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14. ABSTRACT
The goal of this project is to test three classes of compounds in animal models of muscular dystrophy, and evaluate their therapeutic potential in preventing fibrofatty infiltration. Animals and drugs required for the project have been procured. A change has been made to the kinase inhibitor compound to be tested in animal models of disease, as a more efficacious drug was identified with similar substrate specificity.

15. SUBJECT TERMS
Fibrofatty infiltration, drug testing, muscular dystrophy, fibrosis.

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1) INTRODUCTION: In our aging society, degenerative complications of chronic diseases are on the rise and account for a significant percentage of deaths. Among these, fibrosis is the most common, and yet no therapy capable of mitigating its effects is available. Investigating and understanding the signaling pathways that influence fibrogenic progenitor fate will not only elucidate a key component of the regenerative process but may reveal pathways that could be targeted therapeutically to prevent inflammation, fibrosis, and promote regeneration or maintain muscle homeostasis.

Acute tissue injury generates transient inflammation and extra cellular matrix (ECM) deposition, which disappears after the regenerative process is complete. However, under certain pathogenic conditions, persistent damage and inflammation within the tissue generate excessive and chronic deposition of ECM components. This condition is known as fibrosis [1].[2] Fibrosis reduces the amount of healthy tissue regeneration and contributes to organ malfunction in different pathologies such as liver and kidney diseases, idiopathic pulmonary fibrosis, heart failure, and muscular dystrophies. Even though fibrosis contributes to 45% of mortality in developed countries, the mechanisms regulating fibrogenesis initiation and establishment have not yet been completely elucidated [3,4]. In spite of an extensive understanding of fibrogenesis in response to injury, no effective anti-fibrotic therapies are available. Therefore, a better understanding of the cellular effectors and the molecular signals regulating this pathological condition is required.

In skeletal muscle, an organ with a high regeneration potential, fibrosis is a hallmark of severe muscular dystrophies, including the incurable Duchenne muscular dystrophy (DMD), where the lack of dystrophin protein leads to impaired cycles of degeneration-regeneration, and therefore, chronic degeneration of the affected myofibers[5].

At the cellular level, fibroblasts are arguably the most important mediators of tissue fibrosis. In the past years, tissue-resident multipotent mesenchymal progenitors have been described as precursors of fibroblasts. They are named fibro/adipogenic progenitors (FAPs), based on their spontaneous potential to differentiate into myofibroblasts and adipocytes, both *in vivo* and in *vitro*[6-8]. FAPs are also the source of ectopic osteogenesis[9,10]. FAP activation and expansion is induced by damage and highly modulated by inflammatory signals such as Interleukin-4 and 15 (IL-4, IL-15), Tumor Necrosis Factor α, and Transforming Growth Factorα [11-15]. Following acute damage, activated FAPs provide trophic support to satellite cells, which are required for efficient normal regeneration [16,17]. However, the proper tissue clearance of FAPs is defective in degenerative condition such as in chronically damaged muscles of DMD patients. This, primed the cells to differentiate towards both differentiation in adipocytes and fibroblasts, which in turn in fibrofatty infiltration [8,11,18]. Due to their dual role in skeletal muscle regeneration, FAPs seem to be the ideal cellular target to improve regeneration and prevent fibrofatty deposition. Thus, interventions on molecular pathways involved in their activation and/or differentiation are an attractive strategy to successfully combat fibrotic diseases.

Inhibition of TGFβ and PDGFR pathways during skeletal muscle regeneration was shown to reduce FAP number and down-regulate collagen1 deposition, a hallmark of fibrosis

[13,19,20]. TGFβ plays an important role in tissue modeling and remodeling and therefore, it is considered a master molecule in the initiation and establishment of fibrosis. The canonical TGFβ pathway classically transmits extracellular signals via transmembrane serine/threonine kinase receptors and intracellularly via Smads2/3/4 proteins. The non-canonical TGFβ pathways include a variety of intracellular cascades activated by TGFβ independent of Smad2/3/4. These include the molecules TGF-activated kinase 1 (TAK1), p38 mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERK), JUN N-terminal kinase (JNK), and nuclear factor kappa-light chain enhancer of activated B cell (NFkB), among others[21]. Therapeutic manipulation of the canonical, as well as the non-canonical TGF pathway, had was shown to be beneficial in multiple myopathic states and fibrosis of various tissues[22] .

We took advantage of the Collagen1a1*3.6 EGFP mice [23] to isolate, culture, and analyze FAP differentiation into fibroblast, by following the expression GFP as a reporter [24,25]. Also, we performed a drug screening in freshly isolated cells, which allowed us to investigate the role of key molecules in regulating FAP's fibroblastic differentiation.

The goal of this project is to test three different classes of compounds, stemming from a screen for molecules capable of inhibiting the fibrogenic differentiation of mesenchymal progenitors, in a mouse model of Duchenne's muscular dystrophy and thus assess their therapeutic efficiency.

2) KEYWORDS: Duchenne's muscular dystrophy, fibrosis, bromodomain inhibitors, kinase inhibitors, NFkB inhibitors.

ACCOMPLISHMENTS: What were the major goals of the project?

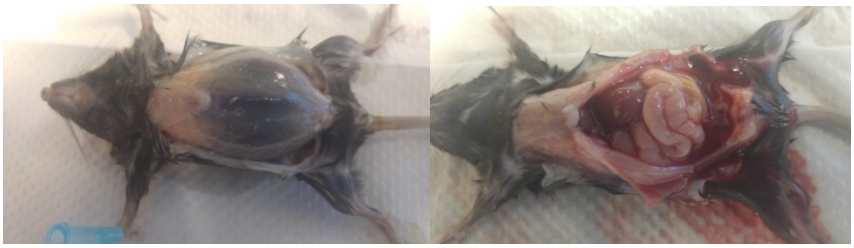
a) There were two major goals of the project:

1) **Testing of candidate compounds in animal models of Duchenne's muscular dystrophy.** This goal has been achieved, and the results are included.
2) **Analyze effects of treatment on FAPs. A secondary goal was to identify theranostic markers**
   This goal is at advanced stages of completion, and its finalization has been affected by the ongoing ban on research activities.

What was accomplished under these goals?

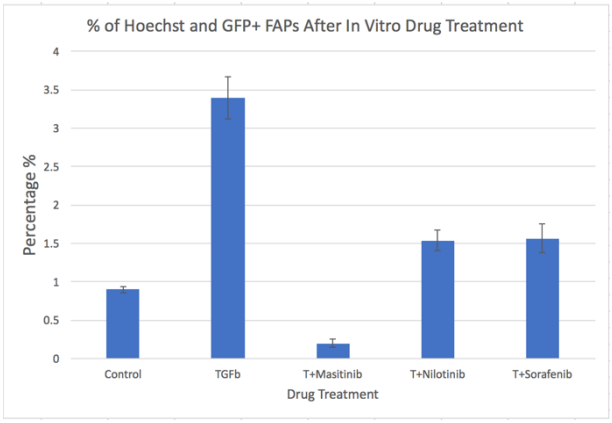
Testing of candidate compounds in animal models of Duchenne's muscular dystrophy.

The first compound proposed for use in the original application was a synthetic mimetic compound derived from the natural compound withaferin, which showed good activity in dampening the expression of fibrogenic genes in the relevant progenitors in response to TGFβ. Unfortunately, preliminary testing of withaferin in vivo led to severe side effects, and multiple animals injected i.p. with this compound met humane endpoints as defined in our animal protocol and had to be euthanized. Upon necroscopy, we found multiple peritoneal adhesions and, in the majority of cases, evidence of profuse peritoneal bleeding (see images below). No such event was observed in vehicle treated animals.



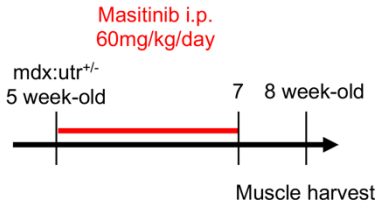
Based on these results, work on this compound has been stopped.

This compound has been replaced with a new candidate, the kinase inhibitor masitinib, that showed excellent ability to inhibit collagen expression in fibrogenic progenitors in vivo. These are represented in the figure below, showing the percentage of cells positive for green fluorescent protein driven by a collagen 1 enhancer. Notice that Masitinib appear more powerful than Nilotinib or Sorafenib in this setting.

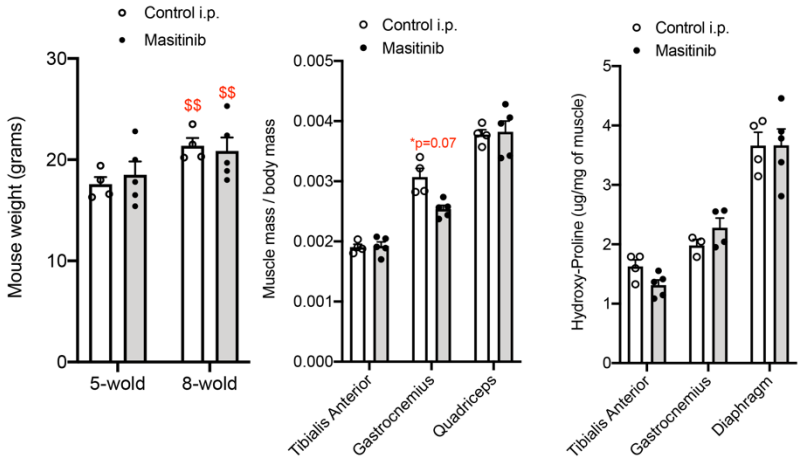


The mdx mouse is the most widely used murine model of DMD, however, it does not completely follow the fibro-fatty progression observed in humans. Indeed, mdx mice show fewer fibrosis later in life and primarily in the diaphragm muscle (Ardite et al., 2012). Other murine models, such as the mdx:utr$^{+/-}$ used above, which lacks one allele of

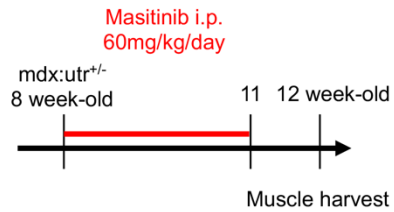
utrophin, a functional analog of dystrophin, have been proposed as a better alternative to mdx for shorter experiments [26,27]. Indeed, mdx:utr$^{+/-}$ mice develop fibrosis earlier after the disease onset (8 weeks), even in the limb musculature. Therefore, this mouse model follows the pathology closer when compared to mdx mice [28,29].Masitinib was injected intraperitoneally every day for two weeks in mdx:utr$^{+/-}$ mice at 60 mg/kg/day.



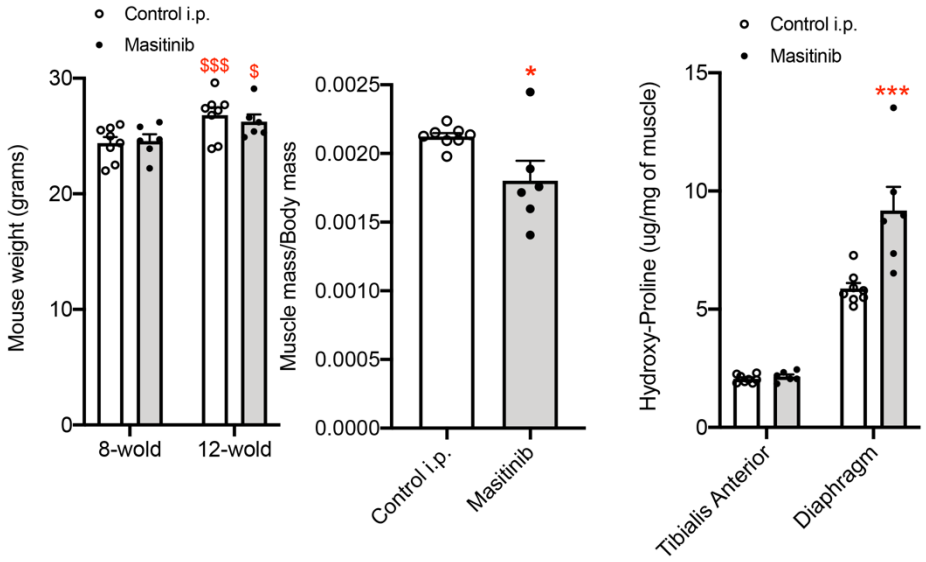
No differences in weight, muscle mass or hydroxyproline content was noticed.



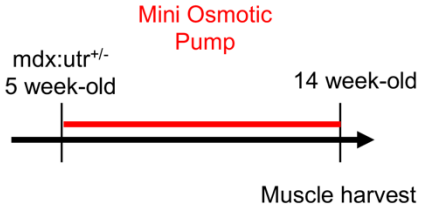
However, it is known that the mdx:utr$^{+/-}$ (as well as mdx) model displays acute phase or inflammation/regeneration at an early stage (4-5 weeks). Thus, it is possible that the effect of Masitinib could be inefficient due to inflammation happening in the muscles. To counteract this, Masitinib injections were performed from 8 to 11-week-old with the same concentration.



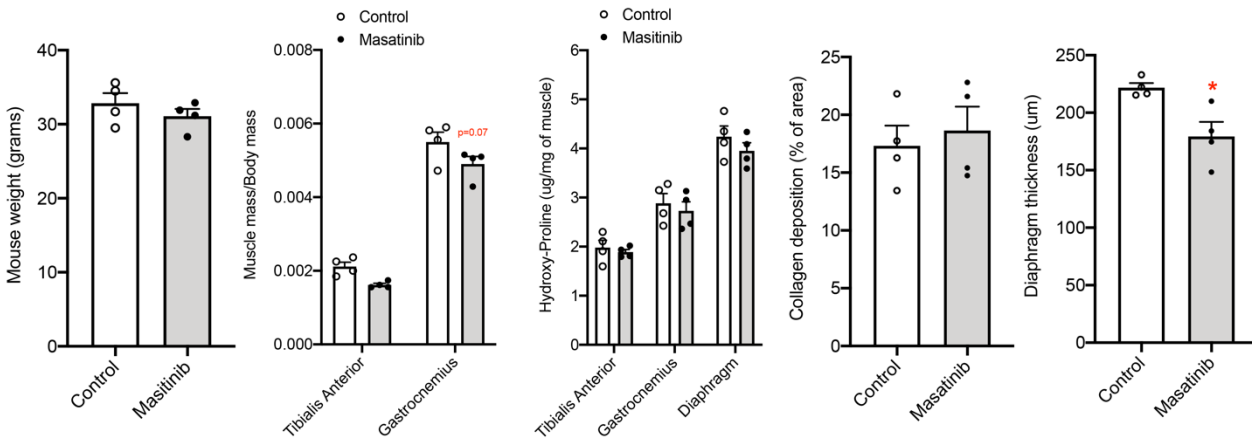
However, while body mass was not affected, we noticed a decrease in tibialis anterior muscle mass (% and $p<0.05$), as well as an increase in fibrosis content in the diaphragm (+% $p<0.001$).



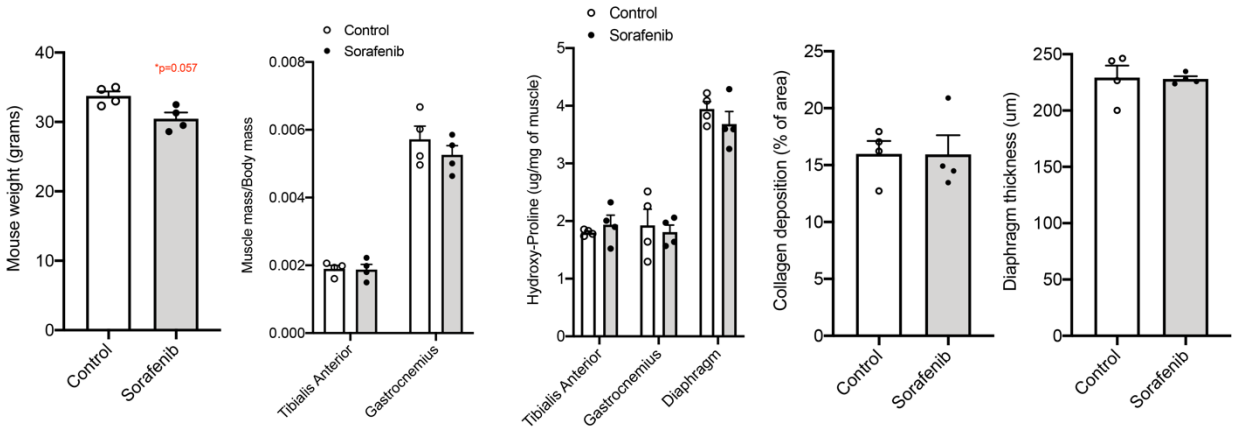
Finally, to determine whether the results we observed were due to the method of drug administration or the drugs themselves, we used an osmotic pump to deliver masitinib for 9 weeks.



However, despite this method, no improvement of fibrosis deposition and muscle histopathology was noticeable.



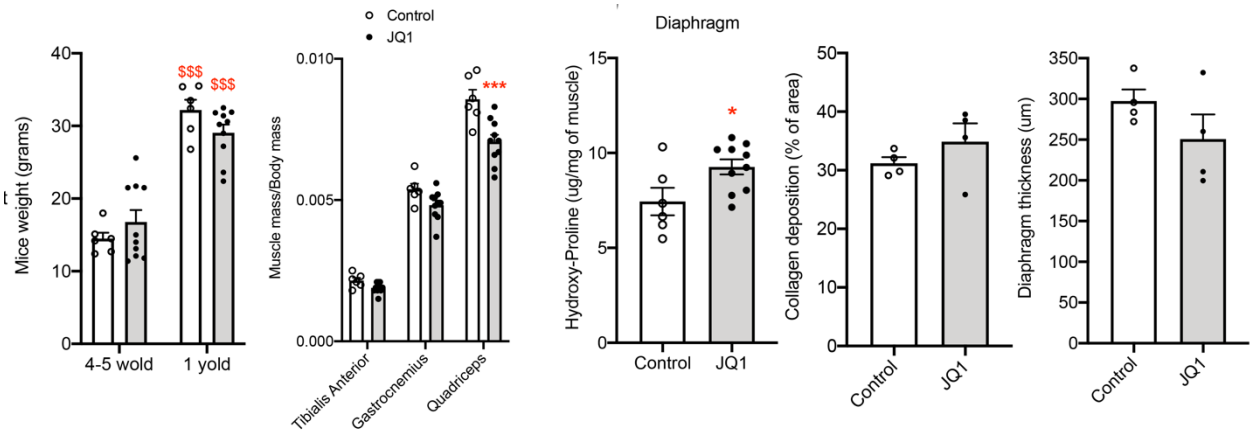
In addition to Masitinib, we also tested the related TyrK inhibitor Sorafenib on muscle fibrosis by using the osmotic pump delivery method for 9 weeks. Overall, no differences were noticeable on Mouse body weight, muscle mass and collagen deposition. Moreover, diaphragm histopathology was not improved by Sorafenib



The final compound we tested was the Bromodomain protein inhibitor JQ1. *mdx* mice (a murine model of DMD) were fed with a diet containing JQ1 from 4-5-weeks of age to 1 year old.

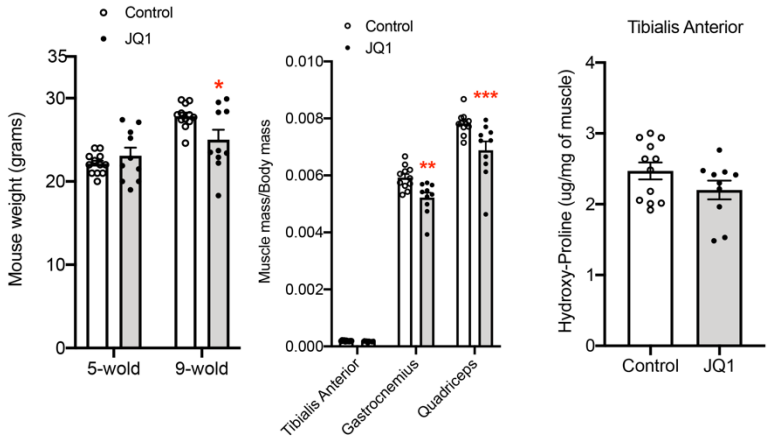
Overall, mice grew up at the same pace. Nevertheless, mice fed with JQ1 display muscle mass loss in the Quadriceps (-18%, p<0.001).

Interestingly, diaphragms displayed more fibrosis, quantified by Hydroxyproline content and Picrosirius Red (PSR) staining.



JQ1 has been previously demonstrated as an antifibrotic drug in heart [30]. A possible reason of a discrepancy in the results is that experimental animal models of injury and repair also may not be as representative of the clinical picture in patients with fibrosis. Thus, we took advantage of the mdx:utr$^{+/-}$ mouse model described above to further test JQ1 efficiency on muscle fibrosis. We tested the use of continuous infusion by implanting mice with subcutaneous drug delivery pumps containing JQ1 from during 4 weeks (5 to 9-week-old). The continuous delivery of JQ1 induced a slower post-natal

growth as the mouse weight at 9-week-old is decreased by 10% ($p<0.05$). Concomitant to this, muscle mass of both gastrocnemius and Quadriceps was strongly affected (respectively -11% $p<0.01$ and -11.3% $p<0.001$).



In summary, despite the promising in vitro results obtained with these compounds, we ddi not observe a dramatic improvement of fibrosis in vivo. In most cases, chronic treatment led to toxic effects evidenced by loss of body mass, and in those cases in which positive effects were seen, they were not consistent across muscle groups.
We conclude that these compounds are unlikely to be effective in reducing fibrosis in human dystrophy patients.

Analyze effects of treatment on FAPs. A secondary goal was to identify theranostic markers.

While the compounds identified may not be promising for use in human patients, the analysis of the responses of fibrogenic progenitors to these treatments in vivo and especially its comparison with the responses obtained in vitro can be very informative.

In line with the proposed activities, we isolated FAPs form treated animals by flow cytometry and we prepared RNAseq libraries that have been quality controlled and are currently ready to be sequenced. Unfortunately, the ongoing shutdown of research activities due to the COVID-19 pandemic has prevented us from actually sequencing these samples and analyzing them. A preliminary analysis on a small number of samples we obtained early shows a strong abatement of inflammatory gene expression in FAPs form animals treated with JQ1, likely through its known inhibitory action on cofactors of NFkB[31]. This analysis will be complete as soon as we can get back to work.

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

ChihKai Chang, our animal surgeon, has now been certified as proficient for osmotic pump implantation by UBC institutional veterinaries.

Elena Groppa, a postdoc involved in the project, has learned significant bioinformatics skills.

How were the results disseminated to communities of interest?

The PI has given talks covering the subject of this award at multiple international meetings, including The Gordon Conference on Myogenesis in Barga, Italy, A Parent Project workshop on the role of inflammation in muscular dystrophy in Chicago, and the Ottawa international meeting on neuromuscular disorders. A paper describing these results is at advanced draft stage.

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

- N/A
-

4) IMPACT:

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

Our work will help others to refine their approach in the search for antifibrotic compounds that could be used chronically. In addition, we believe that the RNAseq datasets, once published, will be highly informative to all working on stromal progenitors

What was the impact on other disciplines?

While these compounds are not appropriate for chronic use, they may still be used topically to prevent fibrotic reactions, for example to implanted medical devices. The data we generated will inform research in that direction.

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

We have changed the kinase inhibitor originally selected for testing in vivo. Instead of using Sorafenib, which was designed for cancer therapy and has significant side effects that may preclude its use in a chronic setting, we have identified a new drugs, Masitinib, as having overlapping specificity and better efficacy but less side effects. In vitro testing using assays described in the original application indicated that Masitinib is superior to Sorafenib in suppressing the fibrogenic differentiation of fibro/adipogenic progenitors. Masitinib is also being tested in human trials in Amyotrophic lateral sclerosis, another neuromuscular disease, for its ability to delay neuronal loss. With the work performed

under this funding, we hoped to expand the range of the use of masitinib to muscular dystrophy and to prove it has a direct antifibrotic activity.

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

The current COVID19 pandemic has caused a significant delay in completing our analysis and publishing our results. I am not sure there is much we can do until the situation is normalized.

Changes that had a significant impact on expenditures

Nothing to report

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

The local IACUC approval has been renewed for the coming year, now expiring in Sept 2020.

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. A publication is being prepared and is in advanced draft form. Another paper reporting the effects of treatment with Nilotinib on cardiac remodeling following MI has now been published in Cell Stem Cell (PMID:32142665).

- **Books or other non-periodical, one-time publications.** Nothing to report

Other publications, conference papers, and presentations.

The PI presented our progress as an invited speaker at the following international conferences:

- Gordon conference on myogenesis, June 2017, Barga, Italy.
- Parent Project Muscular Dystrophy meeting on Inflammation and Immunity in Duchenne, June 2018, Chicago.
- The 4th Ottawa meeting on Neuromuscular Diseases, September 2017, Ottawa.

Website(s) or other Internet site(s)

N/A

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:	*Fabio Rossi*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	orcid.org/0000-0002-0368-2620
Nearest person month worked:	*7.2*
Contribution to Project:	*Dr Rossi is the PI on the project*
Funding Support:	N/A

Name:	*Marcela Low*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*9*
Contribution to Project:	*Dr Low has been working on refining the readout of assays used to test the anti-fibrotic potential of the drugs, which has led to the change, mentioned above, in the kinase inhibitor that will be tested*
Funding Support:	This grant

Name:	*Elena Groppa*
Project Role:	*Postdoctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*27*
Contribution to Project:	*Dr Groppa has replaced Dr Low in coordinating the*

	project as Dr. Low has left the lab
Funding Support:	This grant

Name:	*ChihKai Chang*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*36*
Contribution to Project:	*Mr Chang has been working on procuring the animals required for testing, breeding enough of them, and perfecting osmotic pump implantation surgeries*
Funding Support:	This grant

▪

Name:	*Andrew Wu*
Project Role:	*Graduate Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*8*
Contribution to Project:	*Mr Wu has been working through the summer in support of Dr Low, performing in vitro assays of antifibrotic activity*
Funding Support:	Other PI funds

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

No Change

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

Nothing to report

What other organizations were involved as partners?

We have now MTAs in place with two commercial entities that provided the drugs to be tested. Specifically, Imstar Therapeutics (withaferin analogue) and AB Sciences (masitinib).

Organization Name: Imstar Therapeutics
Location of Organization: Canada
Partner's contribution to the project: providing a proprietary synthetic withaferin analogue for testing in animal models of muscular dystrophy
Financial support; N/A
In-kind support: Providing drug for testing.
Facilities N/A;
Collaboration N/A;
Personnel exchanges N/A
Other. N/A

Organization Name: AB Sciences
Location of Organization: France
Partner's contribution to the project: providing a proprietary kinase inhibitor (masitinib) for testing in animal models of muscular dystrophy.
Financial support; N/A
In-kind support: Providing drug for testing.
Facilities N/A;
Collaboration N/A;
Personnel exchanges N/A
Other. N/A

8) SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A
QUAD CHARTS: N/A

9) APPENDICES:

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