal model. This is, in part, the goal of the grant, and so while the initial results presented here may be negative, there is more to be done to understand why this is the case. Both technical and biological limitations are being probed as underlying causes of these findings, and are being pursued under separate funding initiatives. .

6. Products

Conference presentations:

E.R. Barton. “Development of Orally Bioavailable Therapeutics by Chloroplast Expression Counters Muscle Weakness in DMD.” Oral presentation at New Directions in Biology and Disease of Skeletal Muscle Conference Orlando, FL June 29- July 2, 2016. International meeting.

E.R. Barton. “Development of Orally Bioavailable Therapeutics by Chloroplast Expression Counters Muscle Weakness in DMD” Poster presentation at European Muscle Conference, Montpellier, France, September 2-6, 2016. International meeting.

E.R. Barton. “IGF-I: a mediator of muscle growth and repair”. 15th Annual USADA Symposium on Anti-Doping Science 30 Sept – 3 Oct 2016 Bellevue, Washington, USA. International Meeting.

E.R. Barton. “Chloroplast expression as a drug delivery system”. WADA Annual Meeting, Feb 3, 2017, Montreal, CANADA.

7. Participants & Other Collaborating Organizations

Name	Role	Person Month	Role in Project	Funding Support
Elisabeth Barton	PI	2	Oversee Scientific progress, study design, data analysis and interpretation	DOD, and others
Henry Daniell	Co-Investigator	1	Oversee plant production and quantification	DOD, and others
Hanqin Lei	Research Technician	4	Enzyme Activity, molecular measurements	DOD and other (Barton Lab)
Yuhong Xiao	Research Associate	4	Plant production and quantification	Other (Daniell lab)
Kwang-Chul Kwon	Research Associate	2	Plant production and quantification	Other (Daniell lab)
Ryan Meyer	Undergraduate student	1	animal feeding and handling	Non, research intern
Ray Spradlin	Graduate student	1	animal feeding and handling	Graduate stipend, UF
Chih-Hsuan Chou	Graduate student	2	Enzyme Activity	Graduate Fellowship UF
Michael Matheny	Research Technician	2	muscle function measurements	NIH, part of Muscle core directed by Barton
Jason Puglise	Research Technician	2	muscle function measurements	NIH, part of Muscle core directed by Barton

8. Special Reporting Requirements

NA

9. Appendices

NA