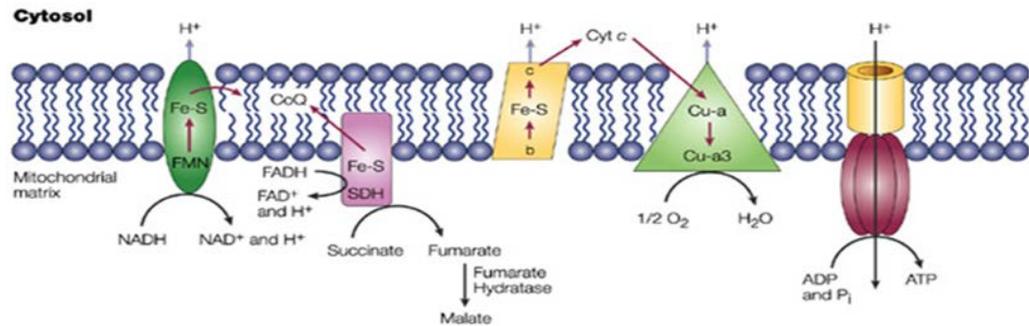


DEVELOPMENT OF AN INNOVATIVE GENE THERAPY METHOD TO CURE MITOCHONDRIAL AGING- "BACKING UP" THE MITOCHONDRIAL GENOME

Background and Overview

Mitochondria are the power plants of the cell and are the only cellular organelle that contain their own DNA. Mutations in mitochondrial genes occur as a result of constant exposure to reactive oxygen species produced by the energy generation process (oxidative phosphorylation).

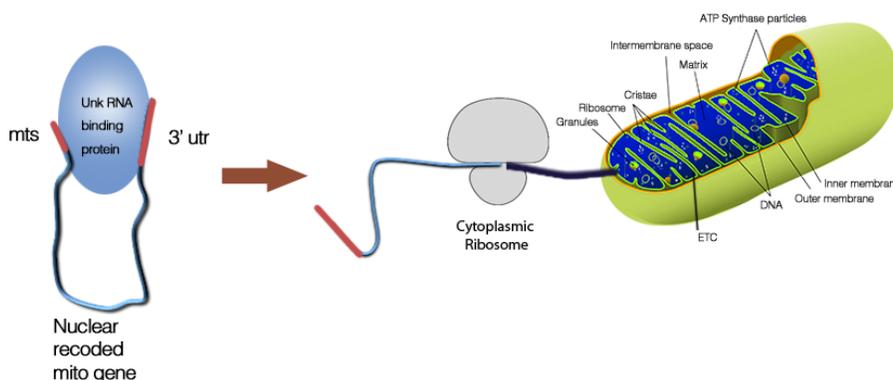


| Complex | I | II | III | IV | V |
|-------------------------|---------------------------------------|----|-----|-------------------|--------------|
| mtDNA-encoded component | ND1 ND4L ND2 ND5 ND3 ND6 ND4 | | CyB | Co1 Co2 Co3 | ATP6 ATP8 |

Unlike the nucleus, mitochondria lack an efficient system to repair damaged DNA, and these mutations accumulate over time. Each cell in the body depends on the efficient production of energy by the mitochondria. As we age, these accumulated mutations result in a damaged system and an increase in oxidative stress throughout the body. It is no coincidence that organisms which age more slowly consistently display lower rates of mitochondrial free radical damage¹. Reversing and/or preventing damage to mitochondrial DNA is a key factor in slowing the aging process. At the SENS Research Foundation, we are in the early stages of creating an innovative system to repair mitochondrial mutations. If this project is successful, we will have demonstrated, for the first time, a mechanism to render the mitochondrial genome redundant by creating a "backup copy" in the nuclear genome. The development of this technology will be a major milestone in the prevention and reversal of aging in the human body.

In this project, engineered mitochondrial genes will be used to restore function to cells that contain defective mitochondrial genes. We are currently developing a unique method for targeting these nuclear encoded genes to the mitochondria; this step has been the bottleneck in research on this topic over the last decade. In our system, the mRNA from the engineered mitochondrial gene is targeted to the mitochondrial surface *before* it is translated into a protein using a co-translational import strategy (see figure below). Once imported, it is incorporated into the correct location in the inner mitochondrial membrane. This precise targeting is achieved by adding a specific tag to both ends of the mRNA to direct the molecule to the mitochondrial surface. Our current results indicate that this system results in a significantly higher import efficiency than

Co - Translational Import



any previously published research. The long-term goal of this project is to utilize this improved targeting strategy to rescue mutated mitochondrial DNA, and thus prevent and cure one of the major causes of cellular aging. If this project is successful, we will have developed a groundbreaking method that will provide us with

the capability to render the mitochondrial genome redundant by creating a "backup copy" in the nuclear genome.

Our six month goal is two-fold. First, we will create cells that are null for two mitochondrial genes: CyB and ATP8. Second, we will "cure" the cells by inserting engineered versions of CyB and ATP8 into the *nuclear* genome, rather than the mitochondrial genome, and then target the functional protein into the mitochondria.

Research Plan

Objective 1 – To generate cell lines that are completely mutant for the ATP8 and CyB genes.

In order to develop a system for the rescue of mitochondrial mutants, the necessary first step is to develop mutant cell lines to rescue. This is complicated by the fact that there are ~5,000 copies of the mitochondrial genome per cell. In a diseased patient, cells contain a mixture of both normal and mutant mitochondrial genomes. We have obtained cells derived from two patients who suffer from null mutations in ATP8² and CyB³, respectively. In order to test our repair system, we must make these cells 100% mutant. To achieve this, a chemical method is utilized to destroy nearly all copies of the mitochondrial genome until few copies remain⁴. The cells are then allowed to recover and clones are screened for a perfectly mutant cell⁵ using a method known as qPCR (quantitative PCR, which we have found to be more sensitive for this purpose than traditional PCR approaches). Once a complete mutant is generated, the integrity of the mitochondrial DNA will be confirmed by DNA sequencing.

In order to functionally confirm that the cells are complete mutants, the generated cells will be tested by incubation in media in which the only provided energy source is galactose. Cells produce energy from galactose using oxidative phosphorylation; therefore, only non-mutant cells will survive, while the complete mutant cells (which lack a crucial enzyme in the oxidative phosphorylation pathway, ATP8 or CyB) will die.

Objective 2 - To rescue mutant cells with our engineered ATP8 and CyB gene constructs.

We have generated and tested retroviral DNA plasmids containing our double targeted versions of ATP8 and CyB in normal cells. The next step is to generate the viruses and infect the mutant cells created in Objective 1. To test the effectiveness of our approach, we will quantify the expression of the mRNA using RT-qPCR (reverse transcription quantitative PCR), evaluate the mitochondrial proteins produced using western blotting, and examine the targeting of the protein to the correct mitochondrial protein complexes using native protein gels and co-immunoprecipitation. Functional rescue will be analyzed by testing the survival of cells in galactose as described in Objective 1 and by performing in-gel activity assays of individual OxPhos complexes (in this case complexes III and V).

Budget

We are requesting a budget of \$21,000 to pay for the supplies necessary to continue this project. We have a talented team of highly trained mitochondrial biologists working on mitoSENS. Right now the rate-limiting factor is the cost of the expensive reagents that we use for these experiments. Increasing our funding will allow us to double the pace of our research and bring results to the public much faster. Two areas which are costly are reagents for operating our qPCR machine and for culturing our mutant cells. The bulk of the money will be spent on reagents for those two types of work. Additionally, valuable hours are spent manually counting cells under a microscope, and the purchase of an automated cell counter would speed up this work significantly and would provide a lasting contribution to lab efficiency.

References

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RESEARCH FACILITIES

SENS Research Foundation, Inc. (SRF), is a non-profit biotechnology research charity based in Mountain View, California. The work in this proposal will take place at our Mountain View research center, which consists of office space we converted to lab space in late 2010. Our researchers can also draw upon specialized equipment and intellectual resources at the nearby universities, including Stanford University, University of California (San Francisco, Berkeley, and Davis campuses), as well as from a number of biotechnology and pharmaceutical companies in the region.

The SRF facility in Mountain View houses both our laboratory and office space. It contains approximately 2000 square feet of laboratory facilities for biochemistry, cell culture, microbiology, molecular biology, and cell biology research. Dr. Matthew O'Connor, the Principal Investigator of this grant, has a dedicated laboratory, as well as access to shared space and equipment. Equipment for cell culture, cell biology, and microbiology research includes incubators, biosafety cabinets, centrifuges, and microscopes, and equipment for molecular biology, biochemistry research includes a flow cytometer, HPLC, gel electrophoresis equipment, and an ABS Real-Time 96 well gradient thermocycler. Cryogenics equipment includes refrigerators/freezers (4°, -20°, and -80°), dry ice, and liquid nitrogen tanks.

DETAILED BUDGET

qPCR experiments

The qPCR experiments are the main focus of Objective 1, and they are also one of the more costly experiments in our research. The bulk of the cost (~83%) comes from the reagent, Qiagen 2x SYBR green master mix. The discounted price for this reagent is \$864 for 10 mls. Each reaction requires 10 ul of the master mix, and therefore each 96 well plate requires 960 ul. This translates to a reagent cost of approximately \$83/plate. We have experimented with less expensive reagents; however, they resulted in inconsistent results which wasted time and money in the long run.

Additional reagents needed for the qPCR experiments include mitochondrial isolation reagents (\$225/kit - Pierce. Also performed manually using a dounce homogenization technique depending on the experiment), Qiagen DNeasy Blood and Tissue kits (\$212/kit), 96-well PCR plates (\$36/10 plates), and Sigma RNAzol (\$299/kit). Overall, the reagents necessary total

approximately \$100/plate. At the research pace outlined in Objective 1, this translates to: 13 weeks x 5 days x \$100/plate = \$6,500.

Cell culture supplies

Throughout the six-month project, it will be necessary to continuously culture cells. Although we have the necessary equipment for cell culture, we will need to purchase the required consumables and reagents. These include sterile petri dishes (\$142/case of 360), serological pipettes (\$29/case), serum (\$400/500 ml), dialyzed serum (\$500/500 ml), and media. It is necessary to use dialyzed serum for our glucose survival experiments because we have determined that the small amount of glucose (~0.5 mM) contained in ordinary cow serum is sufficient for some OxPhos null (Rho 0) cells to survive despite the absence of additional glucose in the growth media. At our estimated research pace, we calculate that the increase in reagents and consumables will cost approximately \$1,250/month. Therefore, over the course of six months the additional supply costs will total approximately \$7,500.

Analytical and functional experiments

Once the mutant cells have been generated in Objective 1, they will be transfected with retroviral plasmids previously generated in our laboratory. In order to monitor the effectiveness of our approach, analytical and functional experiments will include the following (as described in more detail in Objective 2 of our proposal): RT-qPCR, Western blotting, analysis using native protein gels and co-immunoprecipitation, and a functional assay. There are a large number of reagents and consumables necessary for these experiments, including the following: qPCR reagents (as described above), Novex polyacrylamide gels (\$115/box standard gels, \$199/box for gradient and native), PVDF membranes (\$311/roll) and other blotting supplies, primary and secondary antibodies (~\$300/tube), Biohit multichannel pipette tips (\$58/case), and serological pipettes (\$29/case). Other necessary reagents include primers, buffers and transfer reagents for Western blotting, buffers and chemicals for protein gels, staining, and in-gel activity assays, and chemicals necessary for the functional testing of cell survival. It is difficult to determine an exact cost for consumables as the need depends on experimental results. However, we have estimated costs for the consumables based on the prices listed above, as well as a cost analysis of our records from similar laboratory experiments. Our predicted costs for the consumables and reagents for Objective 2 are approximately \$3,000.

Countless Cell Counter

Valuable man-hours are spent manually counting cells under a microscope. The purchase of an automated cell counter would speed up this work significantly and also serve as a lasting contribution to lab efficiency. The cost of the counter (including 750 cartridges) is \$4000.

Total: **\$21,000**