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Chemical Library Screening for Potential Therapeutics Using Novel Cell-Based Models of ALS

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14. ABSTRACT
The leading cause of inherited amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) is a microsatellite repeat expansion in the C9ORF72 gene. This disorder is referred to as C9FTD/ALS. The disease mechanisms are still poorly understood. However, it is clear that the repeat expansion sequence is made into RNA that can aggregate in the nucleus of patient cells. The expansion RNA can also be translated into repetitive polypeptides in the cytoplasm of patient cells. These two processes are expected to play key roles in the initiation and progression of disease at the molecular and cellular level. Drugs that can block or reverse these processes would hold promise as therapeutics to treat C9FTD/ALS. The overall goal of this project is to develop new cell-based models of C9FTD/ALS that recapitulate these two disease processes. RNA foci and repetitive polypeptides will be visible through fluorescence microscopy. These cells will then be used for high throughput chemical library screening to identify and characterize molecules with therapeutic potential. For this Annual Technical Progress Report, we report on progress for year 2 according to the proposed Statement of Work. We have largely completed Major Task 1, preparation of custom expression vectors and model cell lines. We have obtained a no-cost extension and made minor adjustments to our research plan to accommodate completion of Major Tasks 2 and 3 in the remaining year of the project. We expect to identify chemical compounds that will represent lead molecules for therapeutic development of C9FTD/ALS, the leading genetic cause of ALS.

15. SUBJECT TERMS
ALS, repeat expansion, RNA, fluorescence, cell-based models, chemical library, high throughput screening

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1. INTRODUCTION:

We are developing cell-based models of C9FTD/ALS, the most common inherited form of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), for chemical library screening to identify compounds that have promise for therapeutic development. The premise of this project is that cell models should recapitulate the two most basic disease processes, formation of repetitive RNA foci and production of repetitive poly-dipeptides, and these processes must be rapidly detectable through microscopy of live cells to enable high throughput cell-based screening. In this progress report for year 2, some progress toward the sub-tasks were accomplished according to the original Statement of Work (SOW). However, unexpected technical challenges have carried over from year 1 into year 2 for us. We have developed work-arounds for some of these technical challenges. Due to these challenges, we requested and were awarded a one year no-cost extension to complete this project. In the process, we published two manuscripts that developed from our investigations. We have developed a new plan moving forward that will facilitate productivity and increase our chances of successfully identifying lead compounds that could be used to treat C9FTD/ALS.

2. KEYWORDS:

Amyotrophic lateral sclerosis, frontotemporal dementia, C9FTD/ALS, repeat expansion, RNA, RAN translation, fluorescence, cell-based models, chemical library, high throughput screening

3. ACCOMPLISHMENTS:

Year 2 was originally to be defined by completion of Aim 2 and Aim 3, and the completion of this project. However, as described in the previous year 1 technical progress report, we met with significant obstacles in personnel (an 8-month delay in postdoctoral fellow hiring) and experimental outcomes. These setbacks delayed completion of Aim 1 and Aim 2 tasks were not initiated as expected. We made minor adjustments (also described in the year 1 technical progress report) but discovered a new set of unexpected challenges. These will be described below in the appropriate sections.

Accomplishments achieved in this year 2 reporting period were the completion of certain subtasks from Aim 1. We will name those goals, tasks, subtasks and milestones below and describe actual accomplishments toward those goals. We will discuss the remaining goals and our plan for minor changes to the project that will be implemented to manage current setbacks and plan for success in the final year of this project. Finally, we will describe training and professional development for students and personnel, as well as how findings were disseminated.

Major Goals of the Project Based on the Statement of Work (SOW).

Major Task 1: Establish neuronal cells that inducibly express fluorescent expRNA and poly-dipeptides.

Milestone Expected: Generate C9FTD/ALS cell-based model that recapitulates molecular pathology and is compatible with high throughput screening (*6-9 months, projected completion 01-15-17*). (**70% complete**)

Subtask 1: Build custom expRNA expression vectors (*2-3 months, projected completion 09-01-17*). (**100% complete**)
Subtask 2: Stably transform nH9 cells with TetR (*1 month, projected completion 10-01-17*). (**100% complete**)
Subtask 3: Stably transform nH9-TR cells with custom vectors for expRNA expression (*1 month, projected completion 11-01-17*). (**80% complete**)
Subtask 4: Validate c9FTD/ALS molecular pathology of new cell-based models (*2-4 months, projected completion 02-01-17*). (**50% complete**)

Major Task 2: High throughput screening of chemical libraries at Stanford HTBC.

Milestone Expected: Identification of lead molecules for further validation and therapeutic development (*7-11 months, projected completion 09-15-17*). (**20% complete**)

Subtask 1: Cell culture establishment at HTBC (*1 month, projected completion 03-01-17*). (**0% complete**)

Subtask 2: Assay development and high throughput screen workflow protocol (*1-2 months, projected completion 05-01-17*). (**0% complete**)
Subtask 3: Primary high throughput screen and titrations of top 1000 compounds (*4-6 months, projected completion 08-15-17*). (**0% complete**)
Subtask 4: Data analysis, lead compound ranking, and chemical and structural analysis of lead compounds (*1-2 months, projected completion 09-15-17*). (**0% complete**)

Major Task 3: Validate promising lead molecules using biochemical and cell-based assays.

Milestone Expected: Detailed characterization of several lead candidates and identification of top compounds to move into preclinical testing (*6-9 months, projected completion 05-15-18*). (**0% complete**)

Subtask 1: Independently test lead compound effects on expRNA and poly-dipeptide expression/aggregation (*2-3 months, projected completion 12-01-17*). (**0% complete**)
Subtask 2: Determine effect of lead compounds on other disease-associated molecular defects (*4-6 months, projected completion 05-15-18*). (**0% complete**)

Accomplishments under these goals.

Major Task 1, Subtask 1.

Our first major activity was the design and engineering of a custom inducible plasmid for expressing fluorescently-labeled repeat expansion RNA and poly-dipeptides. The specific objective was to create a plasmid with features to enhance the likelihood of developing successful cell-based models. These included i) ability to easily introduce or remove fluorescent tags using traditional restriction enzymes, ii) *in vitro* transcription if needed, iii) a designated site for cloning repeat expansions, and iv) inducible by doxycycline.

Significant results and key outcomes were the accomplishment of all of these objectives. We initially designed these vectors to inducibly express the Broccoli RNA aptamer fused to the repeat expansion followed by LUMIO tags. These were designed to facilitate detection of nuclear foci and poly-dipeptide translation inside of live cells, respectively, both of which are key markers of disease. In our previous year 1 progress report, we described the initial design and custom construction of this plasmid vector (called pINC-3G) and will not repeat those details here. However, in our report from year 1 we had not completed this subtask. What remained were a few key challenges.

First, we realized that our plasmids contained a canonical (standard) AUG start codon upstream of the repeat expansion cloning locus. This will not allow us to recapitulate repeat-associated non-AUG (RAN) translation, which produces the disease poly-dipeptides, but instead will use standard translation mechanisms (Green et al., 2016).

Second, the broccoli RNA aptamer did not appear to be bright enough. Upon closer inspection, we found that brightness issues were due to very rapid photobleaching of the broccoli RNA aptamer. This property has since been revealed in the literature by others (Autour et al., 2018), but was not known to us at the time. So, despite proper cloning and validation, the aptamer itself was intrinsically unstable for live cell imaging.

Third, we discovered that LUMIO tags were not easily detectable in a manner that was suitable for live cell imaging. The fluorescent dyes that must be added were relatively toxic to the cells, despite testing various conditions (data not shown), and were also not bright enough due to low RAN translation expression levels.

Fourth, our plasmids did not contain enough repeats to presumably initiate the RAN translation mechanism (Green et al., 2016). In our previous year 1 progress report we had only cloned 20 repeats.

As a result, in year 2 we spent substantial time redesigning our construct. We performed site-directed mutagenesis to convert the ATG start codon to a CTG near cognate start (NCS) codon to support RAN translation (Green et al., 2016) (**Figure 1A**). Unfortunately, no fluorescent RNA aptamer was deemed capable of replacing Broccoli RNA aptamer and be bright enough or resist photobleaching. Due to the lack of current technology and the difficulties inherent to RNA imaging in live cells, we opted to omit an RNA fluorescent tag altogether. Going forward, we are exploring the use of CRISPR-Cas9 technology to embed fluorescent RNA Mango aptamers (Autour et al., 2018) into the endogenous repeat of patient-derived cells. If this is successful, we could transition over to patient-derived cells for chemical library screening. As part of an initial study, we established the use of CRISPR-Cas9 technology in our laboratory. This work led to a publication in the journal *Biochemistry* (Kartje et al., 2018). At present, we have replaced both the RNA aptamer tag and LUMIO tags with an mCherry red fluorescent protein fusion to the repeat sequences (**Figure 1B**). If RAN translation occurs, this will result in poly-

glycine-alanine (poly-GA) dipeptide repeats being fused to the N-terminus of mCherry. Our rationale is that a simpler and proven protein tagging system can initially be used for screening. Once lead hits are found during screening, we can go back and use traditional FISH probing techniques to determine if lead compounds also affect foci formation (**Figure 1C**). We may also be able to use cells with the Mango RNA aptamer knocked-in to the repeats as well. Once an NCS start site and mCherry were introduced into the vector, we then continued expansion of our $(GGGGCC)_n$ repeat using recursive directional ligation (RDL) as described previously in our year 1 progress report. By this method, we have now expanded our constructs to 40 and 80 repeats, which are sufficient to induce RAN translation (**Figure 1D**). We have now successfully completed the preparation of our custom repeat expansion expression vectors.

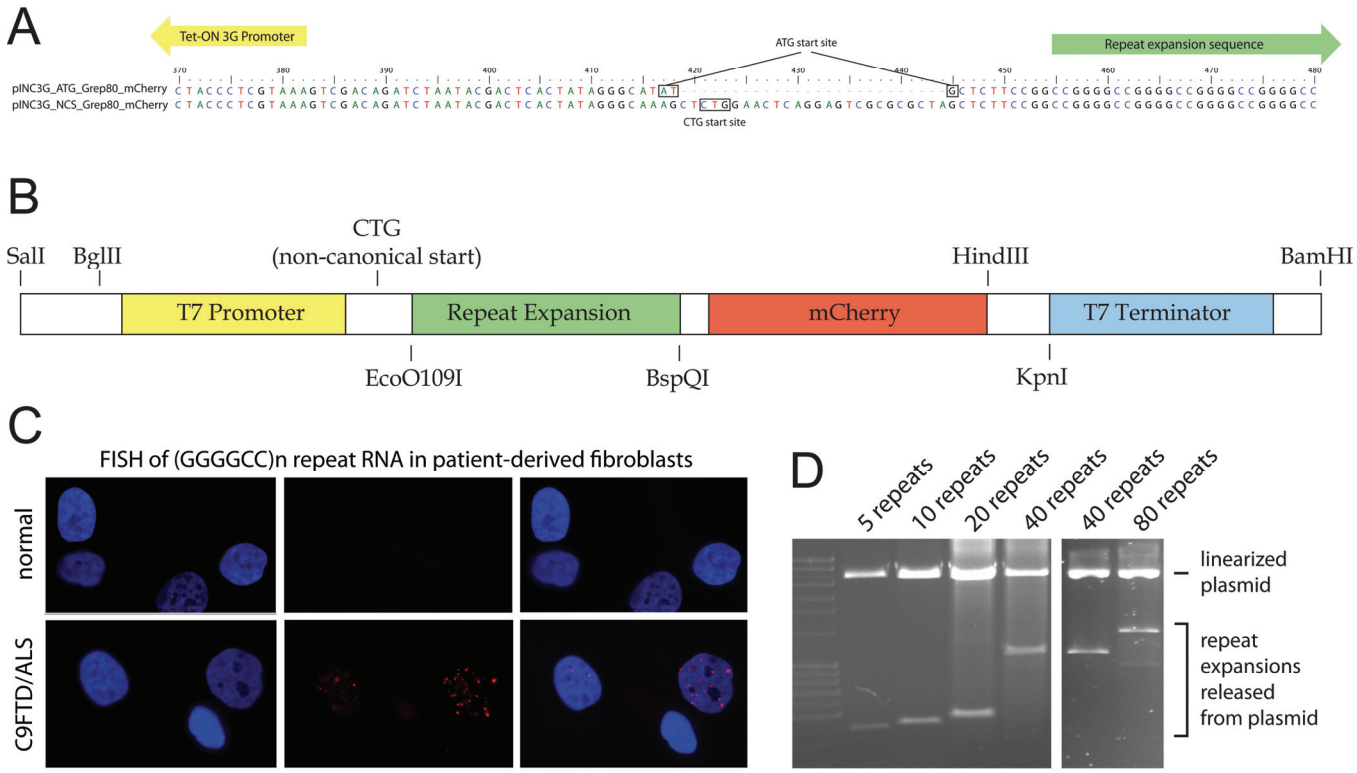

Figure 1. Final cloning of C9ORF72 repeat expansion expression vectors. (**A**) The pINC3G_NCS_Grep80_mCherry vector contains sequence derived directly from that C9ORF72 first intron that replaces the canonical ATG start site with a non-canonical CTG start site to support RAN translation. (**B**) The final repeat construct fuses mCherry in-frame with a poly-GA dipeptide repeat sequence. (**C**) Example FISH data detecting focal aggregates of expansion RNA in patient-derived fibroblasts. (**D**) pINC3G repeat expansion plasmids were treated with restriction enzymes to release a fragment containing the repeats. Separation of repeat fragments on an agarose gel reveals a step-wise increase in size, indicating successful cloning of repeat expansions up to 80 repeats.

Major Task 1, Subtask 2.

Our second major activity was to generate human neural stem cells that stably express a tetracycline receptor (TetR, or TR) protein. The specific objective was to create cells that would support doxycycline-inducible expression of the pINC3G plasmids we engineered. These cells are not commercially available and so must be custom made. This subtask was described in our year 1 progress report and has been completed.

Major Task 1, Subtask 3.

Our third major activity was to generate nH9-TR cells (stably expressing the TR gene) that also contained stably-transfected pINC3G plasmids. The specific objective is to avoid the need to repeatedly transfect cells with the repeat expansion expressing plasmids, which is not feasible for high throughput chemical library screening. These cells, therefore, would contain all of the necessary disease-associated genetic information, but would not present any of the disease-associated biomarkers, including foci and poly-dipeptides, until these are induced with doxycycline.

Significant results and key outcomes are the successful testing of pINC3G-G40-mCherry and pINC3G-G80-mCherry plasmid expression in nH9 cells by transient transfection (**Figure 2A**). These experiments demonstrated that our nH9-TR cells are compatible with our pINC3G plasmids and that the mCherry expression was dependent on the addition of doxycycline. However, stated goals not met for this Subtask are the generation of nH9-TR cells stably transformed with pINC3G plasmid vectors. We have not yet accomplished this goal for two reasons. The first is because the pINC3G vector backbone does not contain an antibiotic resistance marker. Thus, it must be co-transfected with a DNA fragment that does have a resistance gene to facilitate selection. The second reason is because we need a second fluorescent label to mark the cells themselves to enable cell counting during chemical library screening. We initially attempted to stably select nH9 cells expressing a blue fluorescent protein called TagBFP with a nuclear localization signal (NLS). Despite cloning it into an expression construct that should prevent silencing of the gene, we discovered that after selections many of the cells began to lose blue fluorescence (data not shown). To solve these issues, we plan to co-transfect our pINC3G plasmids with an EGFP-expressing plasmid that possesses an antibiotic selection marker. This will allow us to select for cells that fluoresce green and express the 80-repeat mCherry fusions in the presence of doxycycline at the same time. This final step will result in completion of this Subtask.

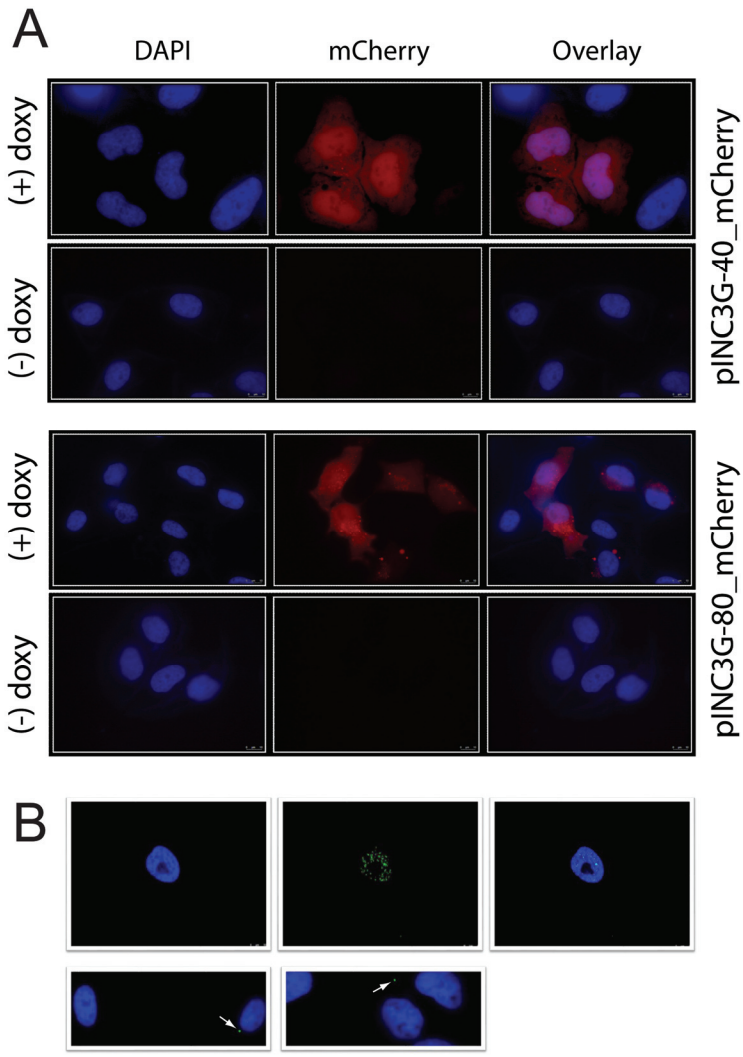

Figure 2. (**A**) Fluorescence microscopy demonstrating expression of 40 repeat and 80 repeat expression constructs when induced with doxycycline. (**B**) Foci detected by FISH in patient-derived fibroblasts (upper panels) and in cells expressing 80-repeat expansions fused to mCherry. A green (fluorescein) probe was used in these experiments.

Major Task 1, Subtask 4.

Our fourth major activity was to validate the cell-based models prepared in Subtasks 1-3 of Aim 1. The specific objective for this activity is to ensure that cell-based models are faithful representations of cellular disease. Positive results for other disease markers, including rRNA defects or TDP-43 mislocalization to the cytoplasm, would be desirable. However, these markers are not required and will not prevent successful screening with chemical libraries as long as fluorescent disease biomarkers established in Subtasks 1-3 are successful.

Significant results and key outcomes are the testing of repeat expansion RNA foci detection with FISH in nH9 cells transiently transfected with pINC3G-G80-mCherry vectors. In these experiments, we observed focal aggregation of repeat expansion RNA when expressed from our custom vectors (**Figure 2B**). These results suggest that our cell model can recapitulate the basic disease mechanisms expected for C9FTD/ALS. Stated goals not met are the testing other disease markers after production of nH9-TR cells stably expressing EGFP and repeat expansions fused to mCherry. These other markers were described in the original proposal and include rRNA disturbances and mislocalization of TDP-43.

Major Tasks 2 and 3.

Our remaining major activities are to establish cell culture and assay development at the Stanford High Throughput Bioscience Center (HTBC), screen molecules, and validate lead molecules and their effects on disease markers. The stated goals for these activities have not been met yet and there are no other achievements to discuss for these tasks. We describe our plans below to achieve our objectives, reach our

milestones and complete this project (*see* “Plans to accomplish goals during the next reporting period” *and* “CHANGES/PROBLEMS” *sections*).

Opportunities for training and professional development.

A variety of students and young professionals have participated in this project in year 2. They include two undergraduate students (Mr. James Donohue and Mr. Anthony Henke), two graduate students (Mr. Zachary Kartje and Mr. Christopher Barkau), and a postdoctoral fellow (Dr. Maria Barton). Mr. Kartje has developed new approaches with CRISPR to be applied toward developing future models of C9FTD/ALS and Mr. Barkau and Dr. Barton have developed cell models and constructed repeat expression vectors. The undergraduates Mr. Donohue and Mr. Henke have participated in cloning repeat expansions. We have a new Ph.D. student that joined the laboratory this past year who will be assisting Dr. Barton in finalizing cell models for screening that the Stanford HTBC. This will provide an opportunity for Dr. Barton to train students and improve her teaching abilities.

Training of undergraduates by graduate students provided a fantastic opportunity for Mr. Kartje and Mr. Barkau to learn how to teach molecular biology techniques. The undergraduates learned important research skills that will serve them well in their future careers. Mr. Donohue is now pursuing his M.S. at St. Louis University and Mr. Henke is now pursuing a Ph.D. at the University of Texas at Dallas. Mr. Barkau and Dr. Barton also learned challenging new skills in cloning repetitive sequences and constructing custom expression vectors.

Mr. Barkau and Dr. Barton both attended an international conference, the RNA Society Meeting, in Berkeley, California recently. They were able to present their progress on this project and related projects. Mr. Kartje presented his project at a local SIU School of Medicine Graduate Symposium and won first place for his oral presentation.

Dissemination of results to communities of interest.

Results were disseminated by conference presentations, abstracts, and publications. Two conferences in 2017 and one in 2018 were used to disseminate research. An oral presentation was given at one conference and poster presentations given at the other two meetings. One review article in 2017 and a peer-reviewed publication in 2018 have been used to disseminate results. Conference and publication abstracts are provided in Appendix I.

Plans to accomplish goals during the next reporting period.

In our first year we encountered significant delays in hiring the necessary personnel and in the design and development of expression vectors and stable cell line selection. These challenges carried over into year 2 and required that we request a no-cost extension. We have been granted the extension and now intend to complete the project in this final year 3.

To complete the project, which requires achieving the final two Major tasks in 12 months, we have made minor modifications to our original SOW. The greatest change is simply the removal of Major Task 3 – Subtask 2, which was to determine the effect of lead compounds on other disease-associated molecular defects. This Subtask is rather open-ended and not necessary to achieve the primary objective of identifying lead compounds for therapeutic development. By removing this aim, we reduce the time required from the original SOW from 16 months to 9-11 months. We have also simplified our approach by using a single fluorescent marker for RAN translation (mCherry). However, this will add an additional small-scale screening to our Subtask 1 in Major Task 3, potentially adding 1 month to our timeline. Nonetheless, this is feasible in the final year with our new adjustments.

Rather than wait until our final cell line selections are prepared, which could require an additional 1-2 months of selection and validation, we will accelerate the first phase of chemical library screening by sending the Stanford HTBC a sample of our nH9 neural stem cells now to establish cell culture conditions. We will also discuss with the HTBC which chemical libraries are best for screening. While up to 130,000 compounds are available, there may be redundancy or low historical likelihood of hits (LHLH) with some libraries. In this case, libraries will be prioritized for screening to increase efficiency and reduce time.

4. IMPACT:

Impact on the development of the principal disciplines of the project.

Our ongoing progress on cell-based models was presented as a poster at the recent RNA Society Meeting in Berkeley, California. Colleagues were interested in our custom expression vectors and cell-based models and their eventual availability. When we publish our cell-based models and the subsequent chemical library screening results, we believe we will make an important impact by shifting research practices. Currently there are no easy-to-access or easy-to-use cell-based models that are representative of the disease. We believe our work will fill this gap. There are also no informative studies using chemical libraries to identify lead compounds for C9FTD/ALS, so we believe we will add a tremendous contribution to the principal discipline when this project is complete by providing detailed insight into potential therapeutics.

We believe our innovative approach of inducible RAN translation coupled to fluorescent live cell imaging in neurons will help transform the standard for basic research in the field. Our characterization of the many moving parts involved in engineering successful cell-based models will provide a foundation for researchers in the principal discipline to build upon. Our cell-based models will also make it significantly easier for researchers outside of the field to enter in and make important contributions.

Impact on other disciplines.

We have published an aspect of this project regarding optimization of CRISPR-Cas9 for efficient catalysis by the Cas9 enzyme. We intend to use our experience in this area to build even better models of C9FTD/ALS in the future and to facilitate the use of patient-derived cells to make models that are more amenable to drug screening and biochemistry.

Impact on technology transfer.

There is nothing to report for this section.

What was the impact on society beyond science and technology?

There is nothing to report for this section.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change.

We have not made any significant changes to our approach or SOW. To meet our objectives and timeline, we have made minor changes in the use of aptamers for fluorescence imaging and in downstream validation of potential lead hits from screening. Changes in our approach have been necessitated by technical challenges, primarily centered around generation of custom expression vectors and the custom cells to use for screening. These have been described above in the Accomplishments section. We have met these challenges with appropriate modifications and have a plan in place to finish this project during our no-cost extension.

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

We have encountered a few problems during the course of this reporting year that have resulted in delays that were not anticipated. These primarily include technical challenges that were enumerated and described in the Accomplishments section above.

We do not anticipate significant delays in the coming year. The remaining unknowns for the project are the establishment of cell lines that stably express repeat expansions and the actual performance of model cell lines in the chemical library screening workflow. To mitigate these unknowns, we plan to employ an additional student during the summer to specifically aid in cell line selection and validation. We also plan to work closely with the HTBC. If the HTBC experiences any difficulties early on that cannot be resolved, Dr. Maria Barton will visit the HTBC for up to 2 weeks and help them troubleshoot cell culture.

Changes that had a significant impact on expenditures.

The technical and personnel issues we encountered during year 1 had a significant impact on expenditures. They resulted in less funds being spent. This is due to the delayed hiring of a postdoctoral fellow and a delay in sending cells to the HTBC for preliminary assay development. We have also yet to spend funds for chemical library screening. Thus, a significant amount of funding has carried over into our no-cost extension that we expect to use completely by the close of this project.

Significant changes in use or care of human subjects

There is nothing to report for this section. Human subjects are not applicable to this proposal.

Significant changes in use or care of vertebrate animals.

There is nothing to report for this section. Vertebrate animals are not applicable to this proposal.

Significant changes in use of biohazards and/or select agents

There is nothing to report for this section.

6. PRODUCTS:

Journal publications.

Rohilla, K.J., and Gagnon, K.T. (2017) RNA Biology of Disease-Associated Microsatellite Repeat Expansions. *Acta Neuropathologica Communications*. 5:63. Support Acknowledged.

Kartje, Z.J., Barkau, C.L., Rohilla, K.J., Ageely, E.A., and Gagnon, K.T. (2018) Chimeric Guides Probe and Enhance Cas9 Biochemical Activity. 57:3027-3031. Support Acknowledged.

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

There is nothing to report for this section.

Other publications, conference papers, and presentations.

13th Annual Meeting of the Oligonucleotide Therapeutics Society, Bordeaux, France, IL, September 24 – September 27, 2017. Invited Lecture. "A 'Guided' Tour of an Early Career in RNA and Nucleic Acid Therapeutics."

28th Annual Trainee Research Symposium, Southern Illinois University School of Medicine, Carbondale, IL, April 21, 2017. Poster presentation. "Nuclear turnover and export mechanisms for repeat expansion RNA in C9FTD/ALS."

28th Annual Trainee Research Symposium, Southern Illinois University School of Medicine, Carbondale, IL, April 21, 2017. Oral presentation. "Chimeric Guides in CRISPR-Cas9."

23rd Annual Meeting of the RNA Society, May 29 - June 3, Berkeley, California. Poster presentation. "Cell-based models of C9FTD/ALS for discovery of small molecule inhibitors of repeat-associated non-AUG (RAN) translation."

23rd Annual Meeting of the RNA Society, May 29 - June 3, Berkeley, California. Poster presentation. "Nuclear Export Mechanisms of Tandem Repeat Expansion RNA in C9FTD/ALS Neurological Disease."

Website(s) or other Internet site(s).

There is nothing to report for this section.

Technologies or techniques

There is nothing to report for this section.

Inventions, patent applications, and/or licenses

There is nothing to report for this section.

Other Products

There is nothing to report for this section.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals that have worked on the project.

Name:	Keith T. Gagnon
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Dr. Gagnon supervised and oversaw research progress, experimental troubleshooting, and dissemination of findings.
Funding Support:	Southern Illinois University School of Medicine, DoD ALSRP (this award)

Name:	Zachary J. Kartje
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Mr. Kartje has performed design and cloning of inducible repeat expansion expression plasmids and CRISPR experiments.
Funding Support:	Gagnon Lab Startup (SIU), Gower Graduate Fellowship in Chemistry (SIU), DoD ALSRP (this award)

Name:	Christopher L. Barkau
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Mr. Barkau has performed cloning of inducible repeat expansion expression plasmids.
Funding Support:	DoD ALSRP (this award), Teaching Assistantship (SIU)

Name:	Maria Barton
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Dr. Barton has performed stem cell culture, cloning of repeat expansion expression plasmids, FISH, stable cell line selections.
Funding Support:	DoD ALSRP (this award)

Changes in the active other support of the PI since the last reporting period.

Previously funded grants that have completed:

Judith and Jean Pape Adams Foundation ALS Research Grant

Gagnon (PI)	0 CY person-months	$60,000	02/01/17 - 01/31/18

Title: “C9ORF72 transcription and splicing as therapeutic targets for a genetic form of ALS”

Newly funded grants that have started:

SIU School of Medicine Discovery Science Grant
Gagnon (PI) 0 CY person-months $15,000 01/01/18 - 12/31/18
Title: “Discovering chromatin-associated long noncoding RNAs acting as mitotic bookmarks in human stem cells”

Other partnering organizations.

We have partnered with Stanford University's High Throughput Bioscience Center (HTBC) to provide high throughput chemical library screening as a service. They will provide us this service as part of the proposed research. The contact person at HTBC is the facility director Dr. David Solow-Cordero. The HTBC did not provide any services in this year 1 reporting period. However, they will be providing their service in the next reporting phase.

Organization Name:	Stanford University High Throughput Bioscience Center
Organization Location:	Stanford, California
Partners Contribution:	
Financial Support:	None.
In-kind Support:	None.
Facilities:	Provides high throughput robotics facility as a service for the chemical library screening phase of this project.
Collaboration:	The facility staff will help the project staff rank results and interpret results to identify promising lead compounds.
Personnel Exchanges:	One project staff from the PI institution may travel to the HTBC facility in reporting year 2 to help establish cell culture and assay conditions.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report.

QUAD CHARTS:

Nothing to report.

9. APPENDICES:

See Appendix I below for publication and meeting abstracts pertaining to the year 2 reporting period. See Appendix II below for additional references pertaining to the year 2 report.

APPENDIX I.

Rohilla, K.J., and Gagnon, K.T. (2017) RNA Biology of Disease-Associated Microsatellite Repeat Expansions. *Acta Neuropathologica Communications*. 5:63. Support Acknowledged.

Abstract:
Microsatellites, or simple tandem repeat sequences, occur naturally in the human genome and have important roles in genome evolution and function. However, the expansion of microsatellites is associated with over two dozen neurological diseases. A common denominator among the majority of these disorders is the expression of expanded tandem repeat-containing RNA, referred to as xtrRNA in this review, which can mediate molecular disease pathology in multiple ways. This review focuses on the potential impact that simple tandem repeat expansions can have on the biology and metabolism of RNA that contain them and underscores important gaps in understanding. Merging the molecular biology of repeat expansion disorders with the current understanding

of RNA biology, including splicing, transcription, transport, turnover and translation, will help clarify mechanisms of disease and improve therapeutic development.

Kartje, Z.J., Barkau, C.L., Rohilla, K.J., Ageely, E.A., and Gagnon, K.T. (2018) Chimeric Guides Probe and Enhance Cas9 Biochemical Activity. 57:3027-3031. Support Acknowledged.

Abstract:
DNA substitutions in RNA can probe the importance of A-form structure, 2′-hydroxyl contacts, and conformational constraints within RNA-guided enzymes. Using this approach, we found that Cas9 biochemical activity tolerated significant substitution with DNA nucleotides in the clustered regularly interspaced short palindromic repeat RNA (crRNA). Only minimal RNA content was needed in or near the seed region. Simultaneous substitution at all positions with predicted crRNA−Cas9 2′-hydroxyl contacts had no effect on enzyme activity. The trans-activating crRNA (tracrRNA) also tolerated >50% substitution with DNA. DNA substitutions in the tracrRNA-pairing region of crRNA consistently enhanced cleavage activity while maintaining or improving target specificity. Together, results point to a prominent role for guide:target A-form-like helical structure and a possible regulatory role for the crRNA−tracrRNA pairing motif. A model chimeric crRNA with high activity did not significantly alter RNP assembly or target binding but did reduce Cas9 ribonucleoprotein stability, suggesting effects through conformation or dynamics. Cas9 directed by chimeric RNA−DNA guides may represent a cost-effective synthetic or molecular biology tool for robust and specific DNA cleavage.

13th Annual Meeting of the Oligonucleotide Therapeutics Society, Bordeaux, France, IL, September 24 – September 27, 2017. Invited Lecture. "A ‘Guided’ Tour of an Early Career in RNA and Nucleic Acid Therapeutics."

Abstract:
This talk will discuss some of the history and future of RNA-guided enzymes in research and therapeutics. It is from the vantage point of a young investigator who has worked on RNA-guided enzymes throughout his career. These opinions may offer different perspectives and spark new ideas for future nucleic acid therapeutics. From RNAi to CRISPR-Cas9, and even RNase H-mediated ASOs, nucleic acid-guided enzymes have been an engine of inspiration and innovation. Nucleic acid-guided enzymes have translated into ground-breaking laboratory tools and a new generation of therapeutics. The more we understand nucleic acid-guided enzymes, the more innovations we can envision. Before RNAi was discovered, complexes of small nucleolar noncoding RNAs and their associated proteins, known as snoRNPs, were the best-known examples of RNA-guided enzymes. Investigations revealed important insight into the structure-function relationships between the RNA guide and the enzyme and established biochemical principles for activity. Their manipulation for therapeutics, however, has never been fully realized. Nonetheless, the knowledge that was gained and the tools developed facilitated the subsequent characterization of RNAi. The utility of RNAi was quickly recognized because it was reminiscent of ASOs, where only a small nucleic acid guide was needed. Chemical modification of the guide RNA has since been the focus of tremendous therapeutic development. More recently, CRISPR-Cas systems have been discovered and their potential to revolutionize gene therapy has become obvious. Knowledge and tools from previous RNA-guided research contributed to rapid progress. However, these systems require introduction of a large RNP. Thus, a logical question was whether such systems would fit into the world of nucleic acid therapeutics? But a little creativity and some nucleic acid chemistry are suggesting ways in which nucleic acid therapeutics and CRISPR can join together for new therapeutic approaches. With siRNAs destined for the clinic, the excitement of CRISPR, and the ability to engineer new RNA-guided systems, nucleic acid-guided enzymes are expected to continue shaping therapeutics for the foreseeable future.

28th Annual Trainee Research Symposium, Southern Illinois University School of Medicine, Carbondale, IL, April 21, 2017. Poster presentation. "Nuclear turnover and export mechanisms for repeat expansion RNA in C9FTD/ALS."

Abstract:
The expansion of microsatellites is associated with over two dozen neurological diseases. A common denominator among the majority of these disorders is the expression of expanded tandem repeat-containing RNA, referred to as xtrRNA, which can mediate molecular disease pathology in multiple ways. To understand disease mechanism, we are focusing on xtrRNA export in C9FTD/ALS, a model repeat expansion disorder that is the leading genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). In C9FTD/ALS, the repeat expansion occurs in the first intron of the C9ORF72 gene yet xtrRNA is somehow exported and translated into toxic repetitive polypeptides. We hypothesize that xtrRNA escapes into the cytoplasm by hitch-hiking with a fraction of intron-retained mRNA. Two main mRNA export pathways may be

utilized: nuclear RNA export factor 1 (NXF1)-mediated and chromosome region maintenance 1 (CRM1)-mediated export. We are knocking-down nucleocytoplasmic transport factors in these pathways, including ALY/REF, NXF1, TREX, CRM1 and UPF1, and monitoring xtrRNA nuclear focal aggregation (by fluorescence in situ hybridization), translation (by Western blot and immunofluorescence) and cellular distribution (by qPCR and North blot). Results and progress thus far will be presented. Identification of the pathway or specific proteins responsible for C9FTD/ALS xtrRNA export should shed light on natural RNA metabolism pathways, disease mechanisms and potential strategies for therapeutic intervention.

28th Annual Trainee Research Symposium, Southern Illinois University School of Medicine, Carbondale, IL, April 21, 2017. Oral presentation. "Chimeric Guides Probe and Regulate CRISPR-Cas9 Activity."

Abstract:
DNA substitutions in RNA can probe the importance of A-form structure, 2'-hydroxyl contacts, and conformational constraints within RNA-guided enzymes. Using this approach, we found that Cas9 endonuclease activity tolerated substantial substitutions in the CRISPR RNA (crRNA). Only minimal RNA content was needed in or near the seed region. The trans-activating crRNA (tracrRNA) also tolerated over 50% substitution with DNA. Substitutions in the tracrRNA-pairing region of the crRNA consistently enhanced biochemical cleavage activity while maintaining or improving target specificity. No specific 2'-hydroxyl contacts for crRNA were critical. Substitutions also suggested induced fit mechanisms during RNP assembly. Together, results point to a prominent role for guide:target A-form-like helical structure and a possible regulatory role for the crRNA-tracrRNA pairing motif. A highly active chimeric crRNA did not significantly alter RNP assembly or target binding but did reduce Cas9 ribonucleoprotein (RNP) stability, suggesting effects through conformation or dynamics. Unexpectedly, gene editing with many of these chimeric crRNAs was inefficient, a phenomenon that did not correlate with nuclease susceptibility or the stability of guide:target interaction. Partially restoring RNA into chimeric crRNAs confirmed the presence of a putative crRNA regulatory element in the tracrRNA-pairing region of crRNA that modulates enzyme activity. Chimeric crRNAs with significant DNA substitutions and robust gene editing were identified. Cas9 directed by chimeric RNA-DNA guides may represent a cost-effective synthetic or molecular biology tool for robust and specific DNA cleavage. Further characterization of the putative regulatory element should help unlock more predictable tuning of CRISPR-Cas9 activity and gene editing outcomes.

23rd Annual Meeting of the RNA Society, May 29 - June 3, Berkeley, California. Poster presentation. "Cell-based models of C9FTD/ALS for discovery of small molecule inhibitors of repeat-associated non-AUG (RAN) translation."

Abstract:
Two dozen neurological repeat expansion disorders are known, many of which are caused by expression and translation of repeat expansion RNA. To better understand molecular mechanisms and screen for molecules with therapeutic potential, we are engineering relevant cell-based models focusing on a genetic form of frontotemporal dementia and amyotrophic lateral sclerosis called C9FTD/ALS. In C9FTD/ALS, large GGGGCC repeat expansions in the first intron of the C9ORF72 gene are transcribed into expanded tandem repeat-containing RNAs which are somehow translated through a non-canonical mechanism known as repeat-associated non-AUG (RAN) translation. Our cell-based models aim to express GGGGCC repeats of varying sizes fused to mCherry to monitor expression. We are collaborating with Stanford High Throughput Bioscience Center to screen chemical libraries and identify RAN translation inhibitors in these model cell lines, thus identifying potential lead compounds for therapeutic development or RAN translation research.

23rd Annual Meeting of the RNA Society, May 29 - June 3, Berkeley, California. Poster presentation. "Nuclear Export Mechanisms of Tandem Repeat Expansion RNA in C9FTD/ALS Neurological Disease."

Abstract:
The advent of high-throughput sequencing technologies has revealed that the human transcriptome comprises mostly non-protein-coding RNAs. A large class of these transcripts, the long noncoding RNAs (lncRNAs) have been implicated in a diverse array of cellular processes, but their many functions are only beginning to be appreciated. How these molecules operate mechanistically is even less well-understood, but many are believed to act in direct proximity to the chromatin. We are using a recently-developed technique called chromatin-associated RNA sequencing (ChAR-seq) to map RNA-chromatin contacts globally. To understand mechanisms of chromatin-associated lncRNA function, we are treating MCF-7 breast cancer cells with 17β-estradiol and will be presenting progress toward applying ChAR-seq to this well-characterized system. Our results will reveal how the RNA composition and localization of the chromatin correlates with a disease-relevant remodeling of gene expression.

APPENDIX II.

References

Autour, A., S, C.Y.J., A, D.C., Abdolahzadeh, A., Galli, A., Panchapakesan, S.S.S., Rueda, D., Ryckelynck, M., and Unrau, P.J. (2018). Fluorogenic RNA Mango aptamers for imaging small non-coding RNAs in mammalian cells. Nature communications *9*, 656.

Green, K.M., Linsalata, A.E., and Todd, P.K. (2016). RAN translation-What makes it run? Brain Res.

Kartje, Z.J., Barkau, C.L., Rohilla, K.J., Ageely, E.A., and Gagnon, K.T. (2018). Chimeric Guides Probe and Enhance Cas9 Biochemical Activity. Biochemistry *57*, 3027-3031.