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TITLE: Identification of Novel Modulators of Metabolic Efficiency

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Identification of Novel Modulators of Metabolic Efficiency

The purpose of this project is to develop a novel cell-based assay that is designed to identify compounds for improving mitochondrial function and metabolic disease. The assay utilizes a dual luciferase reporter system to assess translation regulated by 5’-UTR sequences. During the reporting period, the originally designed assay was implemented, validated, and optimized successfully. A small compound library (~80 compounds) was tested to demonstrate the utility of the assay for phenotypic screening. Nonetheless, the system will likely not be amenable for high-throughput drug screening campaigns. An alternative approach using an inducible expression system is hypothesized to provide better signal-to-noise and will be implemented and assessed during the next reporting period.
Table of Contents

1. Introduction ......................................................................................................4
2. Keywords ..........................................................................................................4
3. Accomplishments .............................................................................................4
4. Impact ...............................................................................................................8
5. Changes/Problems ..........................................................................................8
6. Products ..........................................................................................................8
7. Participants & Other Collaborating Organizations .........................................9
8. Special Reporting Requirements ...................................................................10
9. Appendices ......................................................................................................10
1. INTRODUCTION

The purpose of this project is to develop a novel cell-based assay that is designed to identify compounds for improving mitochondrial function and metabolic disease. The reporter assay is unique in that it uses a phenotypic screening platform for identifying compounds capable of modulating mRNA expression profiles, thereby avoiding a more hypothesis-restricted, target-based approach. After assay development and characterization, the long-term goals of the project are to optimize the assay for potential high-throughput screening.

2. KEYWORDS

Cell-based screening
Drug discovery
Lead molecule discovery
Luciferase
Metabolic disease
Metabolism
Mitochondria
Reporter assays
Translation

3. ACCOMPLISHMENTS

What were the major goals of the project?
1. Prepare and clone 5’-UTR-luciferase reporters.
2. Co-transfect pairs of luciferase reporters and select stably expressing cells.
3. Assess changes in luciferase activity after treatment with control drugs.
4. Optimize assay for dynamic range, kinetics, and miniaturization.

What was accomplished under these goals?

1. Prepare and clone 5’-UTR-luciferase reporters

Subtask 1: Clone promoters into lentiviral vectors. Using standard restriction cloning techniques, we have inserted the CMV or TK promoter into the pLKO-AS2 lenti-based vector. These modified vectors are designed to contain unique restriction sites (NheI and Ascl) for insertion of different UTR sequences.

Subtask 2: Clone 5’-UTR and luciferase coding regions. Five 5’-UTRs representing short or long sequences (short: Cox5, RPL14, or TUBA4a; long: MDM2 or Cyclin D1) have been PCR amplified from either a genomic DNA sample or a fully synthetic oligo. Coding regions of Gaussia and firefly luciferase genes have also been PCR amplified. The luciferase sequences were cloned first into the CMV- or TK-controlled pLKO-AS2 vector using standard restriction cloning to create the Gaussia or firefly plasmids. Each of the five UTR sequences was then cloned into the two luciferase vectors, creating a total of 10 new plasmids for each promoter (CMV or TK). These vectors can be used to establish stable cell lines as well as in transient expression studies.

Subtask 3: Validate constructs. All insertions (UTR + luciferase combinations) have been verified by DNA sequencing of miniprepped plasmids from individual clones. DNA sequencing utilized primers outside of the insertion as well as internal primers to get complete coverage. CMV and TK promoter insertions were also verified by DNA sequencing prior to the cloning of the UTR and luciferase segments.
2. Co-transfect pairs of luciferase reporters and select stably expressing cells

Subtask 1: Co-transfect pairs of luciferase reporters. We co-transfected different pairs of the Gaussia and firefly luciferase vectors (harboring different 5'-UTR sequences) into HEK293T cells using a commercially available, non-liposomal, chemical transfection system. This cell line was selected after transient expression studies comparing different host cells revealed HEK293T to respond best to a positive control (described in Major Task 3). Transfected cells were used to generate stable expression lines in the next subtask.

Subtask 2: Select and amplify stably transfecting cell lines using antibiotics. Taking advantage of the lentiviral system to quickly generate stably expressing cell lines, we selected and amplified stably expressing cell lines. Briefly, HEK293T cells were co-transfected with the UTR-luciferase construct in the lentivirus vector, an envelope vector, and a packing vector. After transfection and virus assembly, recombined lentiviruses were harvested and purified from culture medium. Fresh HEK293T cells were then infected with recombined lentiviruses bearing the different 5'-UTR-fused luciferase open-reading frames. Stably expressing HEK293T cells were then selected with the appropriate antibiotic resistance (neomycin or puromycin). After multiple passages and expansion by standard culture, liquid nitrogen stocks of these lines were established for future use and potential distribution.

3. Assess changes in luciferase activity after treatment with control drugs

Subtask 1: Measure luciferase activity from cultured cells after treatment with selected drugs.
To determine which cell line we would use for subsequent studies, we assessed HEK293T, Hela, and CHO cells transiently expressing a short 5'-UTR-luciferase construct. While all lines showed luciferase activity, HEK293T cells produced the most consistent increase in translation upon application of rapamycin (Figure 1). Thus, all subsequent studies used HEK293T cells.

Figure 1. Fold-change in luciferase activity from indicated cells expressing short 5'-UTR luciferase constructs. Rapamycin treatment is expected to increase translation and activity of mRNAs with short 5'-UTR sequences. HEK293T cells demonstrated the most consistent response to 50 nM rapamycin treatment for all three short 5'-UTR sequences. For each experimental group, luciferase activity is normalized to the non-treated (vehicle) control.

To compare the CMV and TK promoters, we assessed luciferase activity from HEK293T cells transiently transfected with the Gaussia luciferase control vector. CMV-controlled luciferase was produced at higher levels than TK-controlled vector when transfected at similar levels (Figure 2). Thus, we focused our initial studies on the CMV-controlled luciferase vectors.

Figure 2. Luciferase activity from HEK293T cells 24 hours after transient transfection with indicated amount of CMV- or TK-controlled Gaussia luciferase control vector.
We next verified that all the CMV constructs could produce viable luciferase. Compared to a negative control, all 10 constructs (5 UTR sequences × 2 luciferases = 10 constructs) produced significant increases in luciferase signal (Figure 3).

Figure 3. Luciferase activity from individual firefly (left) or Gaussia (right) constructs transiently transfected into HEK293T cells. All constructs showed significant luciferase activity compared to a non-transfected control. Note that luciferase activity is plotted on a logarithmic scale.

Subtask 2: Establish dose-response curves for selected drugs on cell lines harboring different pairs of 5'-UTR-controlled reporters. Having established that rapamycin could impact translation (Figure 1), we next measured the effect of a concentration series of rapamycin on luciferase expression using the most optimal conditions found thus far (see Major Task 4). Namely, individual 5'-UTR-firefly luciferase constructs were transiently transfected with a Gaussia luciferase control vector. Luciferase activity was measured 2 hours post-treatment with the indicated concentration of rapamycin (Figure 4). Using 10-50 nM rapamycin produced the greatest change in translation between short and long 5'-UTR controlled luciferase. Interestingly, as rapamycin concentration was increased to 1 or 5 μM, differences in translation rate were attenuated. Rapamycin is known to induce apoptosis or toxicity in cells at these concentrations. Thus, the reduced difference in translation at higher rapamycin concentrations may reflect poor cell health. This is consistent with our prediction that the dual luciferase assay system would be effective in screening out compounds that affect global translation or cell health and viability.

Figure 4. Shift in luciferase activity after rapamycin treatment at the indicated concentration. Firefly luciferase is under the control of a long (MDM2 or Cyclin D1) or short (RPL14 or TUBA4a) 5'-UTR sequence, and all data are normalized to a Gaussia luciferase control. The difference in translation between short and long UTR-controlled luciferases is maximized with 10-50 nM rapamycin treatment. Error bars are omitted for clarity.

4. Optimize assay for dynamic range, kinetics, and miniaturization

In this Major Task, Subtask 1 was to optimize the use of 96- and 384-well plate formats. Subtasks 2 and 3 would subsequently prepare the assay for a high-throughput screening campaign by optimizing for assay kinetics and dynamic range. Given that the preliminary results using rapamycin (Major Task 3) clearly indicated that the designed assay was not amenable to high-throughput screening due to low signal-to-noise, we instead describe here the efforts made to select the best conditions for future optimization.

Transient transfections led to greater signal than stably expressing cell lines. We generated stable expression cell lines harboring luciferase reporter transgenes and compared results using transiently transfected cells. Transient transfections resulted in more robust luciferase activity for all 5'-UTR sequences tested (Figure 5). We hypothesize that this is due to the better controlled timing of luciferase expression. Thus, the majority of our studies have focused on using transiently transfected cells.
Figure 5. Luciferase activity from stably or transiently transfected HEK293T cells. Firefly luciferase is under the control of a long (MDM2 or Cyclin D1) or short (RPL14 or TUBA4a) 5’-UTR sequence, and all data are normalized to a Gaussia luciferase control. For all 5’-UTR sequences tested, greater normalized signal is observed with transiently transfected cells.

Translation shifts were only observable with single experimental vectors. While we originally envisioned that the dual reporters would include both experimental groups (long and short 5’-UTRs) in a single cell line, translation shifts upon rapamycin application were best observed in cells harboring a single experimental group (long or short 5’-UTR) and a control vector. All results were normalized to the control vector, which subsequently allowed comparison of the effect of drug on translation of luciferase controlled by long or short 5’-UTR. Thus, most of our initial studies used a Gaussia luciferase control vector for normalization.

Optimal plating and drug application. Best results in the 96-well plate format were obtained using an intermediate cell seeding density (10^4 cells/well in 100 μL medium, seeded for 24 h). We measured the shift in luciferase activity at different time points after rapamycin treatment. Maximal differences in activity were observed at 2 hours after drug application (Figure 6). Thus, the majority of our studies have focused on a 2-3 hour time point after drug application.

What opportunities for training/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?
Although the cell-based assay that was originally proposed and characterized is sufficient for screening, it is not suitable for high-throughput work. Thus, we have designed an inducible expression system that we hypothesize will improve signal-to-noise. We have already begun constructing appropriate plasmid vectors using the Tet-On inducible system. During the next reporting period, validated (sequenced) vectors will be transfected into HEK293T cells and assessed as was done in the previous reporting period with the constitutive expression system. Briefly, after verifying induced protein expression in control experiments, we will optimize the 5’-UTR, dual-color reporters for the timing of induction, dosage of inducing reagent (doxycycline), and timing of drug application. Using rapamycin as a positive control reagent, we will assess whether the inducible system holds promise for future high-throughput screening campaigns. If Z scores are greater than ~0.1, we will
continue optimization for dynamic range kinetics, and miniaturization. If the inducible system is an unlikely candidate for future high-throughput screening, we will instead select the best system for optimization for screening approaches using candidate or small libraries (<1000 compounds).

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
There have been no significant changes in the primary objectives and scope of the project. However, based on our results demonstrating that the original assay design will likely be inadequate for a high-throughput screening campaign,

Actual or anticipated problems or delays and actions or plans to resolve them
Despite extensive optimization of conditions, the dynamic range of the originally designed assay will likely be inadequate for a high-throughput screening campaign. As described above, we have designed complementary approaches to the cell-based assay and will assess these methods during the next reporting period, essentially repeating Major Tasks #1-3 for the newly designed reporters. If the new assays hold promise for high-throughput screening, we will optimize them for high-throughput screening, as originally described in Major Task #4.

If none of the assays are adequate for high-throughput screening, we will optimize the best system for a future low-throughput screening campaign (<10,000 compounds). This future screen would be outside the scope of the currently funded project.

Changes that had a significant impact on expenditures
Development of additional reporter assays has primarily made use of previously purchased reagents. Thus, the additional work has not had a significant impact on expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

6. PRODUCTS

Publications, conference papers, and presentations
Nothing to Report.
Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
The cell-based reporter assay has already demonstrated utility for identifying modulators of translate state. Even if the assay cannot be optimized for high-throughput screening, the technique and reagents may be useful in future research activities and will eventually be shared by 1) publication of a research paper and 2) presentation at conferences and invited seminars.

Inventions, patents applications, and/or licenses
Nothing to Report.

Other Products
Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name</th>
<th>Project Role</th>
<th>Researcher Identifier (e.g. ORCID ID)</th>
<th>Nearest person month worked</th>
<th>Contribution to Project</th>
<th>Funding Support</th>
</tr>
</thead>
<tbody>
<tr>
<td>William Ja</td>
<td>Principal Investigator</td>
<td>ORCID ID 0000-0002-4003-7356</td>
<td>4</td>
<td>Dr. Ja is responsible for the overall direction of the project, including experimental design, troubleshooting, and data analysis.</td>
<td></td>
</tr>
<tr>
<td>Erin Keebaugh</td>
<td>Postdoctoral Scholar</td>
<td>N/A</td>
<td>6</td>
<td>Dr. Keebaugh was responsible for assessing the cell-based assay using the constitutive expression system.</td>
<td></td>
</tr>
<tr>
<td>Adwait Godbole</td>
<td>Postdoctoral Scholar</td>
<td>N/A</td>
<td>1</td>
<td>Dr. Godbole is currently responsible for implementing the inducible expression system for use in the cell-based assay.</td>
<td></td>
</tr>
</tbody>
</table>
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?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDICES

None.