2022 | RESEARCH REPORT
SENS Research Foundation

It's been a remarkable year of progress for rejuvenation biotechnology generally and for our work at SENS Research Foundation in particular. At the same time, pandemic travel restrictions and public health measures have kept us from communicating our discoveries in the usual formats: we've adapted by presenting in virtual conferences, but the face-to-face interactions of our scientists with their colleagues and our donors has been lacking. As part of our efforts to close that gap, we here present our first detailed Research Report in several years, bridging the gap between the condensed summaries in our regular Annual Report and our peer-reviewed scientific papers.

We have not only continued to make progress in areas we launched "in the before times," but have been taken in new directions by our own in-house discoveries and by insights from elsewhere in the accelerating fields of rejuvenation research. And we'll be bringing in new projects in the coming year, some of which you'll be hearing about soon.

Thanks to our donors' generous funding, we are always on the lookout for the next project to support. Academic researchers, biotechnology startups, and qualified independent research centers with projects to advance the development of SENS "damage-repair" therapies are invited to submit Letters of Inquiry at https://www.sens.org/our-research/submit-a-grant-proposal . While we apply a rigorous evaluation of proposals for their scientific and practical merits, their contribution to neglected areas of rejuvenation biotechnology, and their relevance to our Mission, our process is much more nimble and far less onerous than typical government or nonprofit grant application procedures.

Every year, we get closer to the first working rejuvenation biotechnologies for the ultimate defeat of human degenerative aging. In these pages are the ways we've worked to bring that future closer. Welcome back — or welcome aboard.

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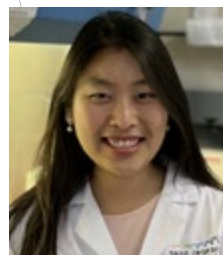
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The SRF team is composed of mission-driven individuals who are the building blocks of our future.
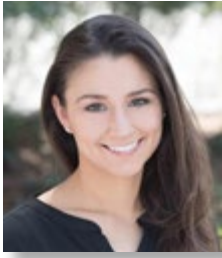
They work tirelessly toward our goal of ending age-related disease.

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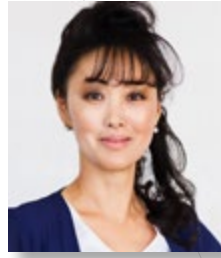**KRISTIE KIM**
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Catalytic Antibodies Targeting Intracellular Tau Oligomers
SENS Research Foundation Research Center

Principal Investigator: Amit Sharma
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Accumulation of tau into insoluble aggregates known as neurofibrillary tangles (NFTs) is a hallmark of several neurodegenerative diseases such as Alzheimer's disease (AD) and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), which are collectively known as tauopathies (1). Importantly, however, extensive evidence suggests that oligomeric and soluble tau aggregates are causally linked to the pathophysiology of tauopathies, and are the direct drivers of cognitive impairment rather than NFTs or other insoluble tau aggregates.(2,3) Even though these species are primarily found within neurons, they can be released into the extracellular space and serve as seeds to spread tau pathology.

Therapeutic interventions with anti-tau immunotherapies have shown promise, but the efficacy seems to vary greatly.(4,5) One possible reason for the disappointing results of human trials may be that the preclinical studies on these antibodies have not focused on whether they engage aberrant tau extracellularly and/or intracellularly. Immunotherapies that only intercept extracellular tau aggregates might slow or arrest the progressive spread of tau pathology across the brain, but might not be expected to rescue neurons whose function is already impaired by existing aggregate burden.

The tau LysoSENS group at SENS Research Foundation is investigating the therapeutic potential of catabodies (catalytic antibodies) targeted to the intracellular compartment to degrade tau aggregates and prevent or reverse tau-associated neurodegeneration.

Figure 1: Advantage of catabodies to ordinary antibodies: Catabodies bind only very transiently to the target aggregate quickly executing direct aggregate destruction without dependence on inflammatory cells. Moreover, catabodies are reused for multiple reaction cycles.

Unlike conventional binding antibodies, catabodies bind transiently to their targets and hydrolyze them into very small hydrolytic end-products, leaving the catabody free to attack the next target molecule.(6) This catalytic mechanism of action constitutes a clear conceptual advantage of catabodies over binding monoclonal antibodies, due to sheer stoichiometry: one catabody can degrade multiple tau aggregates, whereas one binding antibody can only bind and mobilize one aggregate. This is particularly important when targeting the brain, access to which is formidably limited by the blood-brain barrier (BBB), as fewer antibodies will be required to reach the brain to target a given level of aberrant tau.(7)
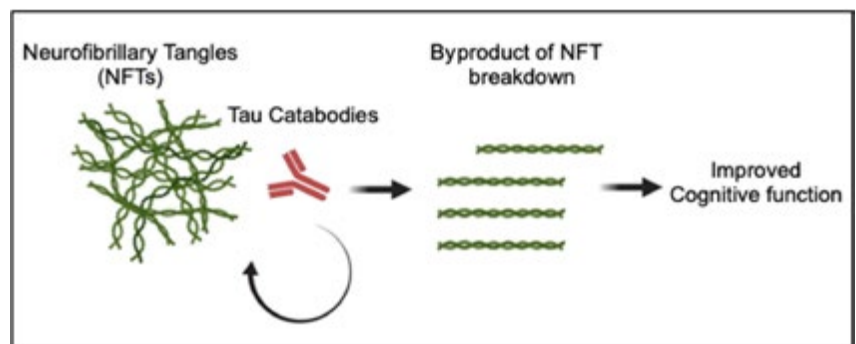
Additionally, catabodies' ability to hydrolyze rather than bind and mobilize aberrant tau species may reduce the risk of side-effects in the form of Amyloid-Related Imaging Abnormalities (ARIA), which have emerged as an important side-effect of binding antibodies used to target β-amyloid (Aβ). While the biological mechanism(s) of ARIA remain to be fully elucidated, they are believed to include iatrogenic cerovascular permeability resulting from antibody-bound Aβ saturating the perivascular drainage pathways while exiting the brain into the periphery (citations in (8)). Catabodies' ability to hydrolyze aggregates *in situ* avoids the need for efflux of antibody-bound aggregate, thus potentially reducing or eliminating the risk of ARIA.

Another innovation of this project is that of increasing the cell penetration of the tau catabodies by covalently conjugating them to phosphorothioated DNA oligonucleotides. This enables the efficient cellular internalization of functionally active antibodies to engage their intracellular target.(9)

The tau LysoSENS group is currently collaborating with Dr. Yue Liu from the Antibody Design Studio, which has developed a rational design approach to modify existing monoclonal antibodies to induce catalytic function without compromising specificity. At the time of this writing, Dr. Sharma is in the process of hiring a talented postdoctoral fellow or other qualified scientist to join the team and conduct studies testing the resulting catabody candidates.

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Rejuvenating Immune Surveillance of Senescent Cells
SENS Research Foundation Research Center

ApoptoSENS

Principal Investigator: Amit Sharma

Research Team: Kristie Kim, Ashley Brauning, Yafei Hou, Tesfahun Admasu

Natural Killer (NK) cells are a known key immune cell type responsible for the immunosenolysis of senescent cells (1). Moreover, NK cells are increasingly emerging as an important defense against several age-related diseases, the best-understood example of which is cancer (2). However, NK cell function declines as part of immunosenescence, and this likely includes immune surveillance of senescent cells, leaving the host increasingly vulnerable to diseases of aging, as recently reviewed in a paper published by the Sharma lab at SENS Research Foundation (2). The loss of NK-mediated immunosenolysis appears to be a highly probable contributor to the rising burden of senescent cells with age. Understanding the impact of aging on NK cell immunosurveillance of senescent cells could enable the development of better-regulated and more selective alternatives to senolytic drugs for senescent cell ablation, and thereby the prevention, arrest, and reversal of multiple age-related diseases.

Given this promise, the Dr. Sharma and colleagues created an improved, easily adaptable, and more physiological co-culture system to increase the fidelity of experimental NK cell immunosurveillance. Previous investigations of this critical function have been flawed due to the use of mixed immune cell populations such as PBMC rather than pure NK cells or defined combinations of leukocytes; unphysiologically high E:T ratios; experiments being conducted for only a few hours, which is inadequate time for the full range of NK cell effector functions to be engaged; and studying populations of ostensibly senescent cells that are in fact a mixture of senescent and unconverted nonsenescent cells. The ApoptoSENS group's new method, recently published in the journal *Aging*,(3) corrects all of these flaws, allowing them and other scientists in the field to gain more reliable knowledge into the phenomenon and its rejuvenation.

Using this new protocol, Dr. Sharma and colleagues have demonstrated the efficacy of freshly-isolated peripheral NK cells in eliminating senescent cells, irrespective of the donor age, or sex, or the mode of senescence induction.(3) The group demonstrates that 50-60% of senescent cells are eliminated following 16 hours in co-culture with pre-activated NK cells (Figure 1). Greater senescent cell killing can be achieved at higher effector:target (E:T) ratios, but at the cost of greater off-target destruction of nonsenescent cells.

Identification of Senescent Cell Surface Markers

As a strategy to enhance NK cell-mediated elimination of senescent cells, the Sharma lab sought to identify proteins selectively present on the surfaces of senescent cells. Such senoantigens could be exploited for a number of therapeutic and diagnostic purposes, including as targets for NK cells engineered for enhanced immunosurveillance, and secondly, to overcome a persistent confounder in senescent cell science by enriching the mixed senescent and nonsenescent cell populations typically present in ostensibly "senescent" cell cultures.

Of note, SENS Research Foundation's Forever Healthy postdoctoral fellow Dr. Tesfahun Admasu has already used a previously-characterized but less specific senescent cell surface protein to make new discoveries about so-called "secondary senescent cells" and their differential vulnerability to senolytics (see "*Identification and Targeting of Noncanonical Death Resistant Cells*" elsewhere in this Report). New and more selective cell-surface antigens could enable even more precise scientific interrogation and therapeutic targeting of bona fide senescent cells.

Figure 1: Activated primary NK cells selectively eliminate senescent cells. NK cells isolated and enriched from different individuals were co-cultured with senescent or nonsenescent IMR-90 cells at the E:T ratio of 1:1 and cytotoxicity was evaluated by LDH release after 16 h of co-culture. The results are plotted as mean % cytotoxicity.

Dr. Sharma and colleagues have used two different approaches to identify senescent cell surface markers. Their first approach employed a proteomic screen of senescent fibroblasts. 35 proteins that are differentially abundant on the surfaces of senescent cells were identified (Figure 2). Through the use of several assays, they have further characterized the five most promising candidates in primary human endothelial cells, and are currently testing the surface expression of these candidates in irradiated and oncogene-induced senescence models. Dr. Sharma has recently filed a patent with the US patent office claiming novel surface proteins as potential therapeutic and diagnostic tools.

In their second approach, a phage display library screening was performed on senescent endothelial cells. Using the phage display technique, 14 motifs that recognized only senescent cells were identified (Fig 3).

The team has identified four highly-conserved peptides that bind to their target on the surface of senescent cells, and have validated those peptides in senescent IMR-90 fibroblasts as well as confirmed it in endothelial cells again (Figure 4).
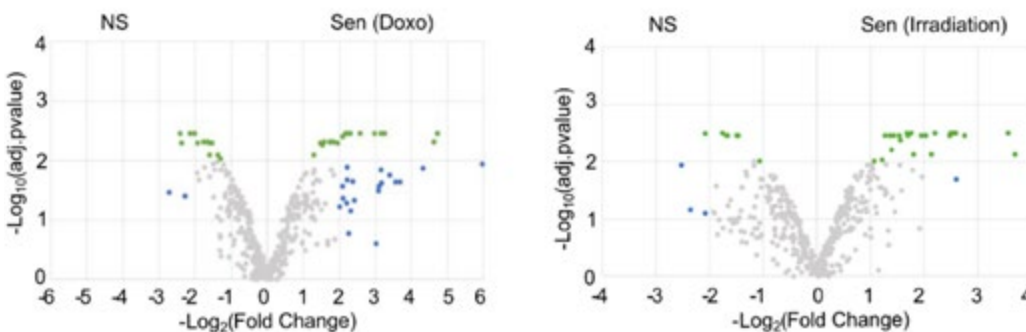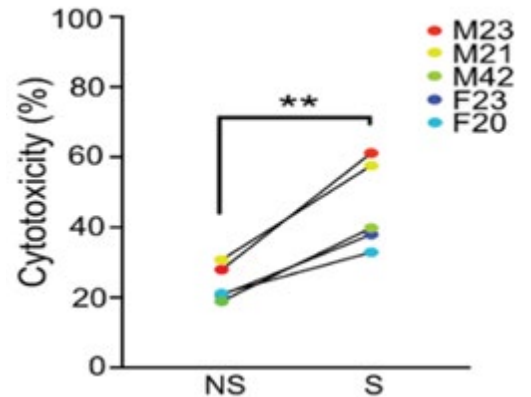

Figure 2: Identification of novel senescent cell surface markers. Volcano plots depicting the abundance of surface proteins affected by doxorubicin treatment or irradiation when compared to nonsenescent cells. Proteins with an adjusted p-value of less than 0.01 were defined as "high confidence" proteins.

These peptides on their own have great potential as either diagnostics, or for improved drug delivery to senescent cells. Identifying the proteins of which they are components could enable enhanced targeting of senescent cells by engineered NK cells; the Sharma lab is currently assessing their potential (see below). Once strong candidate motifs have been identified, a patent will be filed.
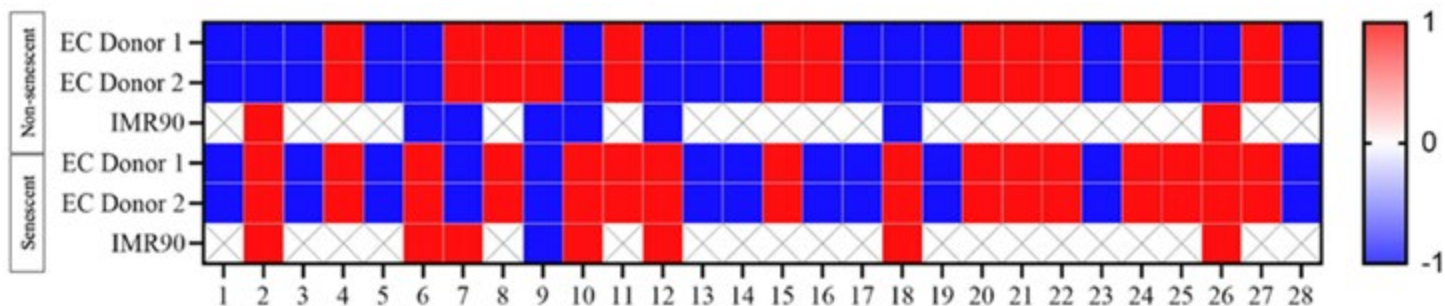
Figure 3: Heat map showing the differences between the nonsenescent and senescent cells when incubated with phage display derived peptides. Peptides were derived via results from phage display analysis. After selection rounds in endothelial cells, only the single condition selective peptides were used for the IMR90 fibroblasts. Red is a positive immunofluorescent signal; blue is a negative immunofluorescent signal; white peptides were not tested under that condition.
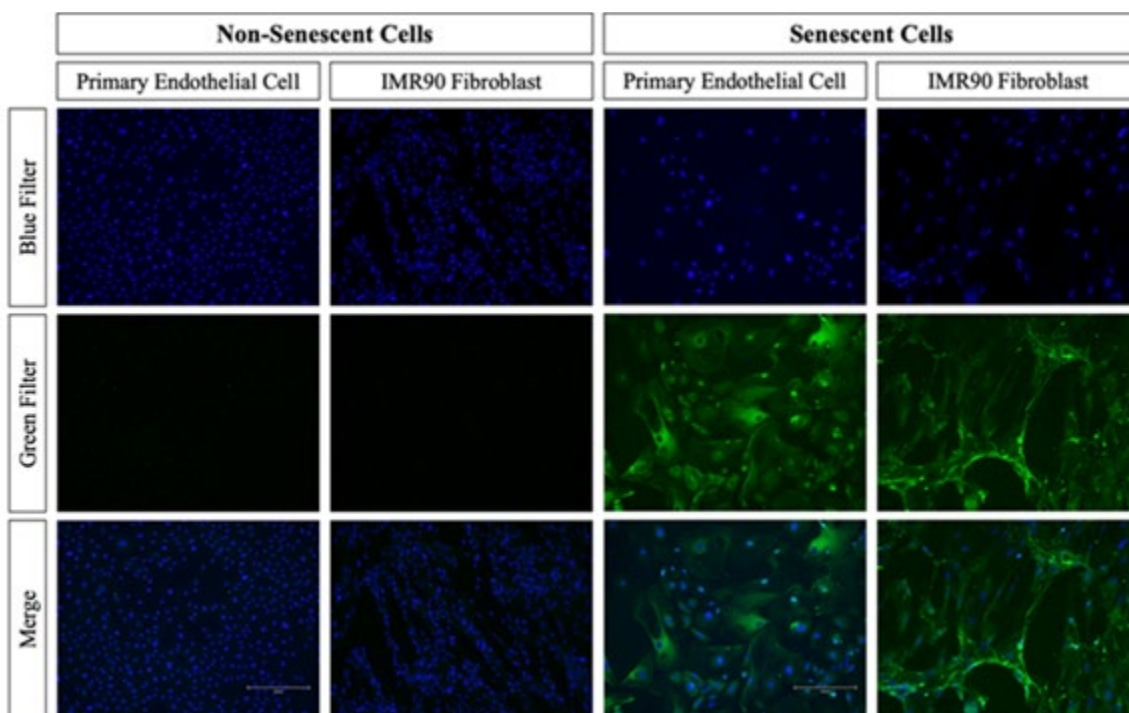

Figure 4: Binding selectivity of peptide ten towards nonsenescent and senescent primary human endothelial cells and human fibroblast cell line IMR90 demonstrated by fluorescence microscopy. Microscopy results expressing the binding of the peptide to A) senescent and B) nonsenescent cells. The control corresponds to cells only stained with DAPI. Blue filter, nuclei stained with DAPI, Green filter, peptide ten and merge, overlapping of all filters. Scale bars represent 300uM.

Reinforcing Senescent Cell Immunosurveillance with Transplanted NK Cells

To understand the potential of NK cell transplantation as an immunosenolytic therapy, the ApoptoSENS team is collaborating with the Campisi lab at the Buck Institute to investigate the effect of aging on NK cell cytotoxicity toward senescent cells. They initially performed cell culture experiments in which mixed mouse spelenocytes were grown in recombinant interleukin (IL)-2, followed by coculture with senescent or nonsenescent mouse fetal lung fibroblasts at an E:T ratio of 1:1. Their results show significantly higher cytotoxicity of splenocytes towards senescent cells compared to nonsenescent control cells (Figure 5).

To determine the effects of aging on NK cell immunosenolysis, the team will be transplanting young or old NK cells into p16-3MR mice that have been treated with the DNA damaging agent doxorubicin. p16-3MR mice, developed by the Campisi lab, have a construct in their cells under control of the promoter for the tumor suppressor p16INK4a, which most senescent cells express. Thus, activation of the promoter drives the expression of both red fluorescent protein (RFP) as a reporter for senescent cells, and also viral thymidine kinase as a drug-inducible "suicide gene." The use of these mice will test the ability of young- vs. old donor derived NK cells to remove senescent cells in a mouse model with high senescent
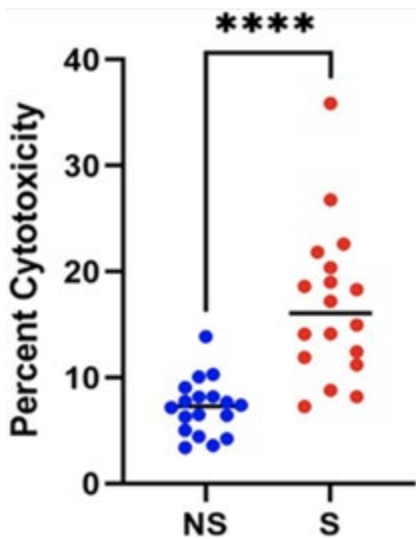
Figure 5: Activated splenocytes selectively eliminate senescent cells. NK cells isolated and enriched from mice were co-cultured with S or NS 3T3 cells at the E:T ratio of 1:1 and cytotoxicity was evaluated by LDH release after 3 h of co-culture.

cell burden, with a convenient reporter system to visualize reductions in senescent cell numbers in different tissues..

Additionally, the Sharma lab is in the process of developing CAR (Chimeric Antigen Receptor)-NK cells with enhanced ability to target senescent cells for adoptive cell therapy, and the above studies will be repeated using CAR-NK cells. CARs, originally developed for adoptive T-cell therapy in cancer, are fusion proteins that combine target-binding and cell-activating functions into a single receptor, by which CAR-NK cells are able to kill target cells based entirely on their presentation of a selected surface protein, without requiring additional signaling to enable target cell destruction. The first constructs have already been created for testing.

Discovery of a T-Cell Subpopulation with Immunosenolytic Potential

Studying age-related changes in the immune cells has led the ApoptoSENS team to discover a sub-population of T-cells that declines with age. FACS analysis of human PBMC indicates that these "X cells" constitute only approximately 5% of total PBMCs, so in order to investigate their interaction with senescent cells, the team established a protocol for enrichment of X cells in culture. This protocol reliably enriched the X cells from less that 5% of PBMCs isolated from peripheral blood to more than 75% of cells, following 10-14 days in a mix of cytokines and growth factors (Figure 6). They then tested the hypothesis that co-culture of X cells with sen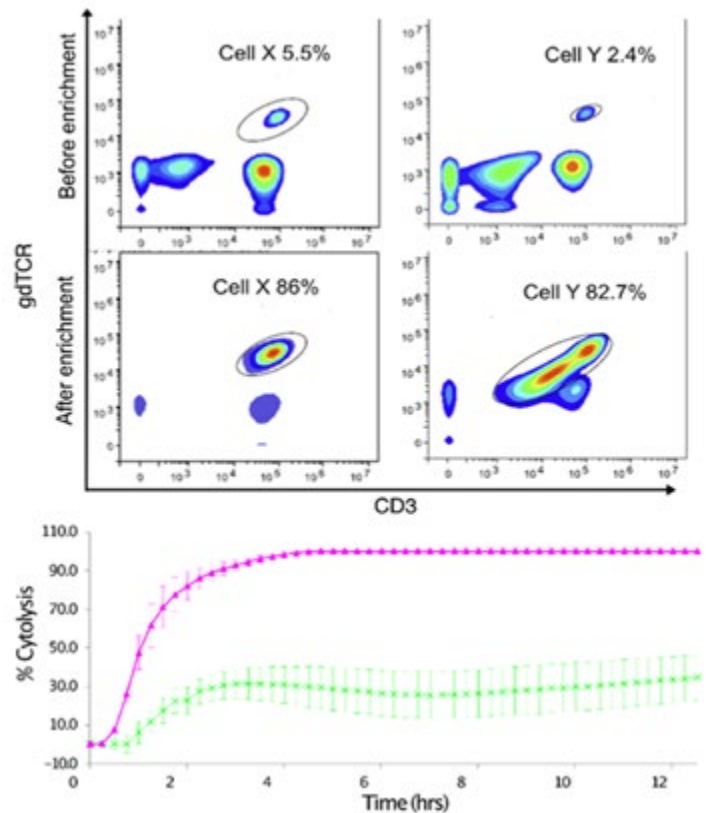escent cells would lead to targeted elimination of senescent cells. To assess immunosenolytic potential, they conducted cytotoxicity assays using impedance measurements.

These experiments indicated that X cells rapidly kill senescent cells (less than two hours of co-culture was required) at E:T ratios of as low as 0.5:1 in a dose- and time-dependent manner. 70% of senescent IMR90 cells were destroyed by co-culture with X cells in just 2 hours, and over 90% were killed within 6 hours, with no significant effect on nonsenescent cells (Figure 6). Based on these promising initial results, Sharma and coworkers are now further assessing the therapeutic potential of this sub-population of T cells, as they appear to be highly selective in eliminating senescent cells in a substantially shorter time than has been reported by others or observed in their own prior work with NK cells. Based on their preliminary data, it appears that X cells may employ an alternative strategy to identify and eliminate senescent cells; Dr. Sharma's team is in the process of investigating the underlying mechanism.

These cells will in the future be tested *in vivo* and compared to NK cells. Depending on the results, the most effective cell therapy product will be selected for preclinical development.

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Figure 6: Isolation and enrichment strategy for "X" cells from human PBMC. (A) Flow cytometry analysis of CD3+ cells and "X" cells. Only 5.5% of the total PBMC were "X" cells (left) and only 2.3% of the PBMC were Vd2 cells (right). (B) Flow cytometry analysis of CD3 and TCR-expressing cells 10 days after enrichment. 86% of cells were positive for CD3 and TCR (left) and 82.6% of these cells were Vd2 (right). (C) % cytotoxicity of "X" cells towards senescent (purple) and nonsenescent cells (green).

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Engineering New Mitochondrial Genes to Restore Mitochondrial Function [MitoSENS]
SENS Research Foundation Research Center

Principal Investigator: Dr. Amutha Boominathan
Research Team: David Begelman, Bhavna Dixit, Carly Truong, Shon Vanhoozer

Cellular energy in the form of adenosine triphosphate (ATP) is primarily generated in mitochondria within cells via the oxidative phosphorylation (OxPHOS) process. The OxPHOS process transfers electrons produced from carbohydrates, proteins, and fats by the citric acid cycle sequentially down the Complexes of the mitochondrial electron transport chain (ETC). Mitochondria are small, reticular organelles, and unusual inasmuch as they house some of their own genes internally, with the mitochondrial DNA (mtDNA) encoding 13 of the proteins of the ETC. A decline in mitochondrial DNA quality and copy number with age has been widely documented, the most notable and consistent example of which is the emergence over time of a small number (≈1%) of post-mitotic cells becoming homopasmic for mitochondria bearing large mtDNA deletion mutations (2,4,6). The age-related decline in mitochondrial quality and function with age has been strongly implicated in several age-related muscular and neurodegenerative diseases such as sarcopenia, Parkinson's, and Alzheimer's disease.

The MitoSENS lab at SENS Research Foundation, led by Dr. Amutha Boominathan, is working to develop rejuvenation biotechnologies to repair or obviate the accumulation of mtDNA mutations with age. Their principal focus is allotopic expression (AE), in which copies of the protein-encoding mtDNA genes are placed in the nucleus, with suitable modifications to allow them to be expressed in the nucleus and translated in the cytosol, following which they must be imported into the mitochondria. There, these gene copies can incorporate into the relevant ETC Complexes and contribute to sustaining OxPHOS. This would allow mitochondria to continue producing ATP, irrespective of the accumulation of particular mtDNA mutations.

In 2022, Dr. Boominathan and her team published a comprehensive review on the current status AE and alternative clinical avenues for mitochondrial DNA mutations (8). In this review, they delineate the requisite features essential for successful allotopic gene expression and the biological and technical hurdles that continue to impede the achievement of these goals. AE can be improved using global strategies such as codon optimization (in which codons are modified not only to minimally sustain proper amino acid sequence in the nuclear genetic code, but opt for preferential synonymous codon substitutions "optimized" for nuclear expression); the inclusion of certain upstream or downstream non-coding regions capable of specifically modulating gene expression; and engineering protein properties for ease of import. However, some individual mitochondrial genes may require additional optimization.

Boominathan and colleagues implemented that strategy in the past to synthesize 2 versions of the 13 mtDNA genes: a) the minimally recoded version that is absolutely required for productive protein translation in the cytosol and b) the codon-optimized version, synchronizing the codon usage in these genes to the mammalian nuclear code. They were able to successfully demonstrate robust transient protein production and mitochondrial association for all the 13 mtDNA genes using the codon-optimized gene expression constructs (5). Cytosolic protein expression under transient expression was substantially higher for the codon-optimized than for minimally-recoded genes, and similarly for steady-state mRNA levels under stable selection. Eight of the re-engineered genes retained expression and targeting to the organelle after stable selection. Building on these early observations, the team validated the utility of these codon-optimized mtDNA gene constructs for additional mitochondrial protein targets that did not work in the past.

Additionally, during these investigations, the team has optimized a simple and inexpensive protocol to enrich mitochondria from mammalian cells for use in the laboratory. They have published these results in order that their improved methods can be of use to the larger scientific community (3).

Allotopic Expression of ND4 in a LHON disease cell model

Leber's Hereditary Optic Neuropathy (LHON) is characterized by any of three mitochondrial mutations that impair OxPHOS Complex I function and reduce ATP production. ~70% of LHON patients are homoplasmic for the ND4 m.11778G>A mutation, with clinical presentations of bilateral vision loss. The MitoSENS team expressed two versions of the allotopic ND4 gene (a minimally-recoded version and the codon-optimized constructs) in a patient cell line homoplasmic for the LHON m.11778G>A mutation. The wild-type ND4 subunit in Complex I has one binding site for rotenone, which is a potent Complex I inhibitor. However, this property is abolished in the LHONmut cells. The team therefore used this rotenone sensitivity parameter to assess functional recovery in their allotopic constructs. Incubating wild-type 143B osteosarcoma cells with increasing concentrations of rotenone inhibited the growth of these cells in a dose-dependent manner (Figure 1). However, the LHONmut cells were resistant to rotenone sensitivity under similar conditions. Incubating LHONmut cells stably expressing either the recorded or the codon-optimized ND4 constructs partially recovered their sensitivity to rotenone, indicating restoration of active Complex I in the respiratory chain — and only in the codon-optimized construct was this effect statistically significant. They are currently optimizing gene constructs to further improve this property and mitochondrial fitness in these cells.

Allotopic Expression of ATP6 in a Leigh's syndrome model

Leigh's syndrome is a neurometabolic disease affecting pediatric populations due to specific mutations in certain nuclear-encoded mitochondrial and mtDNA genes. Mitochondrial DNA-associated Leigh's syndrome is a progressive neurological disorder, and mutations in at least 11 mtDNA genes have been attributed to this disease. The MitoSENS team at SRF
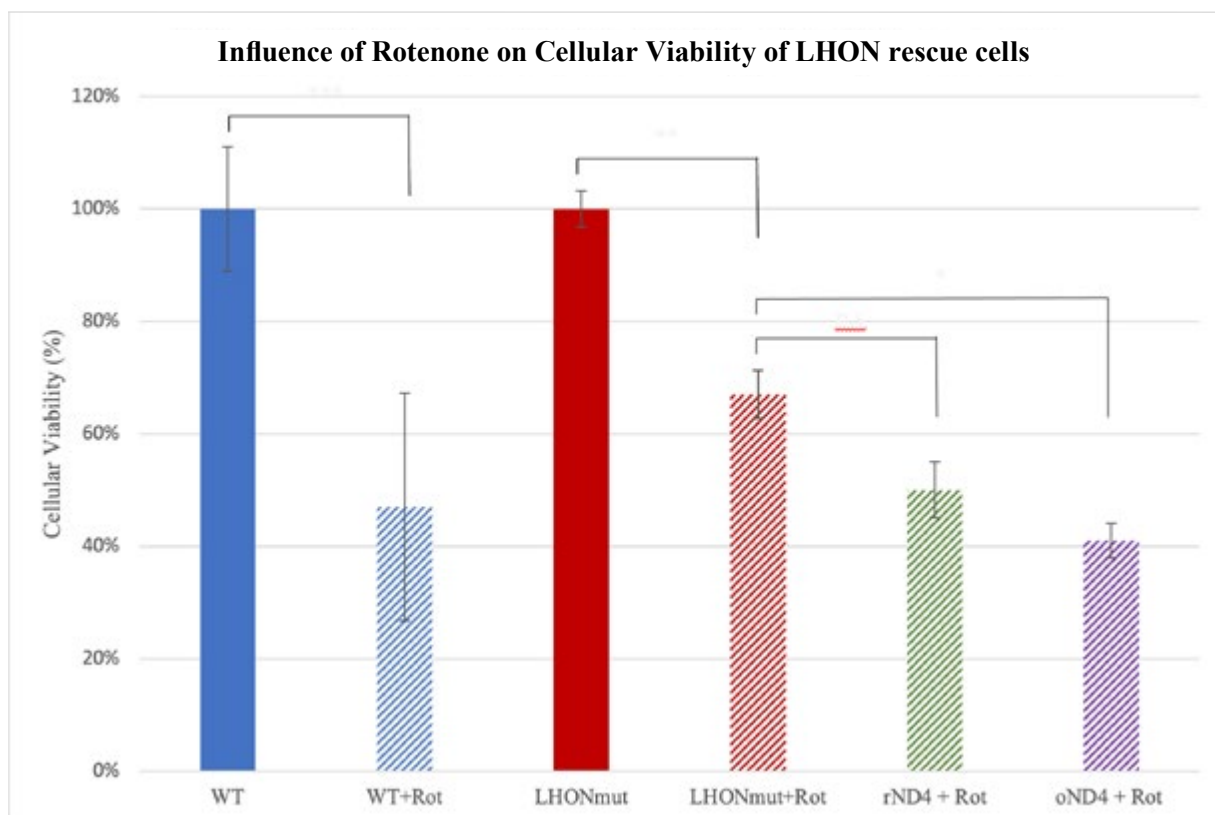

Figure 1. Allotopic ND4 demonstrates some recovery of complex I-dependent cell viability. Cellular viability was assessed by measuring the cellular impedance in the xCELLigence RTCA MP. All measurements were normalized to the untreated condition of the respective cell line. rND4: minimally-recoded ND4; oND4: codon-optimized ND4. Data is presented as cell viability relative to untreated condition of respective cell line +/- SD. N.s. = not significant [rND4 + Rot p = 0.058]; * = p< 0.05 [oND4 + Rot p = 0.032]; ** = p<0.005 [LHONmut + Rot p = 0.0039]; *** = p<0.0005 [WT + Rot p = 0.00031]

expressed different versions of allotopic ATP6 in a Leigh's syndrome patient cybrid cell line with a mutation in the ATP6 gene (m.8993T>G). We observed that allotopic ATP6 can be expressed transiently for up to 2 weeks (Figure 2). However, the expression was not retained stably. Dr. Boominathan and colleagues' current efforts are in expressing the allotopic ATP6 gene and the remaining 4 genes (namely: ND5, CYB, COX1 and COX3) that failed to express stably upon codon optimization alone. We are using computational and virus-mediated directed evolution in addressing these drawbacks.
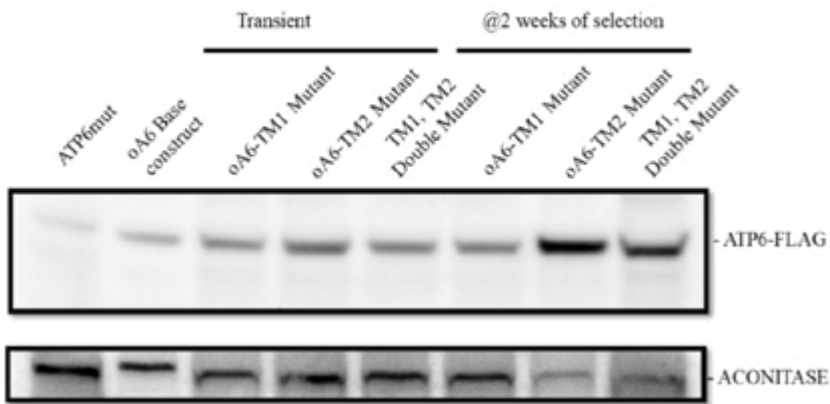

Figure 2. Allotopic ATP6 expression in Leigh's syndrome cybrids.

Variants of codon optimized allotopic ATP6 with a C-terminal FLAG tag were expressed in the m.8993T>G Leigh's syndrome cybrid cell line. Mitochondria were isolated from transfected cells at 72 h (transient) or 2 weeks and analyzed on SDS-PAGE for expression. The matrix protein aconitase was used as the loading control for mitochondrial content.

Allotopic Expression *In Vivo*

Previously, the MitoSENS team demonstrated successful functional replacement of the ATP8 gene in a patient cybrid cell line with a severe mutation (1). To implement AE as a rejuvenation biotechnology or as a treatment for genetic diseases of congenital mitochondrial mutations will require gene therapy, but gene therapy strategies utilizing viral vectors such as adeno- or lentiviruses are limiting in their cargo sizes, and risk disrupting neighboring genes during random integration.

To overcome the limits of these or any other gene therapies, SENS Research Foundation funded the development of the Maximally Modifiable Mouse (MMM), a mouse model in which a "landing pad" for phage integrases has been engineered into a well-characterized safe harbor locus in the mouse nuclear genome. Using these mice, phage integrases can be used to deliver genetic payloads of arbitrary size into a known location in the genome without disruption of native genes.

The MitoSENS team have now used the MMM to create a new transgenic mouse model expressing the ATP8 gene from this safe harbor locus. To achieve this, they crossed the MMM with the only available mouse model harboring a mutation in the ATP8 gene, namely the C57BL/6J-mtFVB/NJ/IbraJ or the "mtFVB" conplastic mouse. This model has a wild-type (WT) nuclear genome, but a polymorphism in the mitochondrial ATP8 gene which leads to mild biochemical perturbations, allowing Dr. Boominathan and colleagues to assess the potential of exogenous allotopic ATP8 to compete with the endogenous mutant ATP8 protein.

To demonstrate safe harbor-based AE, the team first introduced the allotopic ATP8 gene in the nuclear genome of the WT strain of this mouse model and confirmed that the transgene was
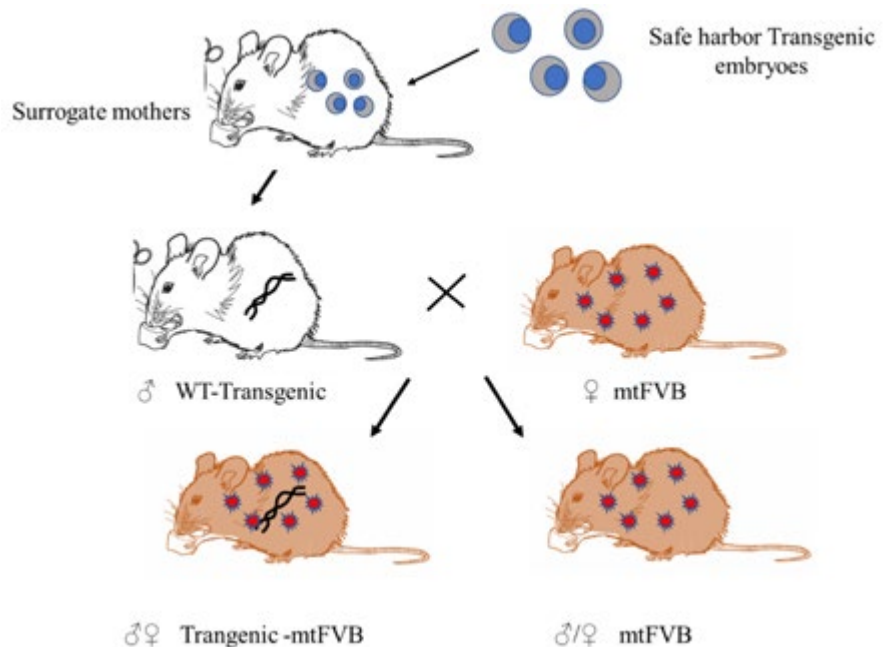

Figure 3A: Schematic to generate the transgenic ATP8 mice. Embryos engineered with the allotopic ATP8 in the ROSA26 locus were implanted into surrogate mice. The male progeny verified for the presence of a single copy of transgene were crossed with female C57BL/6J-mtFVB/NJ/IbraJ (mt-FVB) mice. All the offspring from this cross were positive for the ATP8 mutation mt.7778 G > T due to maternal inheritance.
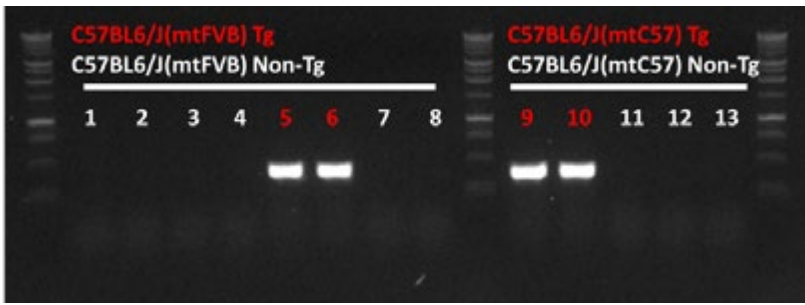
Figure 3B: Transgenic mice with the nuclear allotopic ATP8 gene were identified through genotyping. Fourth-generation positive PCR product of ~447 bp amplifying a region unique to the transgene is shown.

incorporated at the correct location in the nuclear safe harbor. Subsequently, they crossed male MMM mice with female mtFVB mice to transmit the mutated mtDNA genotype across progeny (Figure 3A). After careful genotyping to confirm that these mice had a single copy of the AE transgene in the nucleus (Figure 3B), assessment has shown that germline introduction of the ATP8 allotopic gene in the nucleus of mtFVB mice is tolerated very well and transgene expression is observed across all tissues tested (Figure 4A). The exogenous ATP8 protein efficiently incorporates into the ATP synthase 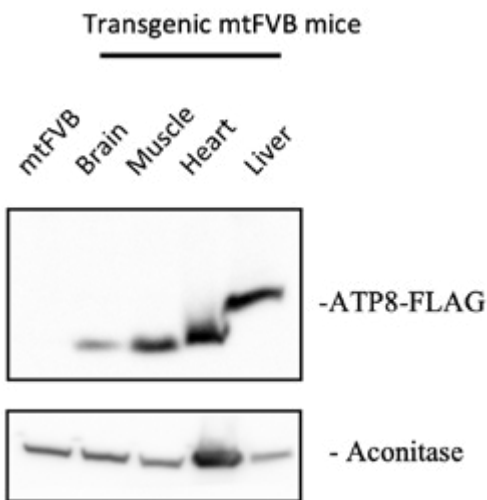complex (Figure 4B). Furthermore, the transgene was inherited faithfully in the progeny up to 4 generations (Figure 3B). Confirmatory studies on the properties of such a model are ongoing and the team hopes to publish these results soon.

Successful demonstration of safe harbor expression for the allotopically-expressed ATP8 gene and its functional integration into the mitochondria will open avenues to test larger transgene inserts and possibly more than one gene simultaneously. Additionally, it will allow for control over gene copy number, regulation, and expression. Moving all the 13 mtDNA genes to the nucleus using this technology will allow us to address larger questions in the field as to the precise roles of mtDNA mutations in aging and age-related diseases, and create proof-of-concept for AE as a treatment or cure for congenital mitochondriopathies and as a human MitoSENS rejuvenation biotechnology.

Figure 4A: Tissue wide expression of the transgene in the mtFVB mice.

Dominant mitochondria transplantation therapy

The mitochondrial DNA deletions that accumulate in aging cells have a strong selection advantage, amplifying within post-mitotic cells to the point of homoplasmy. The MitoSENS team is exploring a strategy to address this issue by transferring exogenous, viable mitochondria modified for sustained retention and for therapeutic activity. While mitochondrial transplantation is already being investigated by several groups and companies as a therapeutic intervention strategy, Dr. Boominathan's team is advancing an improvement on this strategy using mitochondria with genomes engineered for dominance over the native genotype.

SRF has established mitochondrial transfer of both naked and exosome-encapsulated mitochondria. Mitochondria were isolated in their native form from 143B osteosarcoma cells stably expressing mito-mCherry fluorescent signal, which selectively labels mitochondria red within the cell. These mitochondria were purified and overlaid or centrifuged with unlabeled Rho0 cells (which lack mtDNA) and incubated for 24-48 hours. Subsequently, microscopy analysis of the Rho0 cells showed mCherry signal as red clusters surrounding the nucleus,

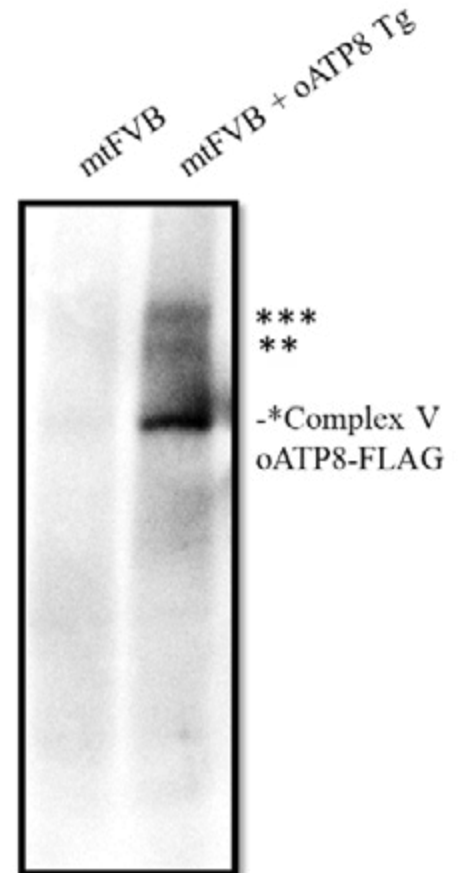**Figure 4B: Allotopic expression of ATP8 *in vivo*.** 4A. Purified mitochondria from various tissues (brain, muscle, heart and liver) in the mtFVB transgenic mice were analyzed for protein expression on SDS-PAGE. The mitochondrial matrix protein aconitase was used as the loading control. 4B. Mitochondria samples from mtFVB and transgenic mtFVB mice were analyzed by Blue Native PAGE and probed with anti-FLAG antibody.
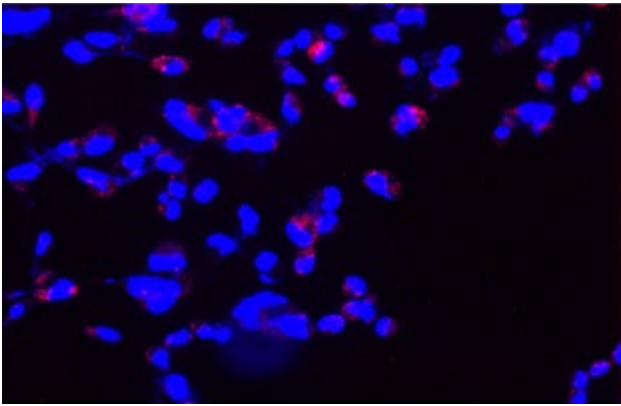
Figure 5: Transfer of mCherry-expressing mitochondria into Rho0 cells. Mitochondria were purified from wild-type cells stably expressing mito-mCherry and overlaid on Rho0 cells. After 48 h the nuclei of Rho0 cells were stained with DAPI (blue) and imaged. Red clusters seen around the nuclei indicate transplanted mitochondria.

suggesting the presence of donor mitochondria within the recipient cell line.

Alternatively, exosome-encapsulated mitochondria were enriched from mesenchymal stem cells and stained with Mitoview Red. Such exosomes were incubated with Rho0 cells pre-stained with Mitoview green. Overlay analysis of the red and green channels via microscopy indicates incorporation of exogenous exosome-encapsulated mitochondria into Rho0 cells (Figure 2). Studies are ongoing in the lab in evaluating the bioenergetics and functional improvement resulting from exogenous mitochondria transfer.

If successful, this alternative MitoSENS biotechnology offers flexibility in options to rescue cells overtaken by mutation-bearing mitochondria, with the advantage of simpler delivery and lower regulatory hurdles relative to AE and potential nearness to the clinic.
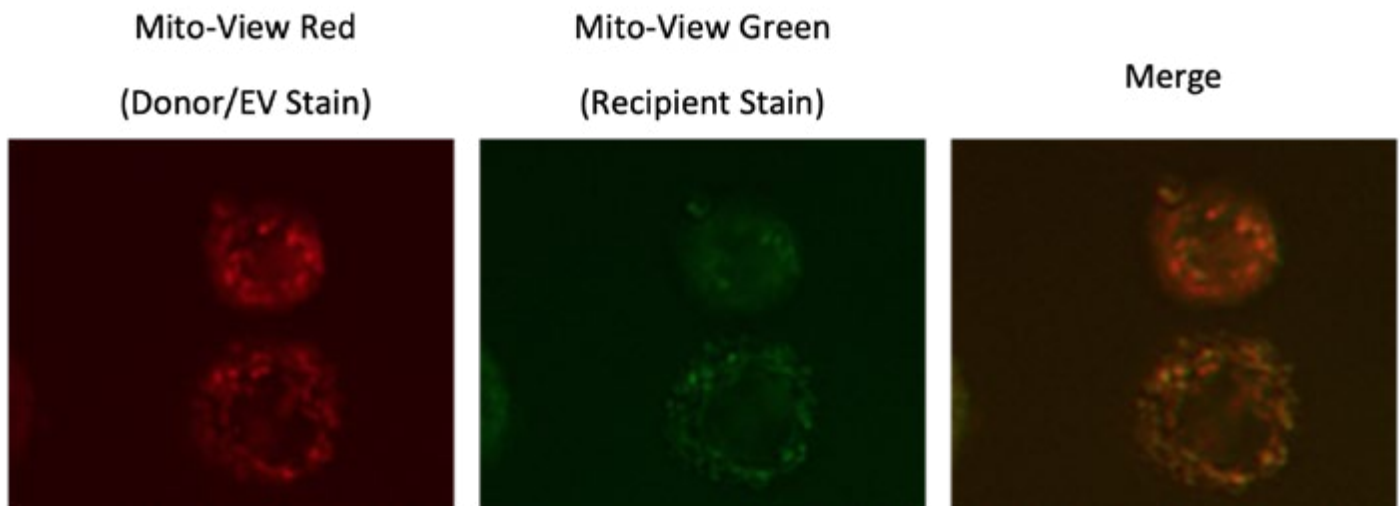

Figure 6: Transfer of MitoView red labelled mitochondria from mesenchymal stem cells encapsulated in exosomes into recipient cells (Rho0 cells) labelled with MitoView green for endogenous mitochondria.

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Target Prioritization of Extracellular Matrix Aging
The Babraham Institute

✂ GlycoSENS

Principal Investigator: Jonathan Clark
Research Team: Melanie Stammers, Izabella S Niewczas, Anne Segonds-Pichon

The extracellular matrix (ECM) is the material found outside cells that forms the support for cells to reside on and "holds" us together. Every organ, whether it is skin, bone, tendon or anything else, is made up of ECM with cells embedded in it. There is a close relationship between the composition of the ECM for a specific tissue and the function of that tissue, with the specific constituent proteins and three-dimensional structure of the ECM in a tissue providing the components of strength, elasticity, and rigidity as required. As we age, changes occur not only in the cells within the ECM, but importantly also in the composition and chemistry of the ECM. Many of the characteristic physical changes that we associate with ageing, such as the changes in skin appearance or the decrease in flexibility of joints, are specifically the result of changes in the structure and composition of the ECM. Two important components of the ECM are elastin and collagen; indeed, collagen is estimated to account for about 30% of the protein in the body. The Clark lab is investigating age-related changes in the chemical structure of elastin and collagen and how these changes impact the mechanical behavior of the tissues.

The tendon is a relatively simple tissue, being largely made up of one type of collagen, with the strands of the collagen lined up in the same direction (as found in rope). Many other ECM tissues are much more complex, with mixtures of collagen types and elastin in layers with different orientations, which makes them much harder to study, and the ensuing data obtained much harder to interpret.

It is therefore perhaps not entirely surprising that there is a high level of conflicting and confusing information in the literature on how the physical properties of the ECM change with age and what causes these changes. Even for an apparently simple question such as "does tendon increase its stiffness with age?", different groups have variously reported increases, decreases, and a lack of change. There are discernible explanations for many of these apparently conflicting results being obtained, but it is only though careful study without a pre-conceived belief in what is happening that the truth can be determined.

The Clark lab's work on aging changes in the tendon illustrate this point. Tendon is a relatively simple tissue, being largely comprised of one type of collagen, with the strands lined up in the same direction as found in rope. Many other tissues are much more complex, with mixtures of collagen types and elastin in layers that point in different directions, which makes them much harder to study and the data obtained much harder to interpret.

Prior to Clark's investigations, the general consensus in the literature was that tendon increases in stiffness with age and that this was due to an increase in crosslinking between collagen molecules. 1 His group has instead found was that it is not possible to say that tendon gets stiffer with age, particularly when comparing mature to genuinely aged animal tissue (rather than comparing tissue from immature, growing animals to that of young adult animals as has often been the case, which is not a valid comparison for probing the mechanism of degenerative biological aging). Instead, Clark and colleagues report an increase in the breaking strain, a decrease in the ability to absorb stress, and an increase in the fragility (chance of rupture) with age. This is clearly a more refined and complex description of the physical properties, and accords better with the orthopedic vulnerabilities of aging human tissues.

Prior groups have concluded that crosslinking increases in aging tissues based on studies involving only one or a small subset of related crosslinks (such as advanced glycation endproduct (AGE) crosslinks). Clark's group has instead studied the level of multiple different kinds of crosslinks and has found a more complex picture, with some crosslinks increasing in number while others decrease.[2] In fact, they found that overall crosslinking decreases with age, and that the different types of crosslinks convey different properties on the tissue. For instance, cyclic stretching and relaxation tests revealed that while unmodified tendon undergoes a substantial reduction the stress-strain profile between the first cycle and second cycle but then is relatively stable through subsequent cycles, reduction of the tendon with borosilicate to fix crosslinks results in progressive increase in stress at higher strains with each test cycle, which indicates an extension of tendon length with each cycle.

Clark's research has also revealed an increase in irreversible crosslinks in the tendon with age, which increase the force required to break a tendon. This increase occurs even as the tendon gradually becomes depleted of reversible crosslinks that allow the tendon to adapt to and absorb force.
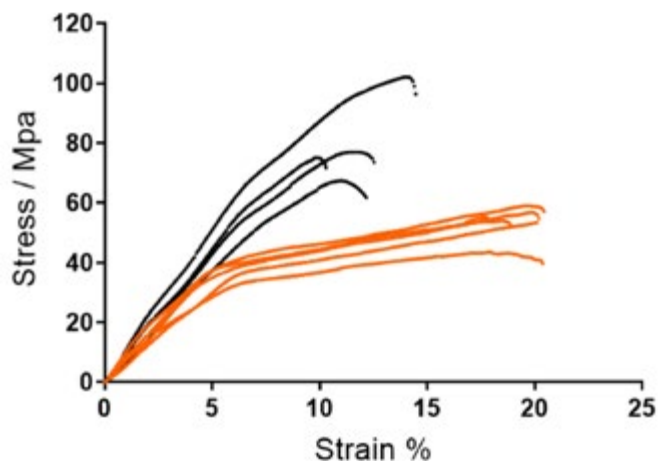

Fig. 1: shows data from 11 week old mouse tail tendon. The traces in orange show the normal response and in black after the crosslinks have been fixed by reduction. This shows that it is important for the bonds to be able to break and re-form in order to absorb the applied stress. As tissue ages, the bonding changes to forms that are chemically stable (fixed) and the tissue response becomes more like that seen for the black traces.

in which the sugar molecule forms an integral part of the crosslink, and non-crosslinking AGE adducts (such as carboxymethyllysine (CML)).

In addition to leading to AGE crosslinking, the Babraham group has found that glycation per se causes stiffening of tendon, and that the level of glycation also correlates with the decrease in reversible crosslinks. Clark's group has noted that glycation is not only initially chemically reversible to some extent, but can be reversed *in vitro* by mechanical stretching; however, we do not yet know how important this might be *in vivo*.

Some important areas of work moving forward are to carry out studies to confirm that the changes Clark and colleagues have reported in mouse tissue are paralleled in human tissue. They plan to initially look at human skin and compare it with their mouse skin data. This work will

Understanding how each of these different crosslinks affect the mechanical properties of a tissue and how they change in number with age will enable more targeted strategies for rejuvenation biotechnologies. Based their findings, Clark and colleagues predict that to rejuvenate youthful tendon function would entail decreasing the number of irreversible crosslinks while greatly increasing the reversible crosslinks. Conceptually, this could be achieved through various means, some of which might not involve directly targeting the crosslinks themselves, but instead cell therapy or other approaches that rejuvenate the behavior of the cells that turn over the ECM. In principle, an unbalanced approach based exclusively on breaking a subset of crosslinks might improve some aspects of tissue function but also cause structural problems.

This approach would also deal with another change that Clark's research uncovered in tendon with regard to collagen glycation. Here we refer to glycation per se: the chemical reaction of sugars in body fluids with proteins. It is an early and easily-reversed modification, only a subset of which goes on to become the more permanent AGE following a series of additional modifications; moreover, AGE includes both AGE crosslinks between adjoining proteins (such as glucosepane),
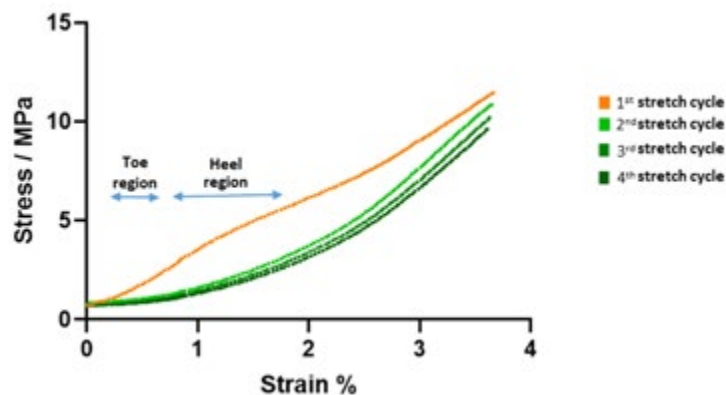

Fig. 2 shows repeated stretching of mouse tail tendon. The orange trace is the first stretch of the tendon and it can be seen that it is much stiffer than subsequent stretch cycles. We have shown that during this stretch that there is a loss of glycation and that this is causing the decrease in stiffness. The subsequent tendon stretches in green can be seen to be very similar to each other and show an elastic behavior.

be facilitated if they are able to show that changes in the skin reflect the changes seen in tissues that are harder to access, such as the aorta. This correlation is seen in mice, but they have yet to show this is the case for human tissues.

The Clark lab plans to continue their studies on different tissues with a focus on the aorta, a tissue of high biomedical significance as the center of arterial stiffening with age. The aorta is a complex, multi-layered structure with two different types of collagen and a high level of elastin. This makes it a much more complex ECM to study and understand how the different changes seen with age contribute to the overall properties of the tissue. Clark's lab have confirmed the widely-reported stiffening of the aorta with age *in vivo*, but the major driver of this process remains to be discovered and targeted with rejuvenation biotechnologies prioritized by causal contribution and ease of therapeutic modification.

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Lipofuscin Degradation by Bacterial Hydrolases

LysoSENS

German Institute of Human Nutrition, Germany

Project Director: Dr. Tilman Grune
Researchers: Annika Höhn, Tim Baldensperger; assistance from Chrisitane Ott, Jana Raupbach, Tobias Jung, Richard Kehm, Patricia Owesny, Vanessa Schnell, Annett Braune

Website: https://www.dife.de/en/research/molecular-toxicology/

Formation of toxic waste products in our cells is an inevitable part of life. Thankfully, most of these hazardous intermediates are either detoxified into less harmful structures or are ultimately degraded, notably in specialized organelles called lysosomes. However, sometimes even our lysosomes fail to degrade highly damaged proteins and lipids, leading to the formation of aggregates. One of best known of these aggregate types is lipofuscin sometimes called "age pigment" (not to be confused with so-called "liver spots" on the skin) because it accumulates with age and is fluorescent. It is resistant to cellular degradation and progressively accumulates in the lysosomes during our lifetime. This effect is especially evident in cells of non-dividing tissues, such as brain and heart, because these cells are unable to dilute their individual lipofuscin content by cell division (1). According to the "garbage catastrophe theory of aging", the accumulation of lipofuscin aggregates limits the remaining life span of the organism by disturbing lysosomal function and inducing cell death (2,3). While humans have no enzymes capable of breaking down lipofuscin, microorganisms possess a wide array of enzymes that allow the degradation of any conceivable molecule formed in nature. Thus, the LysoSENS strategy seeks to identify microbes that are able to degrade lipofuscin via specific hydrolases as lead candidates for potential longevity therapeutics.

To pursue this goal, the Grune lab will use authentic lipofuscin derived from human cardiac tissue. Most experimental work on lipofuscin has resorted to the use of synthetic lipofuscin prepared in culture subjected to e.g. high levels of oxidative stress or proteasome inhibitors, likely creating materials that deviate in important ways from the structural and toxicological properties of lipofuscin *in vivo* and thus generating experimental artifacts. Isolation of sufficient amounts of authentic lipofuscin from human tissue is a mandatory prerequisite to identify enzymes with a high likelihood of degrading the true target substrate. One impediment to the isolation of tissue lipofuscin at sufficient levels has been the strong reliance of most lipofuscin purification protocols on sucrose density gradient ultracentrifugation. This technique is quite time consuming and only suited to isolate rather low amounts of lipofuscin. Based on the observation that the heavily cross-linked lipofuscin core is resistant to ultrasonication and proteinase K degradation, Dr. Grune and colleagues have developed a novel protocol for the isolation of lipofuscin from human hearts. This novel approach is superior not only in yield compared to previous

studies, but additionally reduces the lipofuscin particles to their core components, eliminating potential adsorption of matrix proteins. In total over 1 g of pure lipofuscin was isolated from cardiac tissue (Fig. 1).

In characterizing the isolated lipofuscin, the researchers developed a hydrolysis protocol and analysed the resulting hydrolysate by HPLC-FLD and HPLC-MS/MS (Fig. 2). Structural analysis of the detected compounds is ongoing. Furthermore, ICP-MS detected a strong accumulation of metal ions in lipofuscin, compared to the whole cardiac tissue. Although the results need to be statistically verified, for some metals the concentration factor is in the range of several dozens.

A. Purified Lipofuscin

B. Selected Bacteria

C. Lipofuscin Degradation
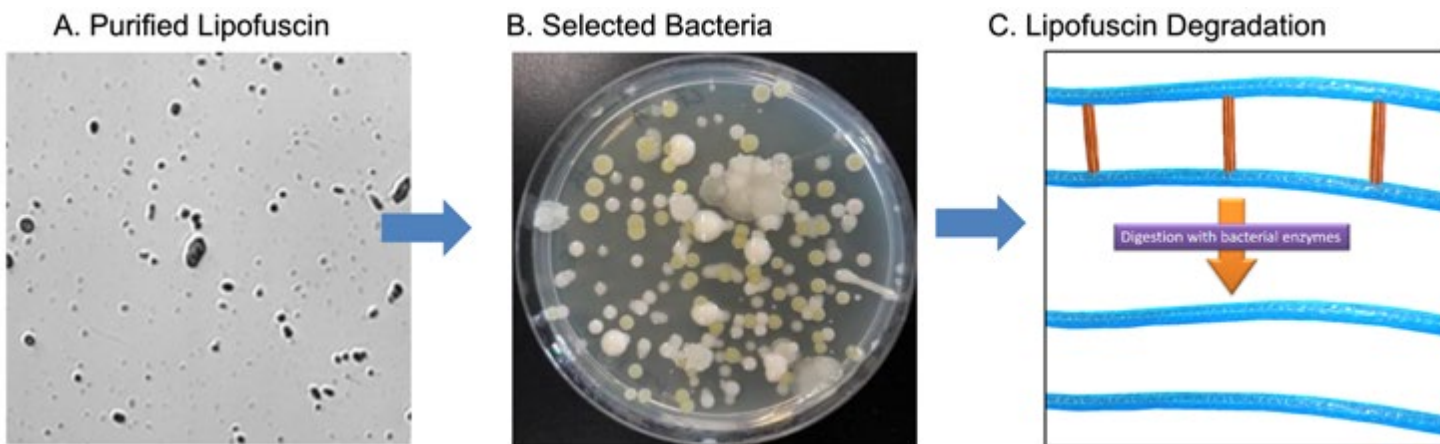
Digestion with bacterial enzymes

Figure 1. Lipofuscin aggregates were isolated from human hearts by a novel purification protocol (A). Lipofuscin was used as the only energy, carbon and sulphur source for selection of lipofuscin-utilizing bacteria (B). Enzymes extracted from these bacteria will be used to degrade crosslinks in the lipofuscin core (C).

Dr. Grune and colleagues extracted microorganisms from different soil samples collected at a residential yard, a forest, a compost heap, and a riverbed. The team used these extracts to select lipofuscin-degrading bacteria by growing cultures on isolated lipofuscin as the only energy, carbon, and sulphur source. Following 20 sets of sub-culture passaging, bacterial mixtures growing on human tissue-derived lipofuscin were extracted, and 12 bacterial strains were isolated. These strains and their specific enzymes will be isolated and further investigated. It bears noting that it is not expected that these bacterial enzymes will prove to be proteases, but hydrolases able to degrade complex crosslinks between proteins, such as cyclic and heterocyclic aromatics. This fact will complicate the identification of lead candidates, but on the other hand, such structures will be unique to lipofuscin and able to function in mammalian cells (after suitable modification) without the danger of digesting functional proteins. A future task will be the targeting of the identified hydrolases towards the lysosomal compartment.
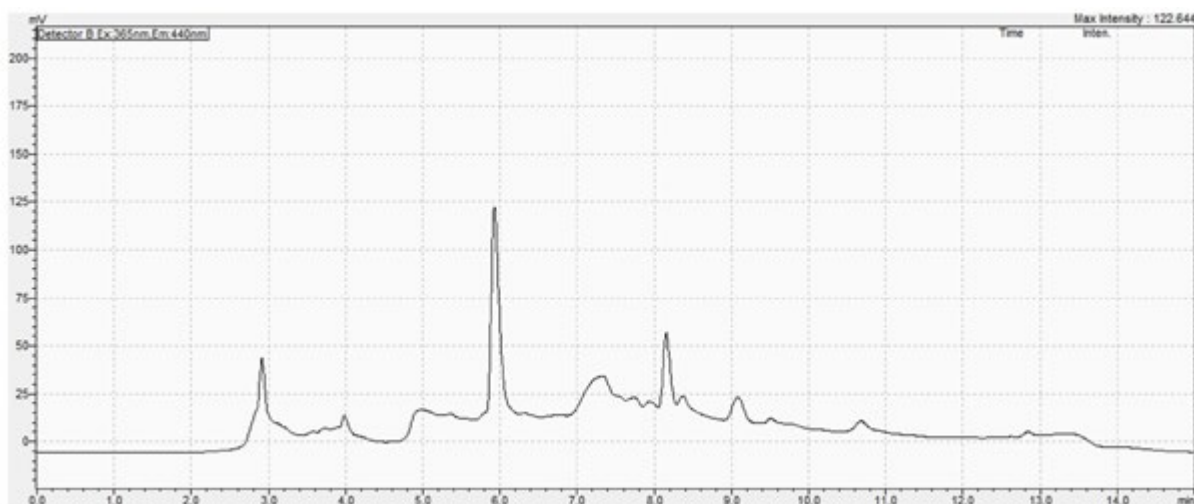

Figure 2. Chromatogram of lipofuscin hydrolysate. Lipofuscin was hydrolyzed and analyzed by HPLC-FLD (Excitation: 365 nm, Emission 440 nm) using a water-isopropanol gradient method.

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SenoStem: Combinatorial Rejuvenation Biotechnologies `ApoptoSENS` `RepleniSENS`
Principal Investigator: Abdelhadi (Hadi) Rebbaa
Research Team: Oliver Frost

Age-related disease and disability results from the complex interaction of multiple forms of cellular and molecular aging damage, and all forms of aging damage accumulate over time and interact with one another, creating both negative synergies and "weakest link in the chain" problems.(12,13) Therefore, comprehensive solutions to specific aging syndromes and the broader process of age-related ill-health are expected to require the repair or replacement of multiple aging lesions in the same organism.(13) However, attempts to combine interventions targeting different classes of aging damage are still rare in the field, because they are commercially challenging (due to complicated intellectual property landscapes), generally disfavored by regulators (which prefer demonstration of safety and efficacy of each therapy individually before allowing human trials or licensing of combinations), academically less attractive (because they use proven methods instead of pursuing complete novelty), and conceptually demanding (as they require domains of expertise and laboratory methods from different research areas).

Prominent examples of this damage are the loss of stem cells and the accumulation of senescent cells. Senescent cells propagate damage and impose systemic metabolic derangement through the secretion of a senescence-associated secretory profile (SASP) including proinflammatory cytokines, profibrotic molecules, proteases, and growth factors, all of which are known to disrupt the function of normal cells and cause functional decline. Interventions that attenuate the harmful effects of senescent cells or eliminate them altogether hold great promise for the prevention and treatment of aging and its associated diseases. Toward this goal, the discovery of senolytics (agents that selectively kill senescent cells) has opened new possibilities for antiaging therapeutic development. For instance, it has been shown that navitoclax (ABT-263) effectively eliminates senescent cells *in vitro* and *in vivo* (1) and ameliorates or reverses the course of a range of animal models of diseases of aging (2,3).

Prior to the discovery of senolytic agents, stem cell transplantation of whole bone marrow (BMSC), haematopoietic (HSC), or mesenchymal (MSC) stem cells into aging hosts was found to ameliorate animal models of a range of diseases of aging(11) and improve overall health and lifespan.(4)(5) A mixture of intrinsic damage (including cell-autonomous mutations and epimutation), clonal haemapoieis of HSC, defects in the aging stem cell niche, and changes in the systemic milieu is responsible for the decline in numbers and functioning of these cells with age.(14-16) Transplants of these stem cells supplement (and to varying degrees replace) lost and/or degraded stem cells. Complicating the effects of these transplants, Preconditioning regimens with total body irradiation and/or myeloablative chemotherapy are normally administered in preparation for HSC transplantation to maximize engraftment, but these regimens necessarily damage the host, including the very niche on which the transplanted cells must rely. As an alternative approach that would not damage the host, recent studies have investigated mobilization of the native stem cells out of the niche using granulocyte colony-stimulating factor (G-CSF) prior to transplant, sometimes in combination with the CXCR4 antagonist AMD3100 (plerixafor)(4).

The SenoStem project at SENS Research Foundation is testing the hypothesis that combination therapy using senolytics and stem cell transplantation will have a synergistic beneficial effect on aging mice and might be able to further improve health and lifespan — literally a remove-and-replace strategy. This approach builds toward SRF's larger long-term goal to develop synergistic combinatorial rejuvenation biotechnology approaches.

One specific potential benefit to this particular combination therapy the rejuvenation of haematopoiesis. MSCs comprise part of the HSC niche and regulate haematopoiesis,(17,18) and both MSC aging(17,18) and experimental bone marrow MSC ablation (19,20) impair and skew the process. A surprising number of otherwise age-retarding and transiently rejuvenating interventions fail to prevent or reverse age-related defects in HSC function in mice.(19) On the other hand, one of the earliest demonstrations of the rejuvenating effects of senolytics was with regard to HSC function in aging mice.(20,21) Yet HSC niche MSCs undergo many age changes other than senescence which impact haematopoiesis.(18) Therefore, treatment to ablate senescent cells (including senescent HSC and MSC) in aging mice followed by replenishment with young-derived MSC free of other age defects offers the potential for a synergistic rejuvenating effect on this core organismal function, amongst others.

Dr. Rebaa and his team are currently conducting *in vitro* and *in vivo* studies to select optimal biomarkers to monitor the response to the anti-aging interventions this project will test, as well as the most effective senolytic from a list of established and in-house candidates.

Senescence biomarker: Among the senescence biomarkers to be tested are Cy7-Gal (the fluorescent dye Cy7 conjugated with a beta-galactosidase substrate (6)) as well as Gal-Nile Blue (galactohexasaccharide-capped mesoporous silica nanoparticles containing Nile blue (10)), both of which were developed by the Martinez-Máñez group. These biomarkers allow for the rapid and non-invasive monitoring of senescent cell burden *in vivo* by measuring the fluorescence of cleaved Cy7 in urine or by imaging the level of Nile blue accumulating in senescent cells (Fig. 1), respectively.
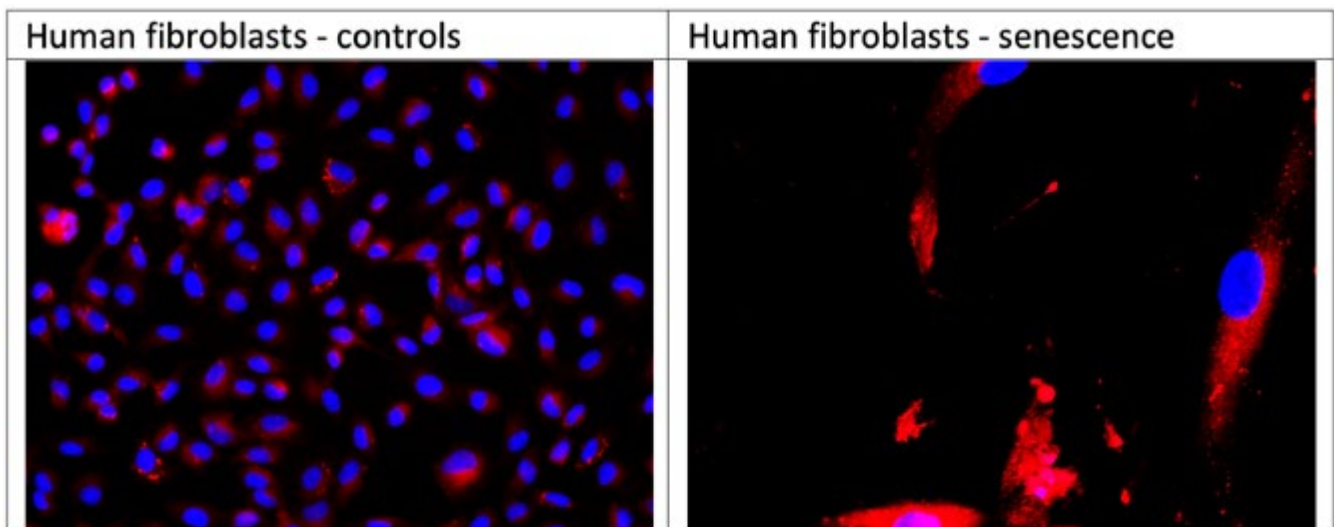

Fig. 1. The tested senescence probe consists of galactohexasaccharide-capped mesoporous silica nanoparticles, which are able to release their cargo, the Nile Blue, in senescent cells due to the hydrolysis of the capping oligosaccharide by senescence-associated beta-galactosidase (SA-β-Gal) activity that is increased in senescent cells. Blue: Nuclear staining with DAPI; Red: Nile Blue Fluorescence.

Senolytic selection: In preparation for the combination senolytic/stem cell therapy, the SenoStem team is carrying out experiments comparing the selectivity and potency of various established and in-house developed senolytics, with the goal of advancing the best candidate for testing in mice. One well-studied senolytic is the Bcl-xL inhibitor navitoclax (1). Although this drug has demonstrated efficacy in selectively eliminating senescent cells *in vitro* and *in vivo*, its ability to induce apoptotic death of platelets and the associated thrombocytopenia (7,8) constitutes a serious limitation for its use. To overcome this challenge, Dr. Rebaa and colleagues are currently testing a Navitoclax-based prodrug (Nav-Gal) (9) that is activated only in cells expressing high levels of the beta-galactosidase enzyme. As platelets do not express beta-galactosidase, and most nonsenescent cells express it only at low levels, this approach may hold great promise in reducing the overall serological toxicity and wider toxicological risks of Navitoclax. Navitoclax and Nav-Gal were compared side by side to assess their effectiveness and potency in reducing senescent cell burden *in vitro* (Figure 2).

SenoStem is an important proof-of-principle in combination rejuvenation biotechnology; positive results hold the potential both to advance translational versions of this specific combination, and to widen interest in this neglected next step in the advancement toward human longevity therapeutics.
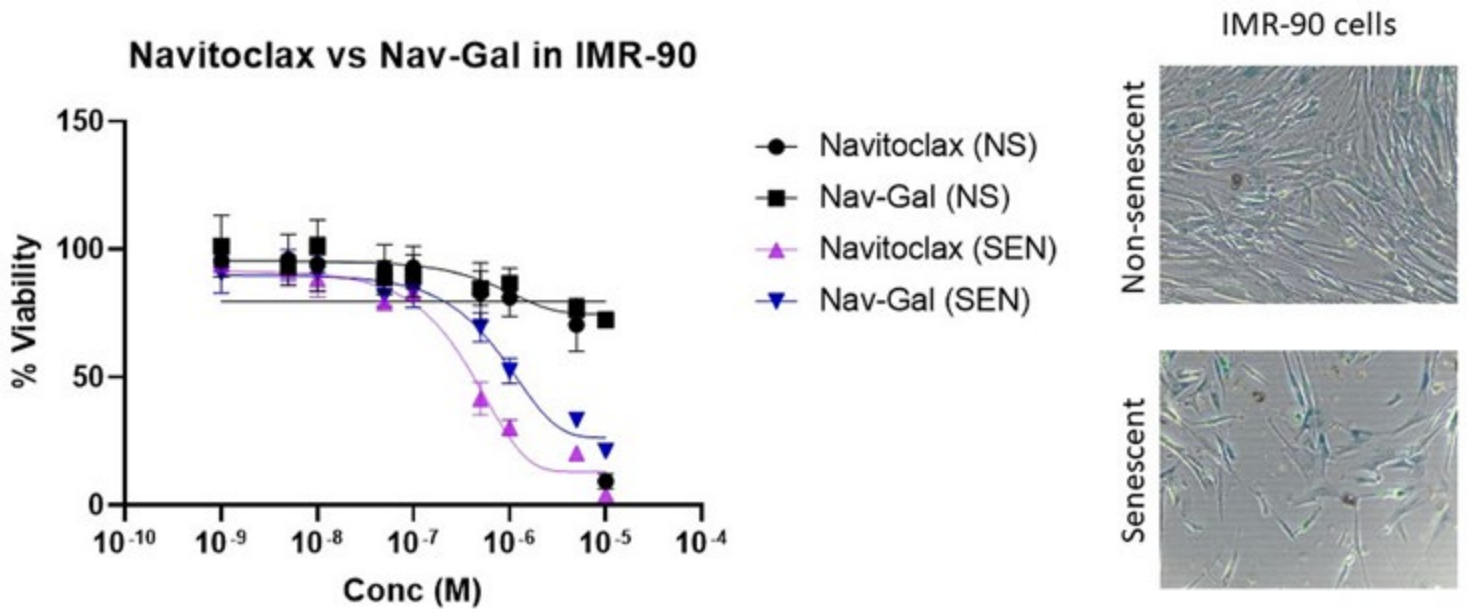
Fig.2 Left panel: Comparison of the senolytic activities of Navitoclax and Navitoclax linked to galactose (Nav-Gal). Nonsenescent (NS) and senescent (SEN) IMR90 cells were subjected to treatment with Navitoclax or Nav-Gal at the indicated concentration for 72 h. Cell viability was determined using the CellTiterGlo assay. Right Panel: Photographs of non-senescent and doxorubicin-induced senescent cells.

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Microglia as a Vehicle for Brain Rejuvenation

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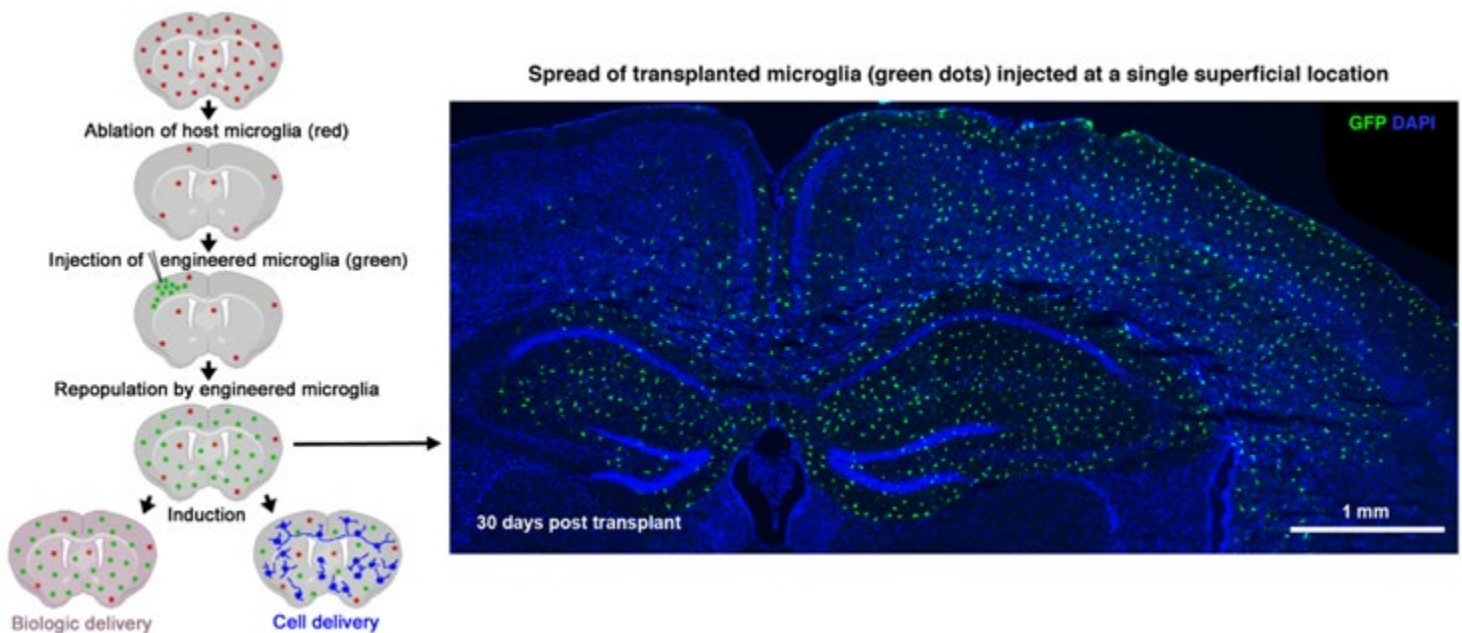
Principal Investigator: Jean Hébert
Researchers: Rohan Hofland, Hiroko Nobuta, Frank Soldner
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https://hebertlab.einsteinmedneuroscience.org/

As our understanding of the processes underlying brain aging and cognitive decline increases, so does the imperative for treating these conditions with biologics, including therapeutic macromolecules such as neurotrophins or anti-inflammatory factors, as well as cell therapy to replace lost cells — most critically neurons. However, the use of biologics to treat brain aging (or any other type of brain disease) has been greatly hampered by the general inability of biologics to cross the blood-brain-barrier (BBB) and their short half-life in circulating blood (1). The current delivery options are injections directly into cerebral tissue (which is limited to local effects due to short diffusion distances, barring highly invasive arrayed injections); injection into the cerebrospinal fluid (which results in limited if any uptake by the parenchyma); intracerebroventricular or intrathecal injection; intranasal delivery (2); or hijacking BBB transporters (which has deleterious side-effects and limited applicability) (1). Therefore, a minimally invasive method for widespread delivery of biologics that circumvents both the BBB and rapid turnover in serum would transform the field of brain health care and rejuvenation by opening the door to innumerable new treatment options.

With SRF funding, the Hébert team has developed a protocol for using microglia as a delivery system for biologics over wide areas of the adult brain (patent filed). With this protocol, endogenous microglia are replaced with transplanted microglia after a single superficial cell injection. Microglia are migratorily more active compared to neuronal progenitors and more easily spread throughout the brain. In addition to therapeutic proteins, this system can be used to deliver new neurons to all areas of the brain to counteract neuronal loss with age. The transplanted microglia can be engineered to produce a secreted biologic, or engineered to be reprogrammed to new neurons. In both cases, normal microglia density is innately re-established, minimizing any effect of transient microglia depletion while providing novel therapeutic support to brain function.

Spread of transplanted microglia (green dots) injected at a single superficial location

Ablation of host microglia (red)

Injection of engineered microglia (green)

Repopulation by engineered microglia

Induction

Biologic delivery

Cell delivery

GFP DAPI

30 days post transplant

1 mm

In 2021, the Hébert group found that transplanted microglia can replace endogenous microglia not only in healthy adult mouse brains, but also in the brains of mice undergoing neurodegeneration, supporting the use of microglia as a delivery system in old brains. The group also showed that replacement microglia can be engineered to secrete the neurotrophin brain-derived neurotrophic factor (BDNF), a factor that causes neurodegeneration when depleted (3,4) and rescues it

when added (4). Together with the group's previous demonstration that they could directly convert microglia to neurons, these findings support the use of microglia in multiple brain rejuvenation strategies.

Over the past year, the Hébert group has been developing the means of bringing this technology to the clinic. First, as part of the collaborative group, Dr. Soldner has established the means of isolating human microglia from human induced pluripotent stem cells (iPSCs) (Fig. 1). These human microglia can repopulate the mouse brain, serving as a platform for further testing and quality control before trials in humans.

So far, Dr. Hébert and his team have used an inducible transgenic mouse model to ablate host microglia (publication pending). To progress to a potentially clinically

Fig. 1: This shows the differentiation protocol of human iPSC into microglia. The differentiation is quite time-consuming, requiring a series of different media with the growth factors and signaling molecules listed on the top. The figure also shows microglia derived from iPSC. IBA1 is microglia-specific surface marker showing the high proportion of microglia in the culture. STEM121 is a stem cell marker showing that the cells are still in the progenitor state.

applicable protocol for humans, his lab is developing a novel method that allows continued pharmacological ablation of host microglia. This, in turn, allows transplanted microglia that are engineered to be resistant to the ablation agent to outcompete residual host microglia for repopulation of host brain tissue (Fig. 2).

Finally, the Hébert group is working on preclinical proofs-of-concept that microglia can be used to curb age-related neurodegeneration. Their first studies will test the ability of microglia engineered to express high levels of BDNF (vs. GFP- expressing control microglia) to retard or reverse neurodegenerative changes in the 5XfAD mouse model of Alzheimer's disease (n ≈ 70), including neuronal density, dendrite complexity, and synapse density. In the meantime, they are building up a supply of otherwise-wildtype aging (>20 months of age) mice
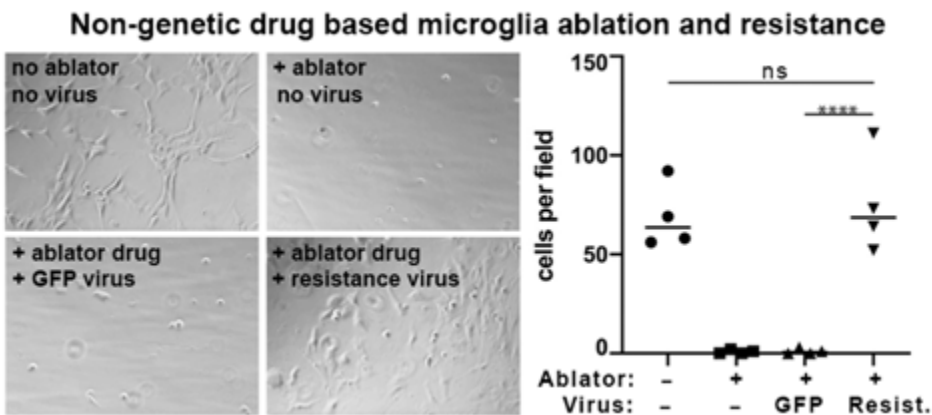

Fig. 2: The pictures on the left show that the genetically-altered microglia are resistant to the drug (bottom right). The right side of the figure shows the quantitative data of the cell cultures.

carrying the transgenes necessary for genetic ablation, for similar studies in this model; these may require only 30 mice. Given positive improvements in neuropathology, these studies will be followed by cognitive tests to detect improvements in treated vs. control mice.

Together, these studies will provide the necessary basis for translating this RepleniSENS biotechtechnology to the clinic, and will open the door to using biologics to treat brain diseases in general and age-related deterioration in particular, for which there is currently no effective treatment.

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Identification and Targeting of Noncanonical Death Resistant Cells

SENS Research Foundation Research Center

ApoptoSENS

Forever Healthy Fellow: Tesfahun Dessale Admasu

It is well established that senescent cells (SCs) can result from a number of stressors, including replicative stress, telomere erosion and damage, and oncogene expression. They are also induced as part of tissue remodeling in wound healing and development. More recently, it was discovered that SCs can spread the senescent phenotype to other cells in the body by secreting factors collectively known as the Senescence-Associated Secretory Phenotype (SASP), sometimes in conjunction with juxtacrine signaling.(1) Surprisingly, this "secondary senescence" can even propagate senescence burden to anatomically remote sites in the body, which can have a significant contribution to SC accumulation with age.(2) Senescent cells are known to have substantial heterogeneity depending on the cell type or the mode of senescence induction, and work to gain a more in-depth understanding of the phenotypes and molecular regulatory pathways of distinctive forms of cellular senescence is still an ongoing endeavor. Characterization of secondary senescent cells might lead to improved senolytic therapies targeting the full spectrum of senescence in aging.

Characterization of secondary SCs and differentiating their biology and vulnerabilities from those of primary SCs is thus critical to developing longevity therapeutics targeting the full spectrum of senescence in aging, and is the central focus of Dr. Admasu's work at SENS Research Foundation.

With his SRF colleagues, Dr. Adamasu developed a novel protocol to overcome one major roadblock in this endeavor, which has allowed him to make new insights into secondary senescence and identify a highly significant therapeutic target for senolytic drugs with broad senolytic activity against both primary and secondary SCs. When researchers induce cells in culture to become senescent, the penetrance is never complete: depending on the cell type, induction method, and other experimental conditions, only approximately 40% of cells in a "senescent" cell culture actually transition into senescence.

In addition to confounding the direct study of primary SCs induced by these methods, the fact that the ostensibly senescent cell culture is actually mixed then propagates an artifact into studies of secondary SCs. Secondary senescence is induced in culture by exposing nonsenescent cells to SASP factors derived from the
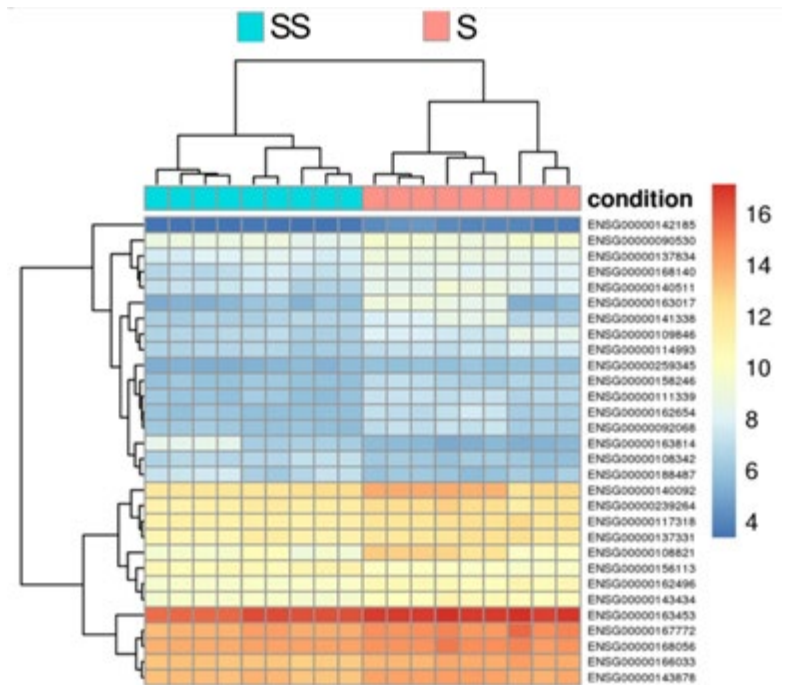

Figure 1: Transcriptome profile of primary and secondary SCs. RNA Sequencing was conducted in primary and secondary senescent human endothelial cells. Heat map of the top differentially expressed genes of primary and secondary senescent cells is shown here. Secondary senescent cells were well-separated from primary senescent cells and clustered together. (LFC >= 1 and P-value < 0.05).

conditioned media (CM) of primary SCs, which latter have been induced into senescence through any of the standard methods. But when the primary SC culture used to induce secondary senescence is in fact only partially composed of SCs, the SASP factors in the CM of such cultures is adulterated with the normal secretory profile of nonsenescent cells, diluting and distorting its effects and limiting its ability to convert normal cells to secondary SCs. The same problem is then perpetuated in the CM of the ensuing culture of secondary SCs, which in turn is itself a mixed population of still-normal cells with cells successfully converted to secondary SC by SASP factors in the original primary SC CM.

To overcome this problem, Dr. Admasu developed a novel technique to enrich primary and secondary SCs using a previously-characterized senescent cell surface

Figure 2: Drug 1 dose optimization. Primary and secondary SCs and their respective nonsenescent cells were treated with range of doses of drug 1 and viability determined 48 hours after treatment (n = 3).

protein. Using this improved culture system, he discovered that secondary SCs engage prosurvival pathways that are distinct from those on which primary SCs rely, and as a result are also resistant to senolytic drugs previously identified to kill primary SCs. Provoked by these differences, he conducted RNASeq analysis and confirmed a distinct molecular signature of secondary SCs (Fig. 1).

Having dissected these differences between primary and secondary SCs, he interrogated the data to identify common prosurvival pathways as targets for senolytic drugs able to kill both cell types. Based on their RNASeq analysis, he and colleagues identified such a target. As a proof of principle, he tested a drug that is a known modulator of their candidate novel senolytic target and confirmed its ability to ablate both primary and secondary SCs, with negligible cytotoxicity towards nonsenescent cells (Fig. 2). Dr. Admasu and colleagues are in the process of filing a patent and publishing their findings in a peer-reviewed journal.

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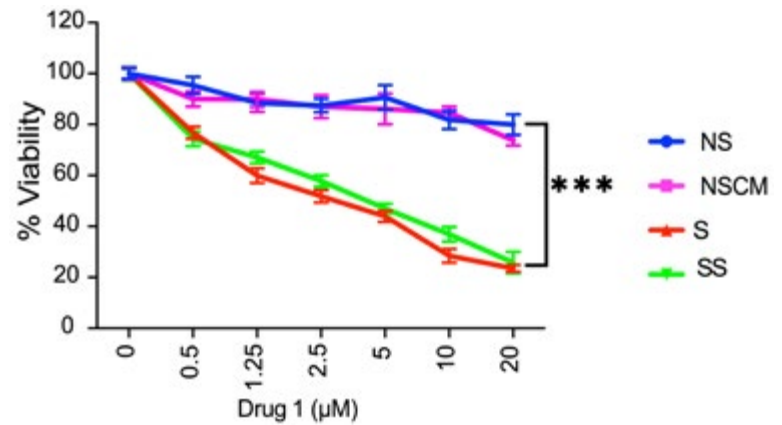
A Reimagined Research Strategy for Aging

Many things go wrong with aging bodies, but at the root of them all is the burden of decades of unrepaired damage to the cellular and molecular structures that make up the functional units of our tissues. As each essential microscopic structure fails, tissue function becomes progressively compromised – imperceptibly at first, but ending in the slide into the diseases and disabilities of aging.

SENS Research Foundation's strategy to prevent and reverse age-related ill-health is to apply the principles of regenerative medicine to repair the damage of aging at the level where it occurs. We are developing a new kind of medicine: regenerative therapies that remove, repair, replace, or render harmless the cellular and molecular damage that has accumulated in our tissues with time. By reconstructing the structured order of the living machinery of our tissues, these rejuvenation biotechnologies will restore the normal functioning of the body's cells and essential biomolecules, returning aging tissues to health and bringing back the body's youthful vigor.

The Targets

Decades of research in aging people and experimental animals has established that there are no more than seven major classes of such cellular and molecular damage, shown in the table below. We can be confident that this list is complete, first and foremost because of the fact that scientists have not discovered any new kinds of aging damage in nearly a generation of research, despite the increasing number of centers and scientists dedicated to studying the matter, and the use of increasingly powerful tools to examine the aging body. Each type of damage contributes in its own way to the rising frailty and ill-health that appears in our sixth decade of life and accelerates thereafter.

Program	Rejuvenation Biotechnology	Aging Damage	Year Discovered
AmyloSENS	Immunotherapeutic clearance	Extracellular aggregates	1907
ApoptoSENS	Targeted ablation	Death-resistant cells	1965
GlycoSENS	AGE-breaking molecules; tissue engineering	Extracellular matrix stiffening	1958, 1981
LysoSENS	Novel lysosomal hydrolases	Intracellular aggregates	1941, 1842
MitoSENS	Allotopic expression of 13 proteins	Mitochondrial mutations	1972
OncoSENS	Removal of telomere-lengthening machinery	Cancerous cells	1959, 1982
RepleniSENS	Stem cells and tissue engineering	Cell loss, tissue atrophy	1955

The specific metabolic processes that are ultimately responsible for causing all of this damage are still only partially understood. The good news is that we don't need to answer the many open questions about the causes of structural decay in order to develop effective therapies to reverse it. No matter what caused a given unit of damage in the first place, the same regenerative therapeutics can be used to repair it. In other words, it doesn't matter how a given microscopic lesion occurred, if we apply rejuvenation biotechnologies that restore the machinery of life to proper working order.

The even better news is that we now understand how to fix all of this damage. For each major class of aging damage, a strategy for its removal or repair either already exists in prototype form, or is foreseeable from existing scientific developments: see the specific "Rejuvenation Biotechnology" listed for each kind of aging damage in the Table.

Even after we have used these new therapies to repair an aging tissue, metabolic processes will continue to cause new damage. This simply means that rejuvenation biotechnologies are not a one-off fix, but will need to be periodically repeated to preserve youthful function. Just as cars need regular rounds of oil changes and spark plug replacements to keep them running smoothly, people will need to go in to rejuvenation clinics to keep up with their regenerative treatments to continue postponing age-related disease.

The entire SENS strategy – from the kinds of aging damage that occur, to their contribution to age-related frailty and illness, to the rejuvenation biotechnologies that can repair and replace the damaged functional units – are described in detail in *Ending Aging*, by SENS Research Foundation Co-Founder Dr Aubrey de Grey and Science Writer Michael Rae. Copies are available from your local bookstore, or from Amazon.com and other online retailers.

Through groundbreaking research in rejuvenation biotechnology, SENS Research Foundation is catalyzing the development of new medical therapies to comprehensively address the disabilities and diseases of aging, leading to a reimagined aging.

SENS Research Foundation PUBLICATIONS

Olgasi C, Borsotti C, Merlin S, Bergmann T, Bittorf P, Adewoye AB, Wragg N, Patterson K, Calabria A, Benedicenti F, Cucci A, Borchiellini A, Pollio B, Montini E, Mazzuca DM, Zierau M, Stolzing A, Toleikis PM, Braspenning J, Follenzi A. **Efficient and safe correction of hemophilia A by lentiviral vector-transduced BOECs in an implantable device.** *Mol Ther Methods Clin Dev. 2021 Nov 3;23:551-566. doi: 10.1016/j.omtm.2021.10.015. eCollection 2021 Dec 10.* PubMed: 34853801

Abstract

Hemophilia A (HA) is a rare bleeding disorder caused by deficiency/dysfunction of the FVIII protein. As current therapies based on frequent FVIII infusions are not a definitive cure, long-term expression of FVIII in endothelial cells through lentiviral vector (LV)-mediated gene transfer holds the promise of a one-time treatment. Thus, here we sought to determine whether LV-corrected blood outgrowth endothelial cells (BOECs) implanted through a prevascularized medical device (Cell Pouch) would rescue the bleeding phenotype of HA mice. To this end, BOECs from HA patients and healthy donors were isolated, expanded, and transduced with an LV carrying FVIII driven by an endothelial-specific promoter employing GMP-like procedures. FVIII-corrected HA BOECs were either directly transplanted into the peritoneal cavity or injected into a Cell Pouch implanted subcutaneously in NSG-HA mice. In both cases, FVIII secretion was sufficient to improve the mouse bleeding phenotype. Indeed, FVIII-corrected HA BOECs reached a relatively short-term clinically relevant engraftment being detected up to 16 weeks after transplantation, and their genomic integration profile did not show enrichment for oncogenes, confirming the process safety. Overall, this is the first preclinical study showing the safety and feasibility of transplantation of GMP-like produced LV-corrected BOECs within an implantable device for the long-term treatment of HA.

Anderson AM, Kirtadze T, Malanga M, Dinh D, Barnes C, Campo A, Clemens DM, Garcia-Fandiño R, Piñeiro Á, O'Connor MS. **Cyclodextrin dimers: A versatile approach to optimizing encapsulation and their application to therapeutic extraction of toxic oxysterols.** *Int J Pharm. 2021 Sep 5;606:120522.* PubMed: 33839224

Abstract

We have developed a novel class of specifically engineered, dimerized cyclodextrin (CD) nanostructures for the encapsulation of toxic biomolecules such as 7-ketocholesterol (7KC). 7KC accumulates over time and causes

dysfunction in many cell types, linking it to several age-related diseases including atherosclerosis and age-related macular degeneration (AMD). Presently, treatments for these diseases are invasive, expensive, and show limited benefits. CDs are cyclic glucose oligomers utilized to capture small, hydrophobic molecules. Here, a combination of in silico, in vitro, and ex vivo methods is used to implement a synergistic rational drug design strategy for developing CDs to remove atherogenic 7KC from cells and tissues. Mechanisms by which CDs encapsulate sterols are discussed, and we conclude that covalently linked head-to-head dimers of βCDs have substantially improved affinity for 7KC compared to monomers. We find that inclusion complexes can be stabilized or destabilized in ways that allow the design of CD dimers with increased 7KC selectivity while maintaining an excellent safety profile. These CD dimers are being developed as therapeutics to treat atherosclerosis and other debilitating diseases of aging.

Dixit B, Vanhoozer S, Anti NA, O'Connor MS, Boominathan A. **Rapid enrichment of mitochondria from mammalian cell cultures using digitonin.** *MethodsX 2021;8:101197.* Document via external site.

Abstract

We describe here a simple method to enrich mitochondrial fractions from mammalian cells for downstream analyses in the lab. Mitochondria purification involves cell lysis followed by separation of the organelles from the rest of the cellular components. Here, we use detergent to rupture the cell membrane of mammalian cells followed by differential centrifugation to enrich the organelles. Optimum conditions with respect to detergent concentration, time, sample size, and yield are discussed. The method's utility in downstream analyses and ease of processing multiple samples simultaneously is also described. All the reagents in this method can be assembled in-house, are economical, and are comparable, if not superior, to commercially available kits in terms of mitochondrial yield and integrity.

• Rapid enrichment of mitochondria from mammalian cells using commonly available reagents.

• Multiple samples can be processed simultaneously.

• Works over a wide range of sample size (1 million to 100 million cells).

Admasu TD, Rae M, Stolzing A. **Dissecting primary and secondary senescence to enable new senotherapeutic strategies.** *Ageing Res Rev. 2021 Sep;70:101412. doi: 10.1016/j.arr.2021.101412. Epub 2021 Jul 21.* PubMed: 34302996

Abstract

Cellular senescence is a state of stable cell cycle arrest that is known to be elicited in response to different stresses or forms of damage. Senescence limits the replication of old, damaged, and precancerous cells in the short-term but is implicated in diseases and debilities of aging due to loss of regenerative reserve and secretion of a complex combination of factors called the senescence-associated secretory phenotype (SASP). More recently, investigators have discovered that senescent cells induced by these methods (what we term "primary senescent cells") are also capable of inducing other non-senescent cells to undergo senescence - a phenomenon we call "secondary senescence." Secondary senescence has been demonstrated to occur via two broad types of mechanisms. First, factors in the SASP have been shown to be involved in spreading senescence; we call this phenomenon "paracrine senescence." Second, primary senescent cells can induce senescence via an additional group of mechanisms involving cell-to-cell contacts of different types; we term this phenomenon "juxtacrine senescence." "Secondary senescence" in our definition is thus the overarching term for both paracrine and juxtacrine senescence together. By allowing cells that are inherently small in number and incapable of replication to increase in number and possibly spread to anatomically distant locations, secondary senescence allows an initially small number of senescent cells to contribute further to age-related pathologies. We propose that understanding how primary and secondary senescent cells differ from each other and the mechanisms of their spread will enable the development of new rejuvenation therapies to target different senescent cell populations and interrupt their spread, extending human health- and potentially lifespan.

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