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TITLE: Persistent Gene Transfer to Mitochondria

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CONTRACTING ORGANIZATION: Weill Medical College of Cornell University

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The major goal of this project was to achieve persistence of gene expression after delivery of an exogenous genome to mitochondria. The project was based on published observations that insertion of a mitochondrial targeting peptide sequence into the capsid of an AAV serotype 2 vector would permit delivery of the AAV2 vector genome to mitochondria. The AAV vector included a gene expression cassette that contained a mitochondrial promoter, terminator, and an open reading frame consistent with the mitochondrial transcription and translation. An AAV2 vector was synthesized, but gene expression was not observed in mitochondria and the vector yield was low. The system was reconstructed using AAV8 as the base serotype. The vector was synthesized, but the project ended before the new system could be tested. As a result, the specific aims of the project that focused on the fate of adeno-associated viral vector backbones were not tested. In addition, while the vector constructs included mitochondrial tRNAs as part of their design, the goal of expressing exogenous mitochondrial tRNAs in models of mitochondrial diseases such as MERRF and MELAS remains to be tested.

15. SUBJECT TERMS

Mitochondria, adeno-associated virus, AAV, MERRF, MELAS, gene therapy

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1. INTRODUCTION:

The major goal of this project was to achieve persistence of gene expression in mitochondria after delivery of an exogenous genome containing a reporter gene and mitochondrial tRNAs to correct mitochondrial tRNA deficiency diseases, MERRF and MELAS. During this project two vector systems were constructed. One was based on AAV serotype 2 and one based on AAV serotype 8. Both vector systems included the presence of a mitochondrial targeting peptide sequence on the AAV capsid to enable interaction of AAV capsids with mitochondria after the capsids accessed the cytosol. Both systems included expression cassettes bearing mitochondrial elements to enable transcription and translation of the expression cassette by mitochondrial proteins.

2. KEYWORDS:

Mitochondria, adeno-associated virus, AAV, MERRF, MELAS, gene therapy

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Tasks	Months	% complete
Specific Aim 1 – Persistence of Genome and Gene Expression		
Major Task 1: Synthesize vector plasmids	1-3	100%
Major Task 2: Produce vectors	4-5	100%
Major Task 3: In vitro binding studies	6-7	50%
Major Task 4: Evaluate vector genome persistence and structure	6-12	5%
Major Task 5: Evaluate transgene expression	6-12	5%
Specific Aim 2 – Impact on Mitochondrial Physiology		
Major Task 1: Confirm tRNA expression	13-14	0%
Major Task 2: Assess mito. protein/autophagic content	13-16	0%
Major Task 3: Assess mito physiological activity	13-16	0%
Major Task 4: Complete documentation and publications	17-18	100%

What was accomplished under these goals?

1. **Major activities.** The major activities included design and synthesis of a set of adeno-associated viruses that contained key elements of the vectors to address the specific aims of the project,.
2. **Specific objectives**. The specific objectives of this project were to synthesize vector plasmids, produce vectors, perform in vitro binding studies, evaluate vector genome persistence and structure in vitro, and to evaluate transgene expression including expression of mitochondrial tRNA molecules in vitro.
3. **Significant results.** According to the project plan, vectors based on the AAV2 capsid were synthesized incorporating an array of control conditions to confirm the veracity of the model and to extend the model to a new AAV backbone (self-complementary AAV) which has superior expression kinetics compared with the conventional single strand vector backbone. The constructs were based on the three plasmid co-transfection system for production of replication deficient AAV. The three plasmids included (1) the helper plasmid that provides replication functions naturally missing from AAV (these functions are normally provided in *trans* by a co-infection with another virus); the helper plasmid was constant throughout the constructions; (2) the rep-cap plasmid that includes the AAV genes that are required by AAV to replicate and the genes that encode the capsid proteins of the virus, and (3) a plasmid containing the viral backbone that includes inverted terminal repeats (ITRs) that enable packaging as well as an expression cassette intended for expression in mitochondria. Plasmids 2 and 3 were synthesized in several different versions. Plasmid 2 included different versions of the cap gene that incorporated a wild type version of the AAV2 cap gene encoding capsid proteins VP1, VP2, and VP3, or a version of the cap gene with a mitochondrial targeting sequence (MTS) inserted at the junction of N-terminal of the VP2 translation start site. Plasmid 3 was synthesized in versions that contained a modified version of the green fluorescence protein (GFP) gene that was optimized for the mitochondrial genome and that contained a single codon that was read as a Trp codon in mitochondria and as a STOP codon in the nucleus. The effect of using this version of mitochondrial GFP (mGFP) is to ensure that any expression of GFP is indicative of mitochondrial protein synthesis, not nuclear protein synthesis. Versions of plasmid 3 also varied in the use of (a) either a conventional single stranded AAV2 backbone (ssAAV2) and a “self-complementary” AAV2 backbone (scAAV2) that loads into AAV capsids as a DNA duplex avoiding the need to synthesize a second strand of DNA before initiating transcription of the payload, and the use of (b) either a cytomegalovirus (CMV) promoter or a mitochondrial heavy strand promoter (HSP) in the expression cassette that should limit gene expression in mitochondria to only the use of the HSP in mitochondria. Each variation was included in combination with all other variables. All plasmids were sequenced to confirm proper construction and fidelity. Despite initial indications of successful mGFP expression, there were no significant differences observed in apparent gene expression from vectors based on the presence or absence of the mitochondrial targeting sequence or the presence of the CMV or HSP promoter leading to a concern that the signal previously thought to be GFP was caused by staining artifacts. An inability to demonstrate MTS binding to mitochondria raised further concerns. The system employed in this project was modified from a series of prior publications of a similar system that successfully transduced mitochondria in vitro. The system differed from the published system in the following ways: (1) the COX8 MTS on the AAV2 was connected to VP2 through a GFP molecule (to enable localization of capsids) while the COX8 MTS in the present system was connected to the capsid through DSRed, a homolog of GFP that produces red fluorescence; (2) the marker gene for the present study, mitochondrially optimized GFP, differed from the ND4 transgene (mitochondrial genetic code) modified with a FLAG peptide tag to enable detection. When gene expression could not be confirmed, the system was recreated using a different AAV capsid from serotype 8. The DSRed was removed and a short FLAG tag was added to the COX8 MTS at the N-terminal of VP2. Finally, tRNAs encoding the mitochondrial Lys and Leu tRNAs were inserted in the 5’ untranslated region of the mGFP to enable a test of the hypothesis that AAV-mediated gene transfer can correct mitochondrial tRNA deficiencies. Two AAV8 vectors were designed and produced. A native AAV8 capsid containing the mitochondrially optimized tRNA/mGFP expression cassette was produced at >10E13 genome copies per ml, one hundred times more vector per prep than was achieved with the

AAV2 vector system. The companion vector, an AAV8 capsid bearing the COX8MTS sequence at the N-terminal of the VP2 protein containing a mitochondrially optimizing expression cassette with both tRNAs and mGFP was also designed and produced. The AAV8 control vector was ready before the AAV8MTS vector and was tested for GFP expression. As expected, the construct lacking an MTS on the capsid did not deliver the AAV genome to the mitochondria, even when delivered to cells in a long-term in vitro culture system (differentiated airway epithelium grown at an air-liquid interface for more than 2 weeks). The production of the AAV8MTS vector was completed as the funding period came to an end. The PI is actively seeking additional funding to continue to project so that the hypothesis can be tested.

Goals not met. While we designed and produced two sets of AAV vectors, we were not able to demonstrate AAV-mediated gene transfer to mitochondria. Because we were not able to achieve mitochondrial gene transfer, we could not test hypotheses related to the long term disposition of the AAV genome in mitochondria, nor were we able to test whether tRNAs could be expressed from an AAV vector in mitochondria for the purpose of treating mitochondrial diseases resulting from mutations of tRNAs in the mitochondrial genome. The inability to test these hypotheses was disappointing, but the, as yet, untested AAV8MTS vector may still succeed in achieving mitochondrial gene delivery.

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

A postdoctoral fellow was recruited for this project. Ciara Agresti, PhD, completed the construction of the initial vector system. In the process, Dr. Agresti received experience in vector design, synthesis, and production. She also receiving mentoring in the cell and molecular biology aspects of vector infection and mitochondrial molecular biology as well as data analysis and presentation and professionalism. After working on the project for 10 months, Dr. Agresti received and accepted an offer of full time employment as a medical writer.

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?

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Not applicable.

4. IMPACT:

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

Nothing to report. Successful gene delivery could not be demonstrated.

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?

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The inability to achieve mitochondrial gene transfer using a system with high similarity to a published model may indicate that there remain unidentified critical parameters in this system that need to be optimized. The majority of mitochondrial diseases result from mutations in nuclear genes that encode proteins that are later imported into mitochondria, and these diseases are well-served by conventional AAV-mediated gene therapy. The thirteen protein encoding genes in the mitochondrial genome are subject to mutation, but in several cases, their function has been supplemented by exogenous AAV-mediated expression of the gene in the nucleus and addition of a mitochondrial targeting sequence. Diseases caused by mutations in mitochondrial tRNA cannot necessarily be solved by gene expression in the nucleus. Therefore, the development of the technology at issue in this project would be of value in the setting of diseases such as MERRF and MELAS, and a successful conclusion of the project is worth pursuing.

5. CHANGES/PROBLEMS:

Nothing to report.

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

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

Construction of the initial vector system based on AAV2 resulted in production and purification of intact AAV2 particles. However, no gene expression was observed in mitochondria using these particles. In addition, the titer of the vector was typical for AAV2, but low compared to other AAV serotype vectors. As a result, the expression system was re-assembled using an AAV serotype 8 vector capsid. The funding period expired as experiments to test the efficacy of the AAV8 expression system were beginning.

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

Nothing to report.

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.

We were unable to demonstrate AAV-mediated gene expression in mitochondria. Therefore, we have not developed a manuscript.

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

Nothing to report.

Other publications, conference papers and presentations.

We were unable to demonstrate AAV-mediated gene expression in mitochondria. Therefore, we did not submit an abstract to a conference. .

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

Nothing to report.

- **Technologies or techniques**

Nothing to report.

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

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Reporting period: last 18 months

Name:	Philip L. Leopold
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-3986-1815
Nearest person month worked:	2.1 (over last 18 months)
Contribution to Project:	Dr. Leopold oversaw the project and performed immunohistochemical staining, light microscopy, and fluorescence microscopy associated with the project. He also participated in the design of the AAV8 plasmids.
Funding Support:	This award.

Name:	Stephen M. Kaminsky
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-1869-834X
Nearest person month worked:	0.54 (over last 18 months)
Contribution to Project:	Dr. Kaminsky aided in the design of plasmids for AAV vector production and provided guidance to Dr. Agresti and Mr. Cisse regarding AAV production.
Funding Support:	This award.

Name:	Ciara A. Agresti, PhD
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	0000-0002-0883-5577
Nearest person month worked:	7.2 (over last 18 months)
Contribution to Project:	Dr. Agresti synthesized plasmids, produced AAV, infected cells, isolated mitochondria, performed binding studies, and evaluated transgene expression.
Funding Support:	This award.

Name:	Georges-Ibrahim Cisse
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.0 (over last 18 months)
Contribution to Project:	Mr. Cisse synthesized plasmids and produced AAV vector using the AAV8 serotype system.
Funding Support:	This award.

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

Leopold other support update

Added

R01 HL134163 (Crystal) **09/15/16-08/31/20 (NCE)** **1.20 calendar**
NIH/NHLBI $300,000
In Vivo Biomarker that Identifies Waterpipe Smoking-Related Lung Health
The focus of this study is to assess airway and nasal epithelial transcriptome and measures of lung health to determine if the nasal epithelial transcriptome can be used as a biomarker of lung toxicity resulting from waterpipe smoking.
Role: Co-Investigator

Kaminsky other support

Added

U01DA048524 (Crystal) **02/01/19 – 01/31/21** **1.2 calendar**
NIH/NIA $336,342
Clinical Assessment of Anti-cocaine Vaccine dAdGNE in Cocaine Addicts
The hypothesis of this project is linking a cocaine analog to Ad capsid proteins would elicit high-affinity, high-titer antibodies against cocaine, sufficient to sequester systemically administered drug from access to the brain, with consequent reduction in cocaine-induced behavior.
Role: Co-Investigator

R01EB027918 (Crystal/Ballon) **07/01/19-03/31/22** **0.6 calendar**
NIH/NBIB $405,157
Rapid Noninvasive Whole-body Imaging of AAV Gene Transfer Vectors
The focus of this project is to develop a noninvasive method to assess the biodistribution of AAV gene transfer using radiolabeling technology to study viral vector biodistribution of AAV administered intravenously or intracisternally to non-human primates
Role: Co-Investigator

The Daedalus Fund for Innovation **07/01/19-6/30/20** **0.6 calendar**
N/A $150,000
Gene Therapy for Chronic Traumatic Encephalopathy
The hypotheses of this project is that generating anti-pTau monoclonal antibodies within the CNS could halt the progression of the disease by enhancing pTau removal via antibody-mediated clearance.
Role: Co-Investigator

Friedreich's Ataxia Research Alliance **10/1/19-9/30/20** **0.6 calendar**
N/A $99,989
The major goal of this project is to carry out a murine dose-ranging toxicology study with intravenous administration of AAVrh.10hFXN to insure safety (scaled to humans), of the proposed doses in the clinical trial; and to leverage the clinical evaluation of individuals with FARA to carry out the cardiac-related screening studies to identify potential candidates for the clinical trial.
Role: Co-Investigator

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

See attached.

9. APPENDICES:

N/A