sens research foundation

reimagine aging

research report 2011

The last twelve months have seen us expand the scope of work being performed both in our own Research Center in Mountain View, CA and in extramural institutions. We've moved significantly forward on planned projects, increased and optimized staffing in key areas, and established firm ties with new research partners worldwide. Given this past year's successes, we're better equipped than ever to perform, support, and promote critical proof-of-concept research into the regenerative medicines that may mean the difference between vibrant good health and painful debilitating disease for all of us as we age.

We're excited to be a part of this revolution in scientific innovation, grateful to everyone who has supported us through their generous gifts of time and funding, and delighted to have multiple exciting developments to report on the research front. This report presents summaries of our intra- and extramural research, followed by more detailed accounts of each research group's achievements and plans for the upcoming year. We're thrilled to have so much to share and hope you'll find it an interesting read. We encourage you to visit out website at sens.org or contact us directly for more information about any of the projects described in this report.

Respectfully submitted,

Tanya Jones
Director of Research Operations

Michael Rae
CSO Team Lead

Aubrey de Grey
Chief Science Officer

MitoSENS

Mitochondrial mutations accumulate as we age, but a mitochondrion is less able to repair itself when damaged than the nucleus is. Since thirteen genes critical to a cell's energy production and respiration reside in the mitochondria, mutation can cause serious harm. The goal of MitoSENS in general, and this intramural research project specifically, is to place copies of these thirteen mitochondrial genes into the nucleus of the cell, as a backup method for production of these proteins.

2011 Milestones Achieved

Our researchers have successfully established several mammalian cell line models, all stably transfected with five mitochondrial gene candidates (ND1, ND4, ATP6, ATP8, and Cytochrome B) modified for nuclear expression. We have also analyzed expression and mitochondrial localization of nuclear-expressed mitochondrial genes by immunofluorescence microscopy and established and optimized protocols for isolation of mitochondrial fractions, extraction of mitochondrial inner membrane proteins, and analysis of oxidative phosphorylation components. Additionally we have now standardized assays to assess the enzymatic activities of Complexes I and V of the respiratory chain and optimized conditions for immunocapture of those complexes.

Upcoming Milestones

In 2012, our efforts will include rigorous biochemical and functional characterization of the oxidative phosphorylation complexes following nuclear expression of all five current gene candidates. Specific goals include the demonstration and confirmation of the successful incorporation of these candidates into their relevant complexes; validation of localization of nuclear-expressed proteins to the inner membrane of the mitochondrion; and then functional rejuvenation of those respiratory complexes.

LysoSENS

Lysosomes are a cell's last line of defense for removing unwanted or damaged parts of a cell, but occasionally the lysosome encounters an object it cannot break down or otherwise eliminate. When such things find their way into the lysosome, they remain there and, when present in sufficient abundance, cause the lysosome to stop working. This is particularly harmful in non-dividing cells, because cells that divide dilute this debris during mitosis. Failure to remove these materials is a key factor in diseases like atherosclerosis and macular degeneration, and we have both intra- and extramural projects investigating methods to clear the lysosomes of this accumulated junk.

2011 Milestones Achieved

At the SENS Foundation Research Center, we are primarily investigating potential therapies for macular degeneration. We have developed protocols for the routine large-scale production and purification of A2E, the major non-degradable compound in the retina, which was used in a series of *in vitro* degradation assays using commercially available laccase and manganese peroxidase (MnP). These assays revealed that both laccase and MnP successfully and substantially promote A2E degradation. We are now producing recombinant laccase and MnP made in yeast, and have discovered that purified recombinant manganese peroxidase is highly mannosylated, potentially facilitating lysosomal importation for therapeutic exploitation.

Our atherosclerosis research is being performed at Rice University, where our team successfully isolated several strains of bacteria that readily degrade 7-ketocholesterol (7KC), the major non-degradable compound in blood vessels, and mapped out the degradation pathway in one strain. A candidate enzyme identified in these studies was re-engineered to target the lysosome, it was tested in human fibroblasts and found to have superior efficacy at preventing cellular 7KC toxicity in experiments of short duration. The team also assayed methods of generating lipofuscin *in vitro* and established a cell-based model for studying lipofuscin, a heterogeneous lysosomal material that accumulates in many tissues in aging, in non-dividing cells. This led, in turn, to the creation of a reproducible means of quantifying lipofuscin using flow cytometry that will facilitate high-throughput testing of treatments intended to reduce or eliminate it.

Upcoming Milestones

At our Research Center, we will complete testing of mannose receptor-mediated delivery of MnP to lysosomes of macrophages and retinal pigment epithelial (RPE) cells loaded with A2E. By the end of 2012, the group will be focusing more on efficient large-scale production of candidate A2E-degrading enzymes, optimizing their catalytic activity towards A2E, and increasing the targeting to the lysosomes of RPE cells. We hope to identify and charac-

terize at least one viable strategy to eliminate A2E accumulation using our cell-based model of age-related macular degeneration by the end of the year.

At Rice University, a manuscript (the fifth from this project) has been accepted for publication in a highly respected peer-reviewed journal reporting the latest results of our work on 7KC. We are also performing additional assays to determine the most effective means of reducing 7KC toxicity, including redesign of our engineered enzyme using different methods of lysosomal targeting and methods to reduce secondary toxicity through export and other means. To facilitate lysosomal efflux of oxysterols, our lab is currently constructing a set of vectors encoding endogenous sterol transport proteins which will be tested both separately and in conjunction with our other strategies. We are also developing cell-based models for atherosclerosis in which we will assay the effect of potential therapeutic approaches to reducing cellular oxysterol burden.

OncoSENS

One tenet of SENS is that a comprehensive cure for cancer, which is the goal of our OncoSENS research program, will obviate the need to address non-specific DNA damage. Existing data suggest that this is indeed true in regard to mutations (changes in the DNA sequence), but the question remains more open in regard to epimutations, which are changes in which genes are on or off in a given cell. A question also exists about whether the rate of epimutation accumulation over the adult lifespan is low enough for the entire SENS strategy to be comprehensive, but in order to answer that question, we needed new tools. SENS Foundation-funded research at Albert Einstein College of Medicine has accomplished precisely this end.

2011 Milestones Achieved

Our research has resulted in the development of a bisulfite sequencing method able to detect DNA methylation patterns within promoter regions of a number of genes within a single cell; additionally, we have done preliminary tests on a genome-wide DNA methylation assay. We are currently preparing a manuscript on the single-cell methylome typing method for submission to a major scientific journal, and a patent for this method is being pursued.

Upcoming Milestones

Work continues to optimize the sensitivity of the assay in detecting alterations in cytosine methylation. Genome-wide experiments are being repeated, and work is underway to expand target coverage; in addition, the work will be expanded to compare cells from young and old animals. Once this is complete, work will commence on the project's ultimate goal -- to quantify genome-wide levels of epimutation in the nuclei of single neurons from aged mice.

ApoptoSENS

Cellular senescence is a genetic program which normal dividing cells invoke in order to prevent excessive cellular division. This is initially a protective mechanism, but some senescent cells persist long after their usefulness has expired, ignoring signals for programmed cell death (apoptosis) while growing larger and, often, secreting various inflammatory molecules that disrupt the environment in which neighboring cells have to function. These inflammatory molecules can have many effects, from the induction of an immune response to the degradation of the extracellular environment and alteration of the behavior of neighboring cells. Targeting senescent cells both for elimination and for the modulation of their secretions is the focus of an extramural project being funded at the Buck Institute for Research on Aging.

2011 Milestones Achieved

Our researchers screened a library of 1400 compounds for their ability to reduce the secretion of Interleukin-6 (IL-6) (as a representative constituent of the pathological secretory phenotype of senescent cells), while maintaining cell viability. Of the 25 most promising candidates to emerge from this screen, a lead candidate (apigenen) was selected based on prior knowledge of its properties, and its mechanism of action in lowering IL-6 secretion was probed. Related compounds with protective effects against protein aggregation in model organisms were identified, tested, and demonstrated to lower IL-6 production in the same DNA damage model.

Upcoming Milestones

Investigation will continue into the role of protein aggregation in senescence and into the mechanisms of action of apigenin and related compounds. Should results warrant, an animal model suitable for studying cellular senescence *in vivo* will provide the ability to look at the accumulation of senescent cells directly and test the effects of suppressing the senescence-associated secretory phenotype against age-related pathology.

GlycoSENS

The elasticity of the artery wall, the flexibility of the lens of the eye, and the high tensile strength of the ligaments are examples of functions that rely on regularity of a protein lattice for maintaining proper performance. But chemical reactions with other molecules in the extracellular space occasionally result in a chemical bond (a so-

called crosslink) between two nearby proteins that were previously free-moving, impairing their ability to slide across or along each other, thereby impairing function. It is the goal of this project to identify chemicals that can react with these crosslinks and break them without reacting with anything that we don't want to break. Our focus is on glucosepane, known to be the most abundant such crosslink in aged human tissues.

2011 Milestones Achieved

This is a new project for the Foundation. In 2011, we established a Center of Excellence for GlycoSENS and other rejuvenation research at Cambridge University and hired postdoctoral fellow Rhian Grainger to design and perform experiments to develop reagents that can detect proteins bearing glucosepane crosslinks, facilitating further studies on its structure, abundance, and cleavage by small molecules. We also established a collaboration with researchers at Yale University, who will lend their expertise in generating advanced glycation end-products and lead efforts in developing agents which may be able to cleave glucosepane.

Upcoming Milestones

Once glucosepane-detecting reagents have been developed, we will work to confirm the distribution and structure of glucosepane in aged tissue and to test potential glucosepane-breaking drugs. Cleaving agents will be developed at Yale and tested both *in vitro* and *in vivo* at Cambridge.

AmyloSENS

Aging bodies slowly accumulate extracellular aggregates around their cells, sticky clumps of proteins that interfere with cells' and tissues' ability to carry out their functions. Many of these aggregates are called amyloids of one variety or another, and these amyloids are involved in a host of diseases, like heart disease, Alzheimer's and diabetes. Removing these aggregates is necessary to rejuvenate the structure and function of the aging body, and we began a project in 2011 to begin addressing this problem.

2011 Milestones Achieved

Researchers at the University of Texas and at Harvard University, with SENS Foundation funding, have optimized methods for generating transthyretin (TTR) fibrils and cross-linked TTR as immunogens. Mice are currently being vaccinated with these TTR immunogens to generate monoclonal antibodies against pathogenic TTR for potential use in diagnosis of senile systemic amyloidosis (SSA) and other diseases of TTR amyloids. Their fibrillar and soluble TTR will also be used as substrates for testing catalytic activity of therapeutic TTR-degrading catalytic antibodies. Indeed, they have already discovered and initially characterized natural human catalytic antibodies specific for fibrillar TTR which have therapeutic potential.

Upcoming Milestones

We will next generate monoclonal versions of the murine anti-TTR antibodies identified in prior work and determine their diagnostic and therapeutic potential. Additionally, we will continue to screen for optimal therapy-grade catalytic antibodies and characterize their specificity for pathogenic TTR. We will determine whether our lead diagnostic and therapeutic antibodies can react with pathogenic TTR present in blood samples and tissues from patients with SSA in ways consistent with their diagnostic and therapeutic purposes and will initiate preliminary studies designed to validate those antibodies in an animal model of senile systemic amyloidosis.

ApoptoSENS

2011 Milestones Achieved

Researchers at the University of Arizona successfully completed the first round of a multi-arm study of potential interventions against immunological aging in the mouse, based on combatting the expansion of anergic killer T-cell clones and of contraction of naïve T-cell production. The team used the results to design and launch a new round of studies using refined protocols to allow for more aggressive intervention of promising therapies, and to home in specifically on adaptive T-cell immunity, the key aspect of the aging immune system that is impaired by these expansions. These new studies should reveal the best-case therapeutic potential of selected interventions.

Upcoming Milestones

In addition to completing studies initiated in 2011 and evaluating prospects for further development, we have initiated a research collaboration with a highly respected tissue engineering center to develop a more advanced approach to the problem of impaired production of naïve T-cells, and are exploring a more robust test of an alternative approach to clearance of anergic killer T-cells.

MitoSENS

SENS Foundation Research Center, Mountain View, CA

Researchers: *Dr. Matthew "Oki" O'Connor, Dr. Gayathri Swaminathan, Daniel Kimbel*

Budget: $210,000 (2011), $260,000 (2012)

Background

Accumulation of mutations in mitochondrial DNA is recognized as a significant consequence of aging and is implicated in the metabolic derangement of aging and in accelerating the course of the degenerative aging process as a whole. Mutations in the mitochondrial genes occur as a consequence of constant exposure to reactive oxygen species resulting from the mitochondrial energy generation process. Because mitochondria lack an efficient repair mechanism, these mutations accumulate over time and compromise respiratory chain function and hence energy generation. One need only look at the mitochondrial genetic diseases to see the similarities to many of the diseases and maladies of aging. For example, mutations in ND1 have been implicated in the development of Parkinson's disease, and Cytochrome B (CYB) mutations can cause muscle fatigue/ exercise intolerance in young patients.

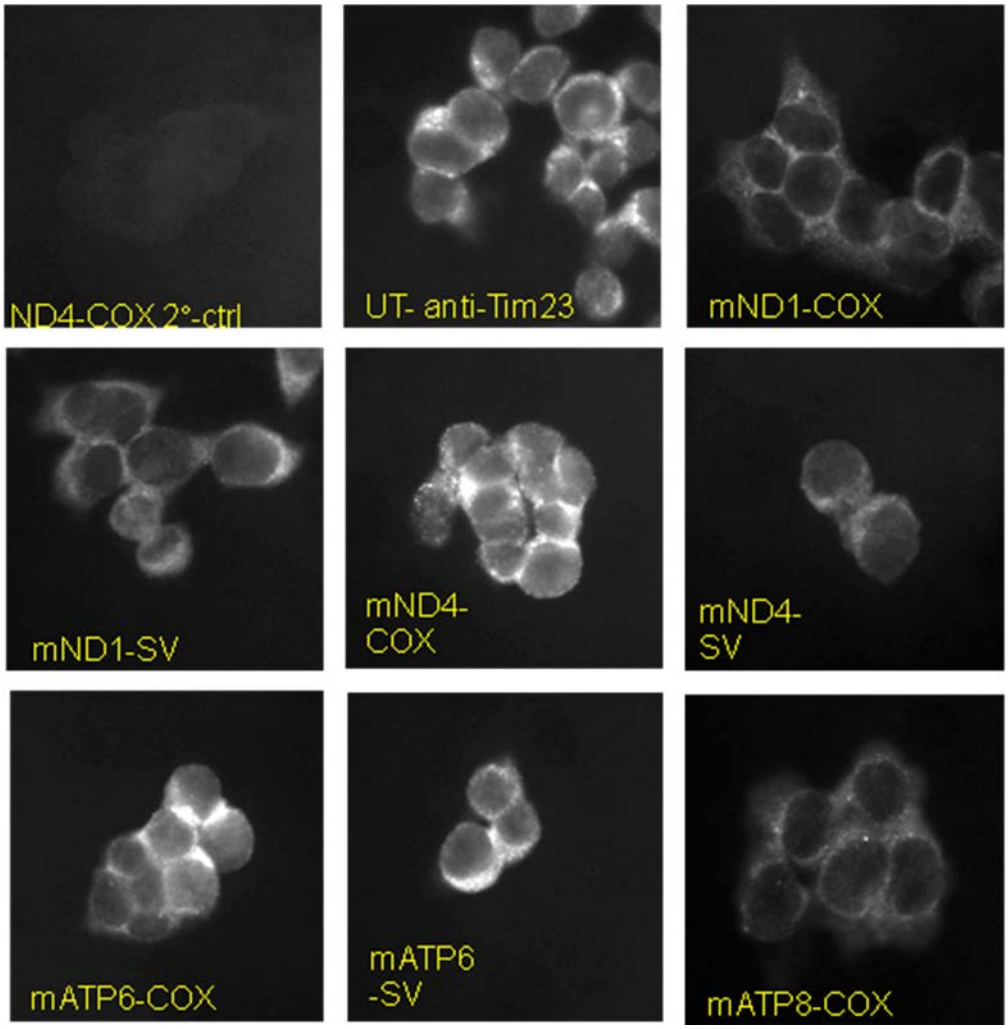
Nuclear Expression

The essential strategy we are using in our approach to MitoSENS is to render the mitochondrial genome redundant. The plan is to engineer versions of the 13 protein-encoding mitochondrial genes involved in respiratory chain function (out of a total of 37 mitochondrial genes) that will be suitable for expression from the cell nucleus and for subsequent delivery of their protein products into the mitochondria. We will then use gene therapy to insert these modified mitochondrial genes into the nuclear genome of the cell and validate the targeting and functional importation of these 'allotopically-expressed' proteins into the mitochondria.

Previous attempts at allotopic expression have experienced only limited success due to problems with import of the proteins into the mitochondria, which were likely caused by the hydrophobic nature of the proteins. In addition to being ineffective, incomplete protein import is likely toxic to the mitochondrion and ultimately to the cell. To sidestep these problems and achieve optimal mitochondrial import, a co-translational import strategy will be used. This strategy has been successfully employed by Professor Corral-Debrinski in work previously funded by SENS Foundation. The improvement that Dr. Corral-Debrinski has pioneered is to tag the RNA of the genes with sequences that not only target the proteins to the mitochondria, but direct the RNA to the mitochondrial surface before it is translated into protein. This approach prevents the convoluted folding of these proteins during translation in the watery environment of the cytosol, improving the efficiency of protein import.

Once we mastered the progress that our collaborators had already made, our next task consisted of extending these advances to all 13 proteins encoded by the mitochondrial genome. During the year, we created many cell lines with our allotopic expression vectors stably integrated into their genomes (along with triplicate versions of negative controls). We have created and produced allotopic expression vectors of 5 mitochondrial genes: ND1, ND4, CYB, ATP6, and ATP8. We have made a complete set (20 stable cell lines, four variants for each of the five genes, including controls) in two different types of cells (HEK293 human embryonic kidney cells and HCA2 primary fibroblasts). We have also created partial sets for specific experiments in NIH 3T3 murine embryonic cells, and for cell lines from patients mutant for ND1 and ATP6. Nuclear integration and mRNA expression were confirmed by PCR and RT-PCR, respectively. Expression and mitochondrial localization of allotopically-expressed mitochondrial genes were confirmed by immunofluorescence microscopy. Specifically, we have allotopically expressed 4 mitochondrial proteins (ND1, ND4, ATP6, and ATP8) in HEK293 cells, and in our preliminary results found them localized to the mitochondria (See illustration on page 6).

To validate and extend our results, we have established a collaboration with Scott Needham, principal of antibody biotechnology firm LifeResearch, to provide us with custom-synthesized antibodies. Commercially-available antibodies against the 13 target proteins are insufficient for the next phases of our research; the high-quality antibodies that LifeResearch can deliver will enable the detection, visualization, and purification of our allotopically-expressed proteins. Scott is a SENS Foundation supporter and has been synthesizing custom antibodies for us at no charge. We work together to design the targets, and he commissions the entire synthesis and purification. So far he has synthesized three antibodies for the MitoSENS project. We have tested two of them and had success with one, and we are planning more work with these antibodies for the future. This is an invaluable collaboration, because it includes not only the synthesis of the antibodies



Preliminary results showing allotopically expressed proteins localized to the mitochondria.

themselves but also Scott's expertise; and we are working well together to optimize the characterization and troubleshooting of new antibodies.

To allow for detection and assessment of the effects of allotopic expression of proteins on the mitochondrial energy-production system, we have established conditions for performing Blue Native Gel Electrophoresis (BNGE) to assess the oxidative phosphorylation (OXPHOS) complexes in essentially every cell line we are working with. We are now able to observe the five complexes of the respiratory chain clearly by Coomassie staining; results using this technique suggest that the complexes are not negatively impacted by the exogenous expression and delivery of the allotopically-expressed proteins we are developing. We have also optimized the conditions for, and performed, 'in-gel activity' assays to assess the enzymatic activities of Complex I and Complex V in different cell lines, and immunoisolated Complexes I and V to analyze the incorporation of the allotopically expressed ND1-FLAG and ATP6-FLAG in cells.

Early in this research, more direct detection of the expressed proteins was problematic. Traditional methods (such as simple Western blotting) did not work. Since then, however, we have succeeded in detecting the FLAG-tagged proteins biochemically by immunoprecipitating the complexes from mitochondrial extracts and then blotting for FLAG. Using this method, we have reproducibly demonstrated the interaction of allotopically-expressed ND1 and ATP6 with proteins of Complex I and V respectively. This a highly positive finding, although it is important for us to confirm that the protein is present at adequate levels – see *"Future Work"* for details on how we are tackling this problem.

Relevance

Using mitochondrial gene therapy as a strategy to correct mitochondrial dysfunction has many advantages. In theory, such gene therapy could be used both to prevent and to correct the effects of mitochondrial mutations. Moreover, any therapy we develop could, in principle, be used to treat any of the known diseases of the mitochondria, such as LHON (Leber Hereditary Optic Neuropathy), Leigh syndrome, and NARP (neurogenic muscle weakness,

ataxia, and retinitis pigmentosa), all of which are debilitating diseases. Indeed, allotopic expression is already being tested in human clinical trials to treat LHON. Treatment of these known and well-characterized diseases is a licensable therapeutic indication under current regulation, which therefore constitutes an entry point for the first human uses of mitochondrial gene therapies that emerge from our work. This foundation, and the experience of patients treated with allotopically-expressed mitochondrial genes for mitochondriopathies, will give rejuvenation researchers an excellent opportunity to develop mitochondrial gene therapies for use against the systemic metabolic derangement imposed by age-related mitochondrial mutation.

Future Work

The most important development goals for our group in 2012 are to develop superior solutions to overcome the current limits on our ability to detect and express allotopically-expressed proteins in situ within the complexes of the respiratory chain and to demonstrate the functional rescue of cells that are mutant and/or null for several of the mitochondrial proteins that we are studying. Research efforts will be focused on more in-depth and rigorous biochemical and functional characterization of the OXPHOS complexes following allotopic expression of ND1 and ND4 in Complex I, ATP6 and ATP8 in Complex V, and Cytochrome B in Complex III, in each of the different cell models that we have generated this year. Subgoals include:

- To demonstrate and confirm the successful incorporation of the aforementioned proteins into the relevant complexes, using a combination of BNGE followed by Western blotting for proteins of interest and Mass Spectrometry analysis of the individual complexes (isolated either by BNGE or immunoisolation of complexes).
- To validate the localization of allotopically-expressed proteins to the inner membrane of mitochondria through colocalization studies with mitochondrial protein markers using fluorescence microscopy (likely confocal or deconvoluting).
- To demonstrate functional rejuvenation of the respiratory chain complex via in-gel activity assays. In addition, we will optimize and perform spectrophotometric assays to analyze Complexes I, III and V, as well as growth assays on selective media to assess the ability of allotopically-expressed proteins to rescue the respiratory potential of the cells.
- To generate and analyze additional cell lines derived from patients suffering from mitochondrial gene mutations, which we will try to rescue through allotopic expression of the relevant mitochondrial genes.

If we advance at the same rapid pace in 2012 that we achieved in 2011, we expect to generate results sufficient for a high-quality publication by the end of the year.

LysoSENS

SENS Foundation Research Center, Mountain View, CA

Researchers: *Dr. Gouri Yogalingam, Max Peto, Lorenzo Albanello, Daniel Kimbel*

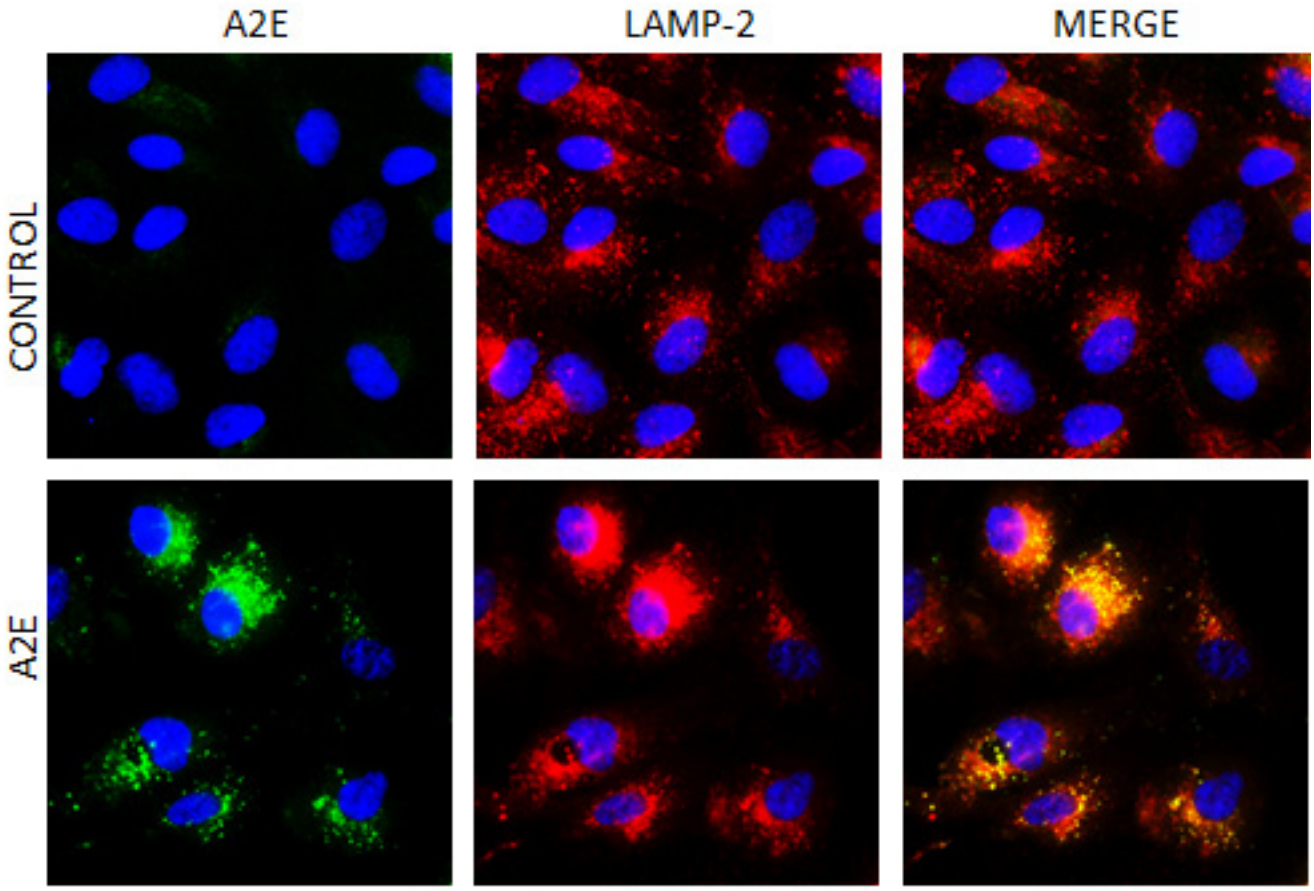
Budget: $200,000 (2011), $200,000 (2012)

Background

Many old and unwanted cellular components – proteins, oxidized lipids, glycoconjugates and even entire organelles – are eventually targeted to lysosomes for degradation into their basic building blocks, which can then be reused by the cell. Despite the existence of over 40 distinct lysosomal proteases, lipases, and glycosidases, some macromolecules that are targeted to lysosomes remain resistant to lysosomal degradation and progressively build up over the lifetime of a cell. In rapidly dividing cells, the material is diluted out and is therefore less toxic. However, in non-dividing cells of organs such as the eye, heart and brain, the progressive lysosomal accumulation of toxic macromolecules with aging can lead to cellular damage and, ultimately, organ dysfunction and age-related disease. For example, accumulation of a form of lipofuscin called A2E and related bisretinoid compounds in retinal pigment epithelium (RPE) cells drives age-related macular degeneration (ARMD), the leading cause of vision impairment and blindness in persons over the age of 65. The LysoSENS group at the SENS Foundation Research Center is working to remove A2E from RPE cells to rejuvenate visual function.

Recent Progress

Under Max Peto, our group has established the routine large-scale production and purification of A2E by flash chromatography and HPLC and has shown that this material is spectrophotometrically identical to that produced by Janet Sparrow's Columbia laboratory. This A2E material is being used by the LysoSENS group to test potential therapeutic interventions aimed at degrading or eliminating lysosomal A2E *in vitro* and in cultured RPE cells. We have demonstrated that the commercially-available enzymes laccase and manganese peroxidase (MnP) both promote a significant reduction in A2E levels *in vitro* as determined by HPLC. We are currently comparing the kinetics of these two candidate enzymes' degradation of A2E as well as three other known A2E-degrading enzymes.



Modeling ARMD in RPE cells. RPE cells were incubated with in-house A2E, fixed and analyzed by immunofluorescence using anti-LAMP-2, a lysosomal marker. Cells incubated with A2E show green autoflourescence of the bisretinoid pigment (green channel), which co-localize with LAMP-2+ lysosomes (red channel) and appear as yellow in the merged image, illustrating that A2 accumulates in the lysosome, which is what happens physiologically in ARMD.

These studies will help us identify a lead candidate A2E-degrading enzyme for further characterization in our cell-based model of ARMD.

Transfection-mediated delivery of A2E-degrading enzymes to lysosomes of RPE cells

To design effective therapies for the treatment of ARMD, it is essential to model the disease in RPE cells. Over the course of the year, we have successfully recapitulated A2E accumulation in RPE lysosomes, as described by Janet Sparrow's lab. RPE cells dose-dependently internalize and target our A2E to lysosomes, as determined by immunofluorescence (see the figure on the previous page). We are now using this cell-based model of ARMD to test the delivery of several A2E-degrading enzymes to lysosomes of RPE cells and the enzymes' efficacy in terms of degrading A2E.

Initial studies using the Bioporter protein transfection reagent in our cell-based model of ARMD suggest that delivery of either laccase or MnP to lysosomes of RPE cells can significantly reduce A2E accumulation. These initial experiments provide "proof of principle" that lysosomes of ARMD cells can be cleared of A2E storage by equipping these organelles with A2E-degrading enzymes. The challenge we now face is finding ways to target these non-lysosomal A2E-degrading enzymes themselves to the lysosomes of human RPE cells in humans.

Therapeutic targeting of non-lysosomal A2E-degrading enzymes to lysosomes.

We are currently producing recombinant laccase and MnP made in yeast and characterizing these enzymes in terms of their ability to be efficiently targeted to lysosomes of RPE cells and clear A2E accumulation.

So far, we have shown that purified recombinant manganese peroxidase is highly mannosylated. We are currently testing to see if we can exploit this feature in mannose receptor-mediated delivery of MnP to lysosomes of macrophages and RPE cells loaded with A2E.

Relevance

Currently there is no effective treatment available to prevent the onset of ARMD. The existing complement of lysosomal enzymes that are present in human cells do not appear to be capable of preventing the lysosomal accumulation of A2E and related toxic bisretinoid species over an entire lifespan, leaving increasing numbers of aging persons visually impaired or blind. Our work aims to equip the lysosomes of aging RPE cells with A2E-degrading capability and thereby eliminate the buildup of these recalcitrant aggregates. It is plausible that just one injection of a lysosomal-targeted A2E-degrading enzyme may be sufficient to degrade the entire pool of aggregated material that has accumulated in RPE over a human life span, reversing the disease. In addition to the intrinsic worth of this goal, a licensable clinical indication for a LysoSENS-based strategy would have the potential to catalyze government and private sector investment into research to extend the strategy to the numerous other age-related diseases driven by intracellular aggregate accumulation. Therefore, identifying novel non-lysosomal A2E degrading enzymes, finding ways to target them to lysosomes, and degrading A2E in situ -- which will ultimately transform this therapeutic strategy into a viable treatment for ARMD -- is of paramount importance to our mission.

Future Work

In 2011, we made considerable progress in establishing the tools necessary to evaluate our therapeutic strategy of targeting A2E-degrading enzymes to the lysosomes of RPE cells and provided initial proof of concept for this approach. In 2012, we will complete testing of mannose receptor-mediated delivery of MnP to lysosomes of macrophages and RPE cells loaded with A2E. By the end of 2012, the group will be focusing more on efficient large-scale production of candidate A2E-degrading enzymes, optimizing their catalytic activity towards A2E, and increasing their targeting to the lysosomes of retinal pigment epithelial cells. We hope to identify and characterize at least one viable strategy to eliminate A2E accumulation using our cell-based model of age-related macular degeneration by the end of the year.

In addition to our intramural research program, SENS Foundation provides funding to a number of centers of excellence around the world. Our collaborations expanded in 2011 to include Cambridge, Stanford, and Yale Universities, University of Texas, and Harvard University. We continue to provide support for productive rejuvenation research projects previously funded at the Albert Einstein College of Medicine, the Buck Institute for Research on Aging, and Rice University.

OncoSENS

Albert Einstein College of Medicine, Bronx, NY

Researchers: *Dr. Jan Vijg, Dr. Silvia Gravina*

Budget: $145,209 (2011), $188,000 (2012)

Background

It has been proposed that one driver of the degenerative aging process is the accumulation of cells that have suffered mutations — permanent damage to their genetic DNA code — and the closely related "epimutations": damage to the "scaffolding" of DNA that helps the cell to control which genes are turned on and off at any given time. Several groups have shown that mutations accumulate with age in a tissue-specific manner. But while accumulation of mutations plays a major role in cancer, its role in contributing to other aspects of aging is less clear, and even less information is available on epimutations.

One of the most important epigenetic modifications is DNA methylation: the reversible addition of methyl groups at cytosines within CpG dinucleotides of DNA, which acts as an on-off switch for gene expression. DNA methylation can suppress gene expression by preventing the binding of transcription factors to their binding motifs and can also recruit proteins that promote transcriptional repression. It is thus conceivable that age-related epimutation accumulation could contribute to age-related functional decline by causing cells to engage in aberrant gene expression, leading to cell death or dysfunction.

If cancer is the sole deleterious effect of age-related mutations and epimutations within the bounds of current lifespans, then a comprehensive cure for cancer, which is another strand of SENS, will be sufficient to obviate their effects for those alive today and into the medium-term future. However, if age-related mutations and epimutations contribute to age-related ill-health in additional ways, then new therapeutic strategies will need to be developed to combat them to rejuvenate the aging bodies. Determining the place of epimutations in aspects of aging other than cancer is thus of paramount importance in determining their place in SENS Foundation's mission and in research priorities going forward.

To answer this important question, it is necessary to have access to procedures that allow the assessment of DNA methylation patterns at the level of the single cell. This is necessary because when epimutations are random, they will show up as low-abundance variants at a particular locus in whole tissue analysis. So, even an age-related load of such epimutations high enough on a per-cell basis to seriously impair function might yet not show up in a whole-tissue analysis, because each individual variant would be present only at low levels across all the cells in the tissue. Unlike mutations, however, no selectable reporter systems for epimutation at the single-cell level are available, preventing the resolution of this key issue. To fill this void, the Albert Einstein College of Medicine (AECOM) group has optimized the first bisulfite sequencing for single cell analysis.

The Single-Cell Bisulfite Sequencing Method

Methods to detect DNA methylation accurately at specific loci typically involve treating DNA with sodium bisulfite or digesting it with cytosine methylation-sensitive enzymes. Applying digestion-based methods at the level of the single cell runs the risk of false positives because digestion may be incomplete. Bisulfite conversion of unmethylated cytosines into uracil, which has become a standard in methylation profiling, is highly accurate, but its sensitivity to DNA degradation is poor due to partial, acid-catalyzed depurination, which leaves a high proportion of the template DNA too fragmented to be analyzed. On the other hand, insufficient treatment results in incomplete conversion and false positives.

Until now, there have been no methods available to detect DNA methylation pattern at the level of the single cell. With SENS Foundation funding, the AECOM group developed, tested, and further optimized a novel procedure for single-cell bisulfite sequencing, using a locus-specific approach. While current bisulfite-based methods require 30 ng of DNA material, the method pioneered at AECOM is optimized to as little as 5 pg of DNA. In this new protocol, single cells are collected under an inverted microscope by hand-held capillaries and either frozen or immediately subjected to proteinase K digestion followed by bisulfite treatment. The converted DNA is subsequently subjected to whole genome amplification using multiple displacement amplification (MDA). The AECOM team was able

to demonstrate that their optimized protocol produces a conversion rate greater than 99%; and it is effective in single cells of several types: hepatocytes, fibroblasts, and neurons.

An example of an experiment in which a series of neuronal nuclei (obtained from mouse brain by sucrose gradient centrifugation and FACS sorting after NeuN staining) were subjected to their novel assay method is shown in the figure below. Using conversion-specific primers, part of the promoter region of the gene Cyp7A1 was assessed in each nucleus for cytosine methylation at CpG sites. FACS-sorted, single neuronal nuclei were subjected to bisulfite treatment and MDA. Each nucleus was assessed for cytosine methylation at CpG sites and compared with the patterns obtained in the bisulfite-treated, nonamplified genomic brain DNA. Fully-methylated cytosines in CG dinucleotides are highlighted in orange. Highlighted in light blue are 3 cases of possible epimutations (demethylating events). The number of converted non-CpG sites are reported in the right column.

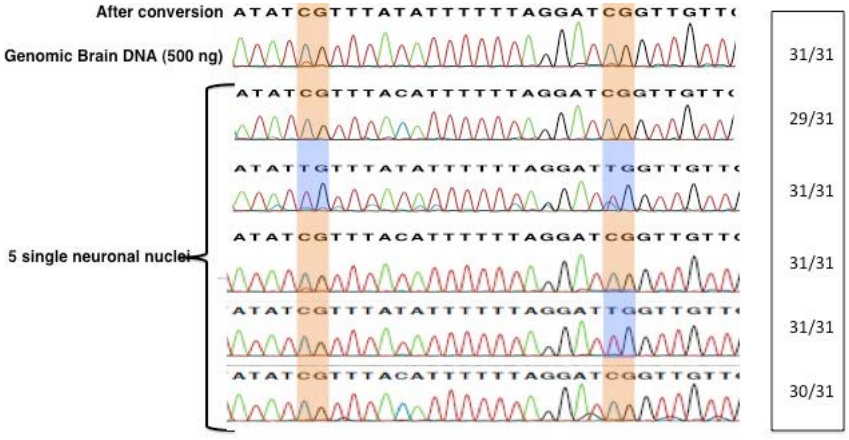
Genome-wide Approaches

While this locus-specific approach allows for the analysis of DNA methylation patterns in single cells within promoter regions of a number of genes, the ability to assess DNA methylation patterns in the entire genome (or a representation of the entire genome) would provide a stronger basis for strategic decision-making in rejuvenation research priorities. The AECOM group is now working on a protocol to use bisulfite-converted material derived from single-cell DNA methylation assaies to make libraries for genome-wide DNA methylome analysis using so-called "next-generation" Reduced Representation Bisulfite Sequencing. In pilot experiments, they have compared the DNA methylation patterns of individual hepatocytes with the methylation pattern of the liver tissue at large in order to identify low-abundance epimutations. While they obtained 95% of coverage of target sites in the liver control, only a small percentage (3.3%) of target sites (average of about 40,000 CpG sites) were covered by reads in the single hepatocytes. As discussed under "Current and Future Work" below, research in 2012 will include methods to enhance coverage.

Relevance

The AECOM team anticipates that the impact of their newly-developed technology for single-cell DNA methylation detection will be broad. Their approach will help resolve the long-standing debate about how important random epimutations are for the degenerative decline in somatic cells that gives rise to chronic pathology and aging, and whether an epigenetic drift may have an impact on healthy lifespan. If the results demonstrate that the rate of accumulation of epimutation-hampered cells over the adult lifespan is sufficiently low, then SENS Foundation's existing strategic research priorities related to age-related (epi)mutations will be validated, and investments will continue to focus exclusively on methods for obviating their contribution to cancer. If, on the other hand, the results demonstrate that the rate of epimutations is unexpectedly high, then new therapeutic strategies will need to be developed and prioritized to rejuvenate aging tissues impaired by such damage.

However these issues are resolved, the fruits of our investments have additional, near-term biomedical potential. A reliable single-cell assay for epimutations could be used not only for basic research on the phenotypic diversity within organs and tissues in relation to aging and age-related disease states, but also could form the basis of advanced diagnostic and prognostic assays. Using a very small number of cells, such epigenetic assays would give clinicians and patients new insights into tissue health,



Assessment of nuclei for cytosine methylation at CpG sites.

disease, and aging. Assays developed from these methods could be used to diagnose current disease states and to predict future disease risk. They could also be used in personalized medicine: One major potential clinical application that comes immediately to mind is to assess DNA methylation patterns in promoter regions of tumor-suppressor genes in circulating tumor cells. The availability of such assays would enable the creation a new class of epigenetically-targeted cancer therapies, with drugs matched to specific patient tumor types, analogous to targeting Herceptin to those breast cancer patients whose tumor growth is driven by HER2 mutations today.

Current and Future Work

The results of research to date is now being written up for peer-reviewed publication, with the intention of submitting it to *Nature Biotechnology* A patent for the single-cell methylome typing method is being prepared. Work in 2012 will include studies to determine the sensitivity of the assay in detecting alterations in cytosine methylation. The group is also working to rule out possible sources of experimental artifact in the protocol, including the possibility of false methylated cytosines generated because of incomplete conversion of unmethylated cytosines, or false negatives resulting from conversion of a small proportion of 5-methylcytosines into thymine. They are testing several approaches to address the limited coverage at the single-cell level encountered in the genome-wide assay, including the use of carrier DNA to limit DNA degradation by bisulfite treatment (which is the most likely cause of the loss of target), and methods to improve the efficiency of multiple displacement amplification (which is reduced when a DNA template is fragmented).

The genome-wide experiment is also being repeated in additional single hepatocytes, and further testing is being done on single cells from young and old animals. Drawing together and optimizing all of these preliminary achievements, work will proceed toward the project's ultimate aim: the quantification of genome-wide levels of epimutation in the nuclei of single neurons from aged mice so that research priorities in rejuvenation of the (epi)genome of aging tissues can be addressed.

LysoSENS

Rice University, Houston, TX

Researchers: *Dr. Pedro Alvarez, Dr. Jacques Mathieu, Rob O'Callahan*

Budget: $84,000 (2011), $98,000 (2012)

Background

As discussed in the background for the intramural LysoSENS project, inherent imperfections and limits to the ability of cellular machinery to eliminate damaged intra- and extracellular components leads to the gradual accumulation of cellular waste products. Deleterious accumulations of such waste products with age in non-dividing cells is widely accepted to contribute to the pathogenesis of several major age-related diseases in humans and is thought to be one of the principal causes of age-related tissue dysfunction, morbidity, and mortality. Accordingly, it has been suggested that removal of such aggregates would attenuate, arrest, or reverse the course of several age-related diseases and therefore delay the global loss of health observed in aging. With funding from SENS Foundation, the Rice University LysoSENS team is working to develop methods to reduce the intracellular accumulation of two classes of such age-related intracellular waste (oxysterols and lipofuscin) that are highly detrimental to cellular function and believed to contribute to atherosclerosis and other diseases of aging.

Targeting oxysterols in atherosclerosis and aging

Cholesterol is an integral component of our cell membranes and is critical for the normal functioning of our cells. Damage to cholesterol by reactive oxygen species generates oxysterols such as 7-ketocholesterol (7KC), which is primarily formed and stored in the endosomal/lysosomal compartment. At low levels, 7KC can inhibit the export of cholesterol out of the lysosome, leading to its accumulation, which impairs normal endo-lysosomal functioning. At higher concentrations, 7KC causes lysosomal membrane permeabilization (LMP) and cellular death. In humans, several enzymes are known to metabolize 7KC and may attenuate 7KC-induced cytotoxicity. However, these enzymes are localized in intracellular compartments other than the lysosome and are limited in their ability to prevent LMP and the subsequent death-signaling cascade. Such damage to arterial macrophages leads to the formation of foam cells and drives the development and progression of atherosclerosis. One arm of the Rice LysoSENS project is focused on the introduction of novel catalytic enzymes into the lysosome to clear accumulated oxysterols and arrest or reverse their toxicity and downstream sequelae.

Previous work at Rice has shown that oxidation of 7KC decreases its cytotoxicity. The Rice team has now investigated the ability of a novel bacterial enzyme, engineered to target the lysosome, to degrade 7KC and other sterols and prevent induction of the LMP-mediated death-signaling cascade in human cell cultures. In comparison to several other 7KC-active enzymes, their engineered construct was found to have superior efficacy in preventing oxysterol toxicity for experiments of short duration (see the figure below).

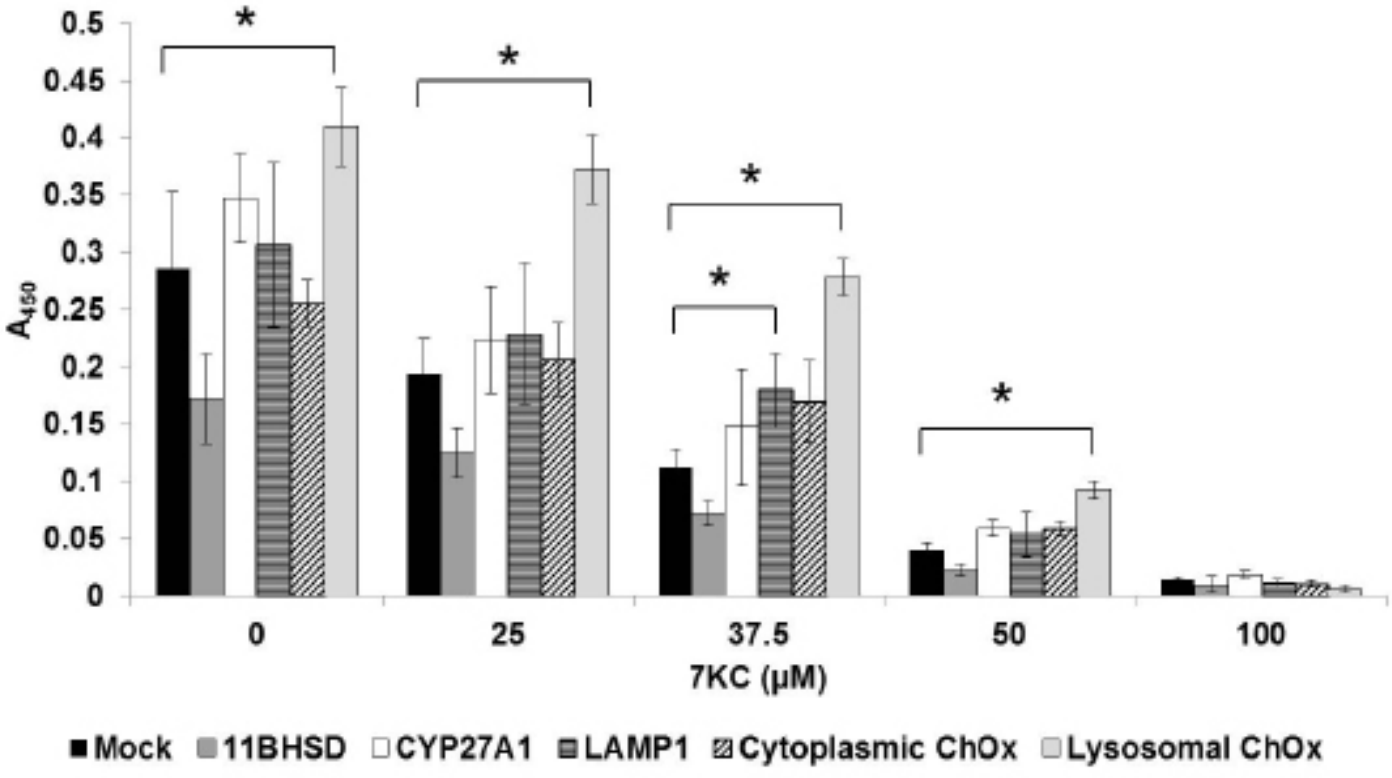
In an additional approach, expected to be complementary or synergistic to the first, the Rice group has probed the potential of 7KC efflux from the lysosome to prevent cytotoxicity. This possibility is suggested by the existence of

several enzymes present in other areas of the cell that are known to transform 7KC. Accordingly, a small molecule was identified that significantly increases cell viability in cultures exposed to high concentrations of 7KC, and its mode of action appears to be the facilitation of lysosomal sterol efflux. This molecule is also believed to increase exocytosis, enabling sterol expulsion into the extracellular space.

Targeting lipofuscin

Lipofuscin is an intralysosomal, polymeric material composed of oxidized protein and lipid that resists degradation and exocytosis. It accumulates at a linear rate with age in the lysosomes of non-dividing cells, ultimately coming to occupy a large percentage of the cell volume, and appears to hinder the recycling of damaged cellular components. Although the full effects of lipofuscin are still being characterized, there is considerable evidence that it may play a causative role in age-related cellular dysfunction, as posited by the free radical and mitochondrial-lysosomal axis theories of aging.

The Rice team has developed three foundational tools necessary to facilitate high-throughput testing of treatments intended to reduce or eliminate lipofuscin from aging cells: methods of generating artificial lipofuscin *in vitro*; a reproducible means of quantifying lipofuscin using flow cytometry; and a model for experimentation on the effects of lipofuscin and lipofuscin-attenuating therapies



Cytotoxicity analysis of transfected human fibroblasts. Normalized changes in absorbance (450 nm) of XTT due to reduction coupled to mitochondrial dehydrogenase that corresponds to differences in cell viability. A greater absorbance indicates more viable cells are present. Cells were transfected and exposed to varying concentrations of 7KC for 24 hours prior to the XTT assay. Significant differences existed amongst treatments up to 50 μM 7KC, with transfection by our lysosomally-targeted enzyme (Lysosomal ChOx) being the most cytoprotective. LAMP1 overexpression also moderately increased resistance to 7KC at 37.5 μM. However, the lysosomally-targeted DS1 ChOx was significantly more protective than all other treatments at 25, 37.5, and 50 μM 7KC. Asterisks (*) denote statistical significance ($p \leq 0.05$).

in non-dividing cells. Many cell-based assays involve the use of dividing cells. These are relatively easy to culture and readily take up transfected DNA. But since lipofuscin, like other age-related intracellular aggregates, accumulates precisely in non-dividing cells such as cardiomyocytes and brain neurons, the Rice team worked to develop a suitable model using cells that do not divide. Moreover, in order to monitor lipofuscin degradation by candidate therapeutic enzymes, they needed to establish methods of introducing exogenous genes encoding those enzymes into non-dividing cells.

To this end, the Rice team constructed two vectors: one for use in a viral transduction system, and the other for use in an mRNA transfection strategy. The viral system uses recombinant adeno-associated virus to deliver DNA to the host cell nucleus. The vector may exist episomally or integrate into the host cell chromosome. The mRNA system uses *in vitro*-generated mRNA to transfect cells directly. The advantage of this system is that the mRNA does not need to enter the nucleus and, as a consequence, is efficiently expressed. Furthermore, the use of mRNA prevents host cell integration and consequent insertional mutagenesis and is therefore considered a safer alternative to gene therapy.

Relevance

We anticipate that the ability to control the intracellular accumulation of either oxysterols or lipofuscin will have far-reaching effects for the prevention of age-related disease and will help determine the potential of these methods for extending youthful human life span. Research into the comparative biology of aging has shown that low lipofuscin loads are associated with "negligible senescence," a state in which an organism experiences no functional decline with increasing age. Additionally, recent investigations into oxysterols have strengthened the view that they may be causative factors in age-related disease, rather than simple biomarkers of oxidative stress. The tools under development at Rice to clear the lysosome of its burden of oxysterols and lipofuscin will therefore serve not only to test the major theories of aging but also these tools may be candidate therapeutics to rejuvenate lysosomal function and correct the age-related conditions that are driven by these loads.

Current and Future Work

The Rice group has recently submitted a manuscript reporting the results of their recent work on oxysterols for journal publication, and it is now undergoing peer review. They are now moving on to research aimed at determining the most effective means of reducing oxysterol toxicity. One aspect of this work is the development of cell-based models for atherosclerosis in which they will assay the effect of interventions that clear oxysterols from the lysosome and/or cell. Amongst the approaches to lysosomal clearance under pursuit is the redesign of their engineered enzyme using different methods of lysosomal targeting and methods to reduce secondary oxysterol toxicity.

Additionally, the team at Rice is currently constructing a set of vectors encoding endogenous sterol transport proteins, with the goal of facilitating lysosomal oxysterol efflux. Moreover, since one effect of elevated 7KC levels is inhibition of some lysosomal enzymes, they will also pursue the engineering of variants of these enzymes that retain activity despite the presence of 7KC. They will also mine further candidates from their previous work, which led to the isolation of several strains of bacteria that readily degrade 7KC. Although they have identified the degradation pathway in one strain, there are many more potential sources of novel enzymes to investigate. Since most of the bacterial strains have not had their genomes sequenced, the researchers at Rice have been developing protocols to create functional expression libraries from bacteria that can be directly screened for their ability to degrade 7KC or any other compound of interest, such as lipofuscin.

Having established methods for producing lipofuscin, quantitating its level, maintaining a suitable model for assessing its effects (and those of lipofuscin-targeting therapies) in non-dividing cells, and transfecting such cells with novel genes, the Rice team is moving to transfect such cells with genes encoding proteins with therapeutic potential for degrading lipofuscin or otherwise reducing its deleterious effects. They are currently generating constructs using each of their vectors to deliver transcription factor EB (TFEB – the master gene for lysosomal biogenesis) into lipofuscin-laden non-dividing cells. This will allow them to determine whether up-regulation of the many genes controlled by TFEB can reduce lipofuscin accumulation in their cellular model. In the process, they will probe whether any reduction in lipofuscin levels is due to its degradation or its exocytosis.

Future avenues of research include the intralysosomal expression of exogenous enzymes, screening of small molecule libraries for potential LysoSENS therapeutics, and the screening of gene expression libraries for the ability of specific gene products to prevent or reverse lipofuscin accumulation in susceptible cell types. Any or all of these multiple approaches could be tested in combination to evaluate the benefits of engaging multiple mechanisms of aggregate clearance. Successful candidates in these initial screens would be tested for acting via deceleration, arresting, or reversing the intracellular waste-driven dysfunction of such cells and the diseases of aging with which they are associated.

ApoptoSENS

Buck Institute for Research on Aging, Novato, CA

Researchers: *Dr. Judith Campisi, Kevin Perrott*

Budget: $60,000 (2011), $60,000 (2012)

Background

Acute inflammation, which occurs in the early stages of injury and resolves shortly after the event that causes it, is an essential feature of the wound healing response. But chronic inflammation – inflammation which does not resolve following an insult – plays a role in the establishment and pathogenesis of numerous age-related diseases, such as atherosclerosis, cancer, and chronic obstructive pulmonary disease. The origins of chronic inflammation are not well understood, but recent studies have indicated that the process of cellular senescence may be involved. Cellular senescence is an irreversible growth-arrested, apoptosis-resistant state, which cells adopt in response to various stressors, such as telomere shortening, DNA damage induced by exposure to radiation, or the loss of tissue integrity involved in trauma and wounding. Although senescent cells do not divide, they are metabolically active, and typically secrete numerous molecules into the extracellular milieu that affect the structure of the surrounding tissue and the function of neighboring cells.

Many cell types have been found to undergo senescence, but fibroblasts have been under investigation for some time and are arguably the best understood model with respect to the senescent phenotype. It has been shown that senescent fibroblasts adopt a pro-inflammatory state, called the "Senescence-Associated Secretory Phenotype" (SASP), in which they secrete pro-inflammatory molecules such as Interleukin-6 (IL-6), chemokines, and matrix metalloproteinases. These aberrant signaling molecules have the potential to negatively impact the surrounding tissue, through degradation of the cellular environment, inappropriate activation of the immune system, and the impairment of the function of resident populations of stem cells in the niche in which they reside. As a result of these effects, senescent cells have been implicated in establishing a permissive environment for age-related degenerative processes in the local tissue environment, such as invasion by metastatic cancer, degeneration of lung tissue in emphysema, and the impairment of the wound healing process.

Targeting the Senescence-Associated Secretory Phenotype

Modulation of the SASP through pharmacologic or genetic means could thus affect the presentation of age-related pathology, potentially providing tools for the prevention and treatment of many age-related disease states, and to rejuvenate the function of tissues dysregulated by the aging process. With SENS Foundation funding, scientists at the Buck Institute for Research on Aging have been screening small molecules for their effects on fibroblasts rendered senescent by ionizing radiation *in vitro*, with the aim of testing the effects of promising candidates on tissue pathology *in vivo*.

In a rudimentary screen of the 1400 FDA-approved drugs in the Prestwick Library, the Buck Institute researchers found no compound that exhibited selective toxicity to senescent cells while leaving normal cells unharmed. However, many of these compounds did reduce the senescent cells' secretion of the pro-inflammatory cytokine IL-6 – a representative constituent of the SASP – while maintaining cell viability.

Since it is generally accepted that it is the aberrant secretory profile of senescent cells, rather than the cells themselves, that contributes to age-related disease and tissue dysfunction, the Buck scientists pursued the alternative strategy of suppressing the SASP as a direct therapeutic modality. Candidates that had exhibited the ability to reduce IL-6 secretion while leaving cells unharmed were therefore prioritized based on prior knowledge of their chemical and pharmacological profiles, including potential mechanisms of action, winnowing the list to 25 of the most promising candidates, which were advanced for additional testing.

Currrent and Future Work

In May 2011, the Buck Institute researchers selected the flavonoid apigenin as the most promising of these 25 compounds and initiated studies to determine mechanism(s) of its action that could be therapeutically exploited in reduction of the proinflammatory SASP. A literature search reveals that the molecule is well-known to have anti-inflammatory effects in the context of infection, but its effects on inflammatory cytokine production in senescent cells are novel to their investigation. One possible mechanism – mediated through a well-known receptor involved in many cancers – was examined, but found to be unlikely to be involved in the cell type used in the Campisi Lab. One current avenue of inquiry is probing a possible effect through activation of this receptor through a non-canonical pathway.

An additional line of investigation was opened by a serendipitous twist. While discussing the effects of apigenin on the secretory phenotype of senescent cells with a colleague, it was noted that other flavanoids had previously been found to prevent protein aggregation in the roundworm C. elegans. These compounds were then tested for their ability to reduce IL-6 expression in irradiated fibroblasts; the results were positive, and have recently been reproducibly confirmed. This is potentially an exciting

area of investigation as protein aggregation may play a pivotal role in senescence and in many degenerative conditions of aging.

In 2012, the Buck Institute ApoptoSENS project will continue to probe the mechanism of action of apigenin and related compounds on the SASP, focusing on the current pathway with the possible involvement of protein aggregation. Negotiations are in place to obtain access to several other libraries for screening for additional candidate compounds, including any that might selectively induce apoptosis in senescent cells directly.

Compounds that emerge out of ongoing work on all of these fronts could subsequently be followed up *in vivo*, exploiting a mouse model under characterization in the Campisi Lab that provides the ability to look at the accumulation of senescent cells directly through luminescence. Promising compounds could be administered to these mice to probe the role senescent cells play in pathology and to test their therapeutic impact on the onset and occurrence of age-related disease and dysfunction.

GlycoSENS

Cambridge University, Cambridge, UK & Yale University, New Haven, CT

Project Director: *Dr. William Bains (Cambridge)*

Researchers: *Dr. Chris Lowe, Dr. Rhian Grainger (Cambridge), Dr. David Spiegel, Dr. Cristian Draghici (Yale)*

Budget: $134,000 (2011), $322,000 (2012)

Background

Elasticity is essential to the function of many tissues, including the walls of our major arteries and the lens of the eye. The stiffening of these tissues with age leads to impairment of their function, resulting (amongst other conditions) in increasing hypertension and risk of renal failure with age. The increase in systolic blood pressure driven by loss of large artery elasticity is one of the major reasons for the increased risk of stroke and the development of dementia in older people.

The stiffening of our tissues with aging is caused in substantial part by the accumulation of chemical crosslinks between proteins of the extracellular matrix (ECM), the network of proteins between cells that gives our tissues their structure. In youthful tissues, ECM proteins are structured in a regular lattice, but subsequent crosslinks that accumulate during aging are located randomly, which causes the loss of elasticity. The creation of these crosslinks involves many chemical pathways and forms many differently-shaped structures, which are collectively called advanced glycation endproducts (AGE). Of these, it has been established that one specific AGE structure, called glucosepane, is the most abundant in aged human tissue.

A method or compound that would unlink glucosepane crosslinks from aging tissues would therefore be of great therapeutic value in the prevention and reversal of age-related tissue degeneration; yet it is not being energetically pursued in any academic institution or biotech lab, which elevates its priority for SENS Foundation research in critical-path analysis. We have therefore established and funded a GlycoSENS collaboration between researchers at Cambridge and Yale Universities, whose aim is to discover and test such a glucosepane "AGE-breaker" therapeutic.

Current and Future Work

This is a newly-launched project. In the latter part of 2011, SENS Foundation secured laboratory space at Cambridge University for our new extramural Center of Excellence and has recruited a post-doctoral candidate to carry out GlycoSENS research. The initial goal of this project is to develop reagents that can detect glucosepane-linked proteins rapidly and specifically as an enabling technology for the development and testing of potential glucosepane-breaking drugs. This work will be carried out as a collaboration between the Cambridge Center of Excellence and Yale University, which has special expertise in making AGEs. In addition to use in SENS Foundation-funded research, the labeling reagents that emerge from the first phase of this work will made available to other scientists, to accelerate research into the role of AGEs in disease and aging. Yale will then move on to developing agents which may be able to cleave glucosepane, which can then be tested *in vitro* and *in vivo* at the Cambridge center.

AmyloSENS

University of Texas, Houston, TX & Harvard University (Brigham & Women's Hospital), Boston, MA

Researchers: *Dr. Sudhir Paul, Dr. Yasuhiro Nishiyama, Dr. Stephanie Planque (UT)*
Dr. Brian O'Nuallain (Harvard)

Budget: $90,000 (2011), $90,000 (2012)

Background

As part of the degenerative aging process, proteins that normally remain dissolved in bodily fluids become damaged and adopt an abnormally clumped form called amyloid. The amyloid clumps are toxic and hard for the body to break down. The accumulation of toxic amyloid deposits in various organs with age disrupts their homeostatic processes and anatomic structure. There is a strong con-

sensus that several specific amyloids are key contributors to major age-related diseases, and they also appear to contribute to a wider range of age-related pathology and ill health. Examples include the β–amyloid peptide (associated with neurodegeneration in Alzheimer's disease) and atrial natriuretic peptide (associated with congestive heart failure).

Another form of amyloid-driven disease is caused by the blood protein transthyretin (TTR), which normally functions as a transporter of the hormone thyroxine and of the vitamin retinol. In patients with senile systemic amyloidosis (SSA), TTR aggregates, forming amyloid fibrils that deposit in the heart and other organs. Certain symptoms of TTR amyloid deposition can first become evident at younger ages (for example, TTR amyloid in joints, which is associated with carpal tunnel syndrome), but the insidious effects of these malformed protein deposits remain mostly silent until after middle age. SSA begins to significantly affect the hearts of persons over the age of 50, and reaches levels sufficient to impair heart function in an estimated 20-25% of individuals over the age of 80 years, and apparently becomes a major contributory factor in the deaths of our elite aging population of "supercentenarians" (persons who achieve 110 years or more of life; personal communication from Stan Primmer, Supercentenarian Research Foundation). Additionally, certain mutant TTR forms display increased aggregation rates, and patients carrying these mutations can develop early-onset familial amyloidosis prior to age 50.

In 2011, SENS Foundation established and provided the funding for a collaboration between researchers at the University of Texas (UT) and Harvard University, the goal of which is the generation of novel antibody diagnostics and therapies for SSA. The immediate objectives are to generate diagnostic antibodies that bind to pathogenic TTR and to develop novel catalytic antibodies that can destroy TTR amyloid.

Antibodies for Diagnosing SSA

The standard diagnostic technique for diseases of TTR amyloid is biopsy of the affected tissue, dye staining of amyloid deposits, and protein sequencing to determine the protein that has formed the amyloid deposits. These procedures are invasive, expensive, and error-prone: patients with SSA can be misdiagnosed, and early diagnosis is difficult. There is an urgent need to develop a simple diagnostic test that is less invasive, relatively cheap, and capable of specifically detecting pathogenic TTR. We believe that antibodies have unique potential for diagnosis of SSA.

Antibodies that are specific for their target can be developed readily, and various technologies enabling the use of antibodies for sensitive diagnosis of various diseases have been developed. In preparation for generating anti-TTR antibodies with potential for diagnosing SSA, Dr. O'Nuallain and coworkers have optimized various laboratory methods to generating fibrils and chemically cross-linked TTR species expressing epitopes that can be recognized by antibodies. The Harvard component is to generate antibodies that recognize the unique epitope present in fibrillar TTR and the misfolded soluble form of TTR, without cross-reacting with the normal, soluble tetrameric form of TTR found physiologically in blood. They will employ various forms of TTR immunogen to stimulate the formation of antibodies against pathogenic TTR. They have also developed procedures for detecting and isolating such antibodies for later production and use.

Antibodies for Treating SSA

In his lab at the University of Texas, Dr. Paul has discovered that older humans produce novel enzyme-like antibodies that catalyze the cleavage of the Alzheimer's disease-associated β-amyloid peptide, disaggregating their fibrillar structures and blocking the neurotoxic effects of the soluble β-amyloid oligomer species. Catalytic antibodies combine the binding specificity of traditional antibodies with the ability to fragment their protein targets. Thus, a single catalytic antibody can be used again and again to destroy thousands of target molecules. In comparison, traditional antibodies can only bind to and inactivate one target molecule each. Moreover, traditional antigen-antibody complexes are long-lived, and can induce undesirable inflammatory effects and vascular damage, whereas catalytic antibodies cleave their targets and release the breakdown products without forming stable immune complexes. For these reasons, these catalytic autoantibodies appear to be a beneficial immune response that slows the onset of Alzheimer's dementia. Dr. Paul is working to develop these antibodies as a therapy for Alzheimer's disease.

The UT/Harvard team and SENS Foundation believe that the body may similarly develop beneficial catalytic autoimmunity against other amyloidogenic proteins, including TTR. The immune system is tolerant to physiologically folded self-proteins, but the "foreign" amyloid-specific epitopes can induce an adaptive antibody response. Promiscuous catalysis is an innate function expressed by antibodies prior to their specialization for recognition of individual antigens, and adaptive improvement of the antibody's catalytic function is expected to be favored by amyloid carbonylation reactions that are characteristic of the aging process: protein carbonylation can generate electrophilic antigenic epitopes that may induce adaptive strengthening of the nucleophilic sites responsible for catalysis.

Initial immunochemical studies performed at UT support the existence of native autoantibody catalysis of fibrillar TTR. The UT researchers biotinylated TTR at low

density and permitted it to form aggregates as measured by the Thioflavin T and turbidity assays. An electrophoresis method was developed to measure the cleavage of biotinylated fibrillar TTR (F-TTR) and soluble tetrameric TTR. Screening of panels of human monoclonal antibodies identified several IgM-class antibodies and an IgA-class antibody, each with the ability to cleave F-TTR. Remarkably, F-TTR was susceptible to the catalytic antibodies despite its comparative resistance to digestion by the powerful proteolytic enzymes trypsin and endopeptidase Asp-N. The frequency and magnitude of the observed catalytic activities suggest that clearance of F-TTR may be a physiological function of catalytic antibodies.

Promisingly, extensive fragmentation of F-TTR into small peptides was evident, reducing the likelihood that the F-TTR reaction products might themselves be amyloidogenic. Moreover, the antibodies are selective to F-TTR, leaving physiologic TTR intact; this suggests that the antibodies are specific for one or more epitope generated upon formation of the amyloid fibrils. Catalytic antibodies targeting β-amyloid did not cleave F-TTR, implying that the F-TTR and β-amyloid peptide epitopes recognized by the catalysts are structurally distinct. IgM from aged humans cleaved F-TTR at rates superior to IgM from younger humans, suggesting an age-induced adaptive improvement of the catalytic function, consistent with the theoretical expectations outlined above.

Current and Future Work

The researchers are currently immunizing mice with TTR fibrils and cross-linked TTR, to generate novel hybridomas (fused murine B- and myeloma cells) that secrete antibodies capable of specifically targeting the pathogenic forms of TTR. The suitability of various antibodies as candidate diagnostic reagents will be determined using their standard *in vitro* binding assays and tests of antibody reactivity with TTR amyloid derived from SSA patients. Monoclonal antibodies will also be tested for their therapeutic potential by measuring their ability to catalyze the breakdown of TTR fibrils. Supplementing native anti-amyloid immunity with therapeutic infusions of exogenous monoclonal TTR-targeting catalytic antibody may be a route to prevent and reverse this insidious disease. Dr. Paul's arm of this SENS Foundation project is developing candidate therapy-grade catalytic antibodies to F-TTR using hybridoma and recombinant antibody library technologies.

The project developmental plans are based on the same experimental algorithms that previously permitted them to identify lead catalytic antibody fragments specific for amyloid β peptide. They will select human antibodies with superior catalytic turnover and specificity for F-TTR. Certain technological innovations favoring isolation of therapy-grade catalysts will be exploited, including the use of antibody constant domain scaffolds that support expression of efficient catalysis and possess a sufficiently long half-life in the body. An electrophilic TTR containing phosphonate groups at Lys side chains has been synthesized to help identify antibodies with the greatest catalytic activity. In addition to wild type F-TTR, cleavage of certain mutant forms of F-TTR responsible for hereditary amyloidosis will also be tested to assess the target patient populations suitable for catalytic antibody therapy.

ApoptoSENS

Arizona Center on Aging, University of Arizona, Tucson, AZ
Researchers: *Dr. Janko Nikolich-Zugich, Dr. Megan Smithey*
Budget: $45,000 (2011) $35,000 (2012)

Background

As part of the degenerative aging process, the immune system becomes progressively impaired with age, leading to reduced responsiveness to vaccination, higher risk of infection, and greater morbidity and mortality from such infections with age. One contributor to this "immunosenescence" is thought to be the accumulation of anergic T-cell clones that are no longer capable of clearing their original target pathogen, and whose population expansions may "crowd out" T-cells specific to other pathogens and impede the maturation of naïve T-cells. This project seeks to test whether removal of accumulated age- and virus-related T-cell clonal expansions and/or rebalancing the T-cell repertoire by introducing more naïve cells that can target other pathogens can restore the youthful functionality of the T-cell compartment in aged organisms and improve the immune defense against new infection.

To that end, a series of longitudinal experiments was designed in the laboratory of prominent immunosenescence researcher Dr. Janko Nikolich-Zugich, Co-Director of the Arizona Center on Aging at the University of Arizona. These studies were conducted using two mouse models of persistent viral infection known to result in the development of virus-specific CD8+ (killer) T-cell expansions (TCE): infection with either HSV-1 or murine cytomegalovirus (MCMV). Both of these infections lead to the lifelong accumulation of CD8 T cells that target these pathogens specifically, resulting in a CD8 population largely dominated by these antiviral cells at the expense of naïve T-cells or alternative target pathogen antigens. Evidence from Dr. Nikolich-Zugich's laboratory suggests that such TCEs have the potential to impair protective immunity upon challenge with a new pathogen. SENS Foundation-funded research aims to test several ways to remove and/or neutralize these expansions, to see whether the old immune system can thereby be rebalanced and rejuvenated to preserve robust immune defense.

In the first round of studies testing this "rebalancing" concept, Dr. Nikolich-Zugich's lab evaluated several interventions for their ability to establish a more "youthful" immunological profile in aging mice, and protect against novel infection: (a) partial depletion of the aged, effete CD8+ T-cells, to test the degree to which removal of their repressive effects alone might allow the expansion of alternative T-cell populations and improve host defense against new infection; (b) administration of Kepivance® / palifermin (KGF) – a modified version of a human growth factor that is known to expand the thymus in various conditions of immunological impairment, and is licensed by FDA as a treatment for some blood cancers, to improve the host's ability to produce new T-cells; or (c) adoptive transfer of naïve T-cells from young animals.

Each of these interventions demonstrated some modest ability to restore a more balanced CD8+ cytokine profile, and KGF treatment of old mice significantly improved thymic cellularity overall, including increasing the frequencies of both CD4+ and CD8+ T-cells to the approximate level seen in untreated adult mice. However, the effects on host defense against novel Listeria monocytogenes infection were limited to nonexistent. Studies launched later in 2011 were designed to re-evaluate these interventions using improved protocols. Animals were again infected with HSV-1 or MCMV to generate anergic TCE, and given one of several interventions (see the table below). Group A began continuous treatment with the antiviral drug Famvir in their drinking water in order to test the ability of antivirals to prevent infection-associated TCEs in the first place. Animals will be challenged at 20 months of age.

Group	Infection
A (n=20)	HSV-1
B (n=20)	HSV-1
C (n=20)	MCMV
D (n=20)	MCMV
E (n=20)	MCMV
F (n=20)	MCMV
G (n=20)	MCMV
H (n=20)	None

In the new protocol, KGF treatment was initiated earlier than in the first round of testing, at 17 months of age, allowing 10 weeks for the thymus to regenerate prior to pathogen challenge. The dose of young-derived transferred T-cells was increased, and two arms testing the effects of combination therapy with CD8+ T-cell depletion with adoptive transfer of young CD8+ T-cells were added; one group will be challenged with the pathogen soon (one week) after transfer, to test for immediate effects of rebalancing, while the other will be tested later (two months) to evaluate its persistence. The challenge pathogen against which these interventions will be tested was switched to murine West Nile Virus instead of Listeria. West Nile Virus constitutes a superior model for testing therapies intended to remediate age-related defects in adaptive immunity, as host adaptive immune response to this pathogen has been shown to decline with age in mice, while little such effect is manifest in Listeria.

Relevance

The decline in immunity with aging leads to substantial morbidity and mortality in aging persons. To cite one prominent example: Influenza, and influenza-associated pneumonia, is the eighth leading cause of death in the United States, and nearly 90% of these deaths occur in persons over the age of 65. Worldwide, the World Health Organization estimates that 250,000 to 500,000 deaths each year are attributable to seasonal influenza.

These numbers actually underestimate the toll wrought by influenza, because infection in the biologically aged also leads to increased risk of death from cardiopulmonary and other chronic diseases, which influenza infection exacerbates. Researchers believe that influenza is the probable cause of most of the excess mortality that occurs during the winter in the elderly not only from pneumonia, but from cardiovascular disease, stroke, and diabetes. Influenza is also responsible for approximately 200,000 hospitalizations annually, during which elderly subjects can lose up to half of their lower limb strength, and when combined with cardiovascular complications, these incidents are placed among the top six causes of "catastrophic disability" in older adults. And perversely, the age-related decline in immunity means that the most effective bulwark against infection is greatly diminished in effectiveness compared to younger adults: influenza vaccines provide, on average, 59% protection from infection in persons aged 18 to 65, while estimates using more generous criteria for effectiveness place the effectiveness of vaccines in older adults at just 35%. Rejuvenation biotechnologies to restore the robust immunity of youth can thus be expected to significantly improve the quality and quantity of life in the seventh decade and beyond.

Future Work

In this new round of testing, animals will be challenged at 20 months of age, following at least 14 months of aging- and infection-associated TCE accumulation. This will occur in approximately March 2012. Depending on the results, different interventions may be advanced for further testing. Additionally, we will also be exploring more aggressive approaches to the decline in naïve T-cell production and the accumulation of anergic T-cells that have emerged from previous SENS Foundation research and from independent work.

Extending Our Research Program

The in-depth project descriptions you have just read tell only part of the story of SENS Foundation's plans for 2012. We would thus like to conclude this report with a short preview of several exciting new efforts currently underway.

Thymus Project

We've begun a collaborative effort to create an artificial thymus, as part of our program to rejuvenate the immune system. This project will exploit the "decellularization" technology that others have used for heart, trachea and other organs in recent years, but for the purpose of rejuvenating the organ that suffers the most dramatic structural degeneration during human adulthood.

OncoSENS Expansion

We have initiated new intramural and extramural projects focusing on the implementation side of our OncoSENS program. Specifically, our SENSF-RC team seeks to elucidate the genetic basis of ALT (Alternative Lengthening of Telomeres), the mysterious telomerase-independent method that 10% of human cancers use to maintain telomere length. Meanwhile, a planned collaborative effort with one of our extramural partners will explore the ability of circulating stem cells to infiltrate the gut wall and repopulate the intestinal lining when stem cells there are failing, a problem that is expected to occur as a side-effect of the OncoSENS anti-cancer strategy.

RepleniSENS Development

Our RepleniSENS program is likewise moving forward. Stem cell therapy often works less well in older organisms, and this may largely be due to changes to the composition of the plasma. A multi-site project involving several prestigious laboratories is set to explore the impact of a rejuvenated circulation on improving the effectiveness of delivering stem cells to a variety of tissues in the elderly.

Nematode Longevity

While the majority of our efforts remain directed toward testing and development per the SENS proposal (as we believe this plan to have the best chance of delivering the comprehensive damage-repair therapies necessary to effectively mitigate the diseases of aging), we remain open to other possible strategies that might prove effective. We are therefore initiating a project to determine whether a recent spectacular longevity result in nematodes is actually as remarkable as it seems by revisiting some older work which may have underestimated the malleability of nematode longevity in response to famine.

Moving Toward an Improvised Mouse Model

We are getting closer to where the various genetic interventions we are currently exploring in cell culture can move to live mice. Projects involving genetic manipulation of mice are by nature lengthy efforts, and those attempting to intervene in aging are even more time-consuming, for obvious reasons. If a mouse could undergo significant genetic modification after reaching adulthood, this could hugely accelerate the pace of productive experiments and, accordingly, contract the timeline needed to gather critical data regarding the effectiveness of applied interventions. Within the next few months we will begin a project to investigate an intriguing newly-discovered approach to possibly achieving this.

2013 and Beyond

Given the tremendous recent increase in the breadth of SENS Foundation's research efforts, we plan to focus the majority of our energies and resources over the next few years into driving existing projects beyond the "entry level" status that most of them occupy now. We will be redoubling our fundraising efforts so as to allow that process to occur as rapidly and productively as possible.