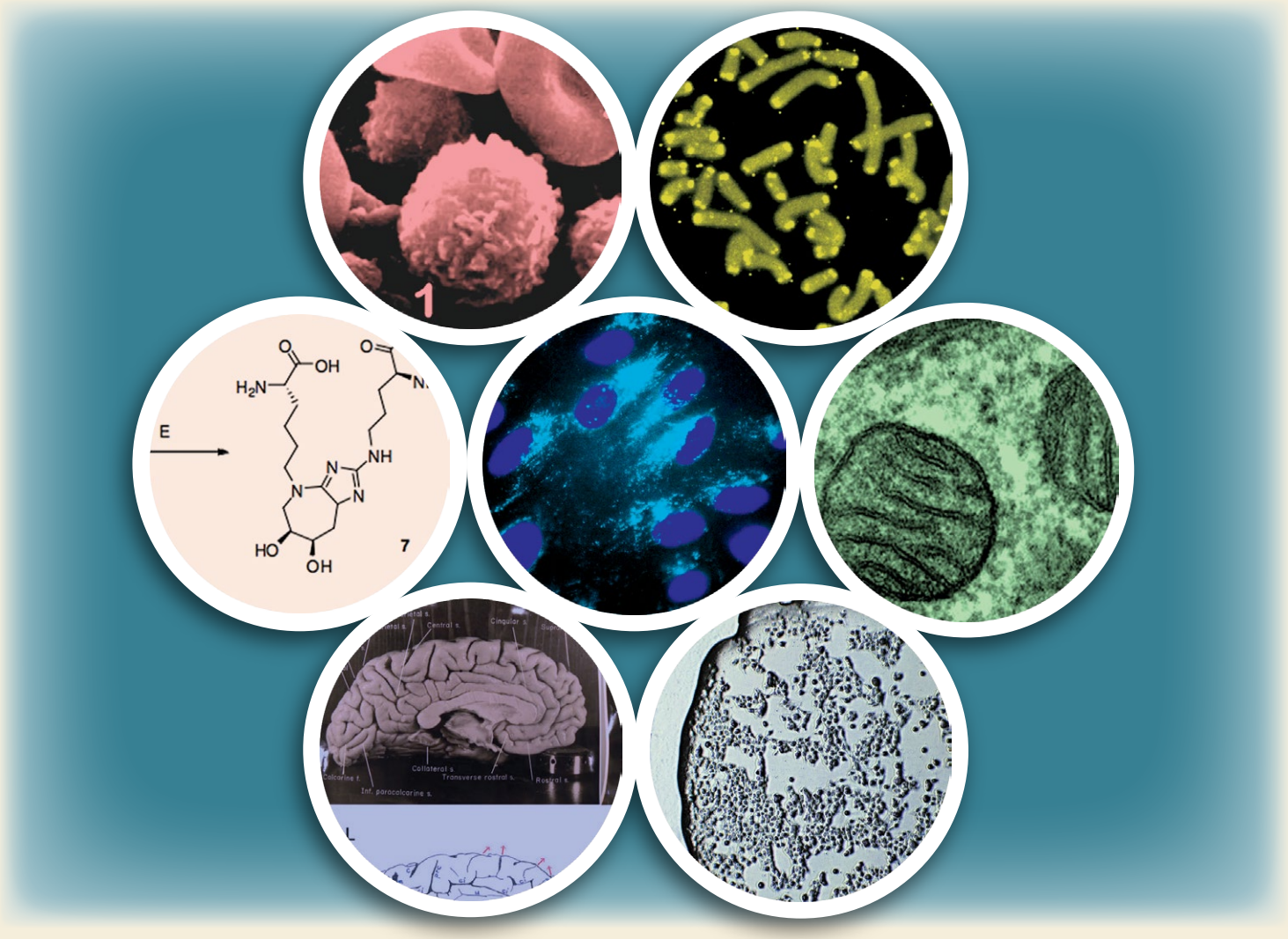
sens research foundation

reimagine aging



research report

august 2013

research report: contents

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rejuvenation

introduction

In May of 2012, a 10-year-old girl was suffering in hospital with a blockage in her portal vein — the major blood vessel that brings nutrients from the digestive tract into the liver. Bypassing the risks of transplantation, Swedish doctors engineered the girl a new, custom replacement. Drawing from earlier human successes with engineered tracheas, a donor's blood vessel was stripped of its cells. The resulting scaffold was seeded with stem cells from the girl's bone marrow, and chemical signals were then used to encourage the cells to grow into a functional portal vein. Thanks to the engineered vessel, the girl's blood tests have normalized, and she is now capable of light gymnastics and mile-and-a-half walks.

This groundbreaking achievement is just one example of what can emerge from a few years of rapid progress in animal models and other precedent-setting tissue engineering projects. It is also an example of the type of work SENS Research Foundation is funding with the goal of applying it to a primary problem of aging: the decline of the immune system. Only a few of us will ever need a new portal vein or trachea — but nearly all of us will need a new thymus, which plays an indispensible role in the immune system. The fine structures and functioning cells of the thymus we were born with will slowly degenerate between our teen years and our sixties; as the organ begins to fail with age, we become increasingly vulnerable to influenza and other common infectious diseases. With SRF support, Wake Forest Institute of Regenerative Medicine researchers are now making rapid progress (see page 19 for their report) in work to apply the decellularized-recellularized scaffold method to the thymus in animal models. SRF is excited to be spearheading the adaptation of existing techniques to geriatric medicine, where innovation is so sorely needed. But we know that this alone is not enough to address the emerging health crisis posed by age-related disease, which has surpassed infectious disease as t*he most pressing health problem facing humanity today*[1].

SENS Research Foundation is currently the only research nonprofit pushing the boundaries of the field toward the molecular level, where much of the damage of aging resides. Treating the symptoms of the resulting pathologies can only take us so far, because the body's repair and maintenance mechanisms continue to deteriorate. SRF's unique dedication to identifying and alleviating the damage that long precedes pathology serves as the basis for much of our work. Our longest-running project in this vein targets age-related macular degeneration, the leading cause of blindness in people over the age of 65. Macular degeneration is caused by the accumulation of a toxic byproduct of the visual cycle called A2E, which builds up in the retinal pigment epithelial (RPE) cells responsible for maintaining the light-sensing cells of the eye. We are working to preserve and restore the health of these cells by fortifying them with new, engineered enzymes capable of clearing A2E deposits. In 2012, scientists in our Research Center identified an enzyme (SENS20) that has since demonstrated efficacy in degrading A2E not only *in vitro*, but in RPE cells administered an A2E "stress test." For more on this work, turn to page 3.

These two critical-path projects, as well as the others described in this Research Report, reflect our ongoing mission to transform the way the world researches and treats age-related disease. Our commitment to developing the industry of restorative medicine begins with proof-of-concept work -- but ultimately rests upon creating the rejuvenation biotechnologies that can actually *cure* these diseases. To accomplish this, in addition to funding and conducting more research into the health problems of aging, we must realize a shift in how these health problems are conceptualized. We must move from seeing age-related illnesses as discrete entities to acknowledging that as we grow older, we become progressively more vulnerable to *every single age related disease* that exists, because we are all accumulating damage at levels no form of medicine can presently touch. When we *reimagine aging*, we envision a world where the damage *preceding* pathology is recognized as a treatable condition in and of itself, and addressed accordingly. SRF is delighted to share our progress toward this end in this Research Report. We hope you will join us in taking the tremendous opportunity at hand to set the new standard for twenty-first century medical research and development.

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Tanya Jones, COO 

Michael Rae, Science Writer 

Mike Kope, CEO 

1 - Pg. 2, World Health Organization, *Global Health and Aging*: http://www.who.int/ageing/publications/global_health.pdf

Research Report
intramural projects

Lysosomal Aggregates

SENS Research Foundation Research Center, Mountain View CA

Researchers: Gouri Yogalingam, Ghezal Beliakoff, Ehud Goldin, Maximus Peto

Cells are equipped with specialized "incinerators" called *lysosomes*, where they send damaged or unwanted material for destruction. Some cellular wastes, however, are so chemically snarled that even the lysosome is unable to shred them. With no way to eliminate these compounds, the cellular garbage simply builds up over time, progressively interfering with cell function. The disabling of specific cell types by their characteristic waste products drives numerous age-related pathologies.

For instance, age-related macular degeneration (AMD) — the primary cause of blindness in persons over the age of 65 — is believed to be primarily caused by the progressive disabling of retinal pigment epithelial (RPE) cells in the eye, resulting from their accumulation of *A2E*, a kind of waste specific to RPE cells. Currently, there is no effective treatment for this form of AMD.

Clearing these accumulating "garbage" compounds out of the cells where they occur would maintain or restore cell function, preventing or reversing the age-related diseases that arise when these cells are overcome with waste. Identifying enzymes that can degrade specific harmful accumulations, and modifying these enzymes for delivery to and function in our own lysosomes, would provide a way to achieve this goal.

At the SENS Research Foundation Research Center (SRF-RC), our Lysosomal Aggregates team is working to efficiently deliver novel enzymes into the lysosome to degrade A2E. Extensive protocols have been developed which employ RPE cells derived from humans to be used as cell lines for the study of AMD. In our prior research, we identified many enzymes (e.g., manganese peroxidase) capable of degrading A2E *in vitro*, but were unable to efficiently deliver most of them to the lysosome.

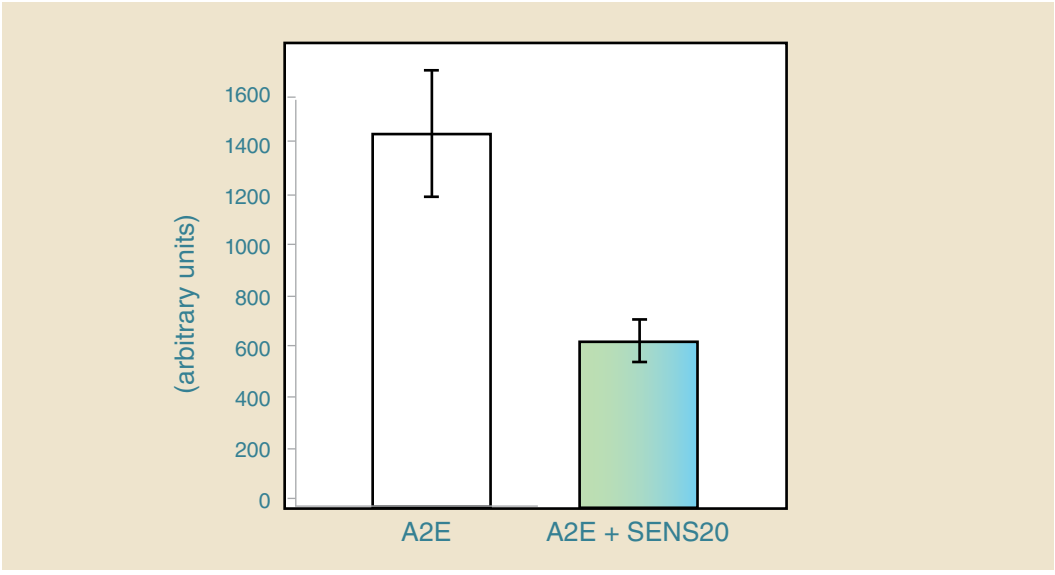


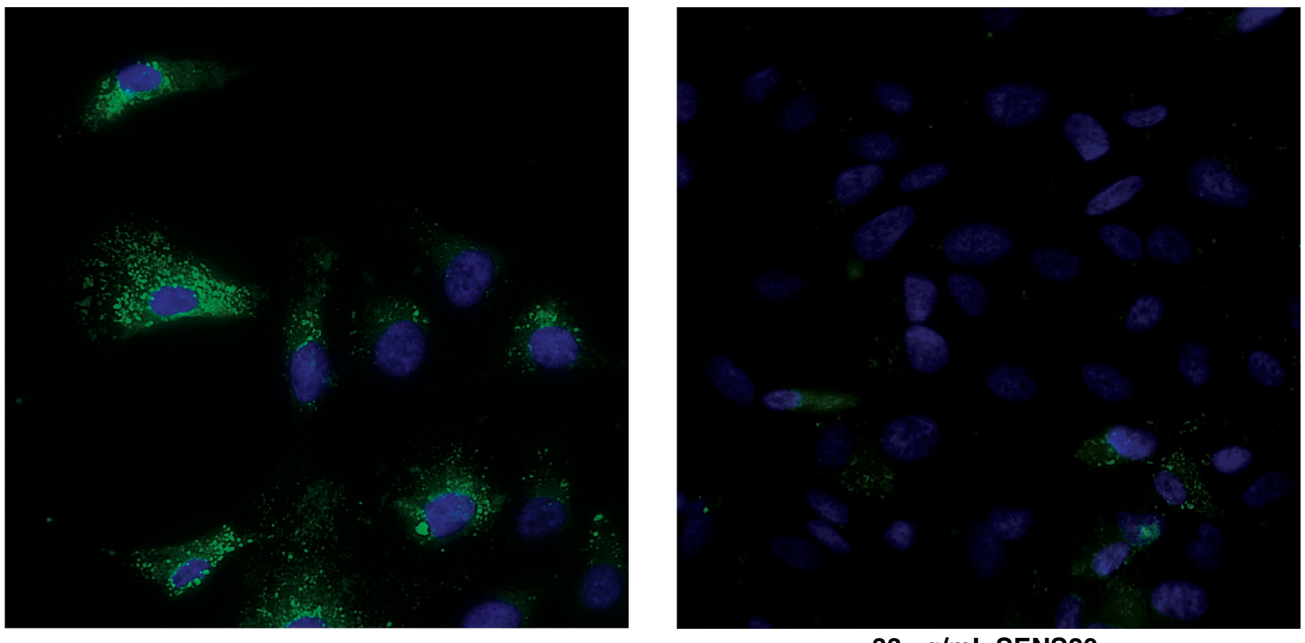
Figure 1: Efficacy of SENS20 enzyme in degrading A2E in vitro.

We are now working to develop ways to efficiently deliver the most promising identified enzymes into the lysosome of cells. One in particular (which we are calling *SENS20*) has demonstrated efficacy in degrading A2E not only *in vitro* (see ***Figure 1***) but in A2E-loaded RPE cells (see ***Figure 2***).

In 2013, the SRF-RC team is in the process of putting SENS20 to the test, assessing its ability to degrade A2E *in vitro* and in RPE cells. We are also performing a variety of tests to assure ourselves that the enzyme and its activity are not toxic to the cell. The studies will build toward eventual testing of candidate enzymes in animals that develop A2E-driven blindness and — if successful — eventually towards human clinical trials. We are advancing toward preventing or curing macular degeneration with the first-of-class regenerative therapy for this debilitating disease.

Meanwhile, we are continuing to screen and develop other enzymes capable of degrading A2E, and working to improve methods for targeting engineered enzymes to the lysosome.

MICROSCOPY ANALYSIS OF A2E



Control **33 μg/mL SENS20**

Figure 2: Reduction of A2E in enzyme-treated RPE cells. *Green indicates presence of A2E; note considerably greater A2E presence/density in Control image (left) vs. following treatment with SENS20 enzyme (right).*

Mitochondrial Mutations

SENS Research Foundation Research Center, Mountain View CA

Researchers: Matthew O'Connor, Amutha Boominathan, Jayanthi Vengalam

Our cells' energy-producing *mitochondria*, unlike most internal cellular machinery, house within themselves genes that serve as "blueprints" for thirteen key proteins. These proteins in turn serve as necessary components of mitochondrial energy-harvesting machinery. Keeping these genes inside the mitochondria themselves is precarious, because it exposes them to constant bombardment by the toxic byproducts of energy production.

Over time, these toxic byproducts damage the mitochondrial genes in more and more of our cells, resulting in the loss of the ability produce the very proteins that keep their energy-production lines running. Such cells not only suffer a kind of cellular "brownout" for lack of power, but they also export toxicity to the rest of the body, distorting the body's finely-tuned self-regulating cycles.

The goal of the RC Mitochondrial Mutations project is to engineer a way to let mitochondria keep producing energy normally, even after such mitochondrial mutations have occured, keeping our cells and our metabolisms healthy with age.

Damage to mitochondrial DNA is inevitable so long as it remains housed in the mitochondria, but the harmful effects of mitochondrial mutations can be bypassed by engineering "backup copies" of the genes for the thirteen key proteins into the cell's nucleus. In the nucleus, these *"allotopic"* gene copies could continue producing the needed proteins even when the mitochondria are no longer able to produce them themselves. The genes would moreover be far better shielded from damaging toxins, and better maintained when they are damaged.

Of course since the majority of mitochondrial proteins are naturally nuclear-coded, the "import machinery" necessary to accomplish our goal already exists; our task is therefore to devise a means of co-opting this machinery for the remaining proteins.

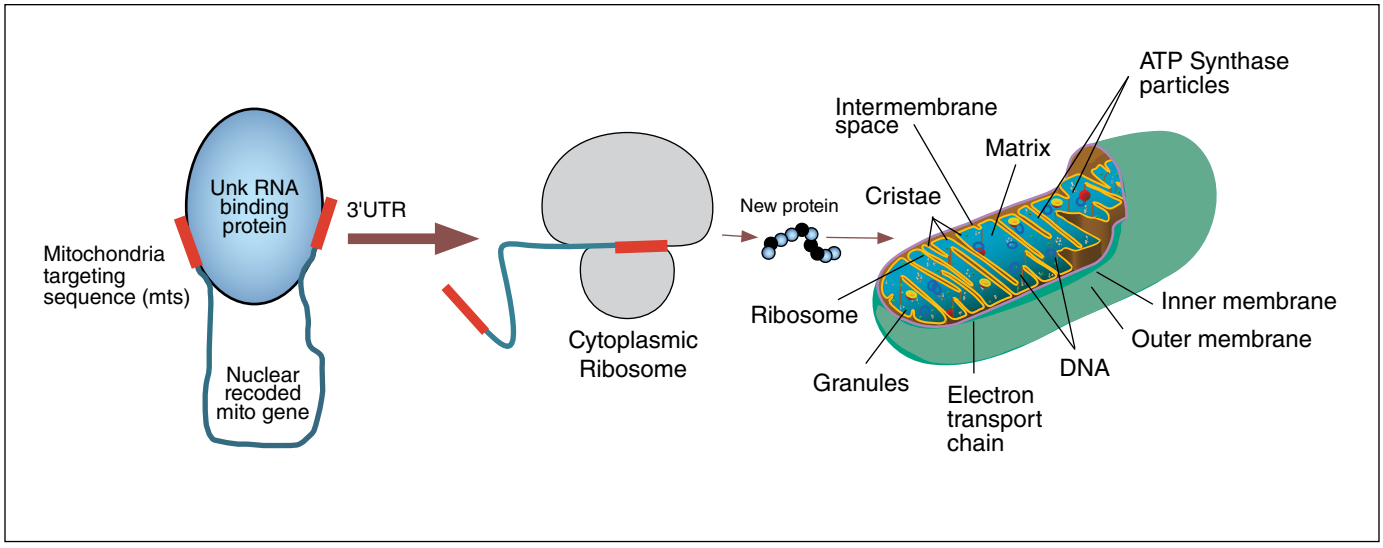


Figure 1: Co-Translational Import.

With funding from SENS Research Foundation, Professor Marisol Corral-Debrinski developed an innovative system for improved delivery of allotopically-expressed proteins into the mitochondria (co-translational import; see ***Figure 1***). SRF-RC scientists are now working to master and refine this system and to extend its application to all thirteen of the proteins encoded by the mitochondrial genome.

Our team has produced stable cell lines expressing our improved mitochondrial gene constructs in four lines of cells that were taken from patients suffering from severe diseases caused by inherited mitochondrial mutations. Some of the inherited mitochondrial genetic diseases bear a resemblance to many of the diseases and maladies of aging. For example, mutations in the ND1 gene have been implicated in the development of Parkinson's disease and Cytochrome B (CYB) mutations can cause muscle fatigue / exercise intolerance in young patients.

We have begun collecting data to show that instructions copied from these genes are indeed being targeted to the surface of the mitochondria and have characterized a working antibody to detect the presence of the engineered mitochondrial protein ATP8, custom made for SRF by our collaborator Scott Needham of Life Research.

In 2013, our two primary goals are to definitively confirm the localization of allotopically-expressed proteins at the inner membrane of mitochondria and to demonstrate that our allotopic expression systems can functionally rescue cells with each of several missing or severely mutated mitochondrial genes. Research efforts will be focused on more in-depth and rigorous biochemical and functional characterization of the mitochondrial energy-production chain following allotopic expression of the five missing or defective proteins in our re-engineered mutant cell lines, showing the assembly of the full chain.

In order to directly demonstrate the restored metabolic activity in these re-engineered cells, we have established a collaboration with Professor Gino Cortopassi at UC Davis, a top researcher in mitochondrial diseases, who is using a state-of-the-art Seahorse Biosystems XF Analyzer to measure the restoration of cellular metabolism. Additionally, we have now completed submission of our first National Institutes of Health (NIH) grant for additional funding to expand our work.

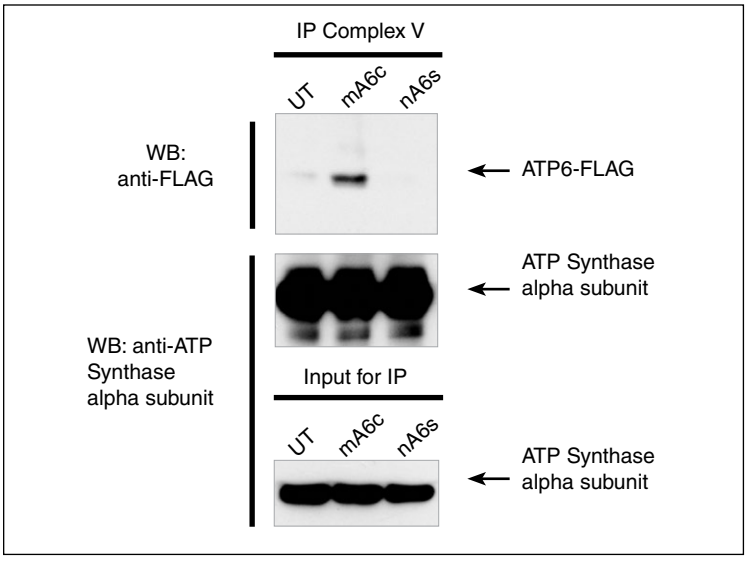


Figure 2: Incorporation of exogenous ATP6 into complex V in transfected HEK 293 cells.

Cancerous Cells

SENS Research Foundation Research Center, Mountain View CA

Researchers: Haroldo Silva, David Halvorsen, Kelsey Moody, Thomas Hunt

Cancer is a disease of unlimited cellular division. Healthy cells have a built-in limit on the number of times they can divide: with each division, a stretch of DNA called the *telomere* (***Figure 1***) progressively shortens, until it becomes so short that it triggers either the destruction of the cell or a program to stop the cell from dividing. This limit on cell division prevents the runaway growth of cancer cells.

To bypass this limit, would-be cancer cells must exploit one of two escape systems. The most common method is to activate the gene for an enzyme called *telomerase*, which re-lengthens shortened telomeres. While the gene for telomerase is present in all cells, it is suppressed in the great majority of normal cell types and only activated under strictly-regulated conditions in our stem cells.

The other, less common escape route is to activate a poorly-understood system known as *alternative lengthening of telomeres* (ALT). Many labs are now working to develop cancer-fighting drugs that work by suppressing telomerase alone, but cancer cells may be able to evolve resistance to these drugs by turning to ALT as a backup telomere-lengthening system.

The goal of the SRF-RC Cancerous Cells project is to determine the mechanisms of ALT and to use this information to disrupt the system, opening up the power to strongly suppress cancer.

Based on genetic studies performed on yeast, worms, and human cells, we are now identifying several gene candidates thought to be necessary for the emergence and maintenance of the ALT pathway. We are screening several cancer cell lines (either immortalized *in vitro* or derived from human tumors) for cells that display ALT behavior, in order to test whether they are exploiting specific candidate genes.

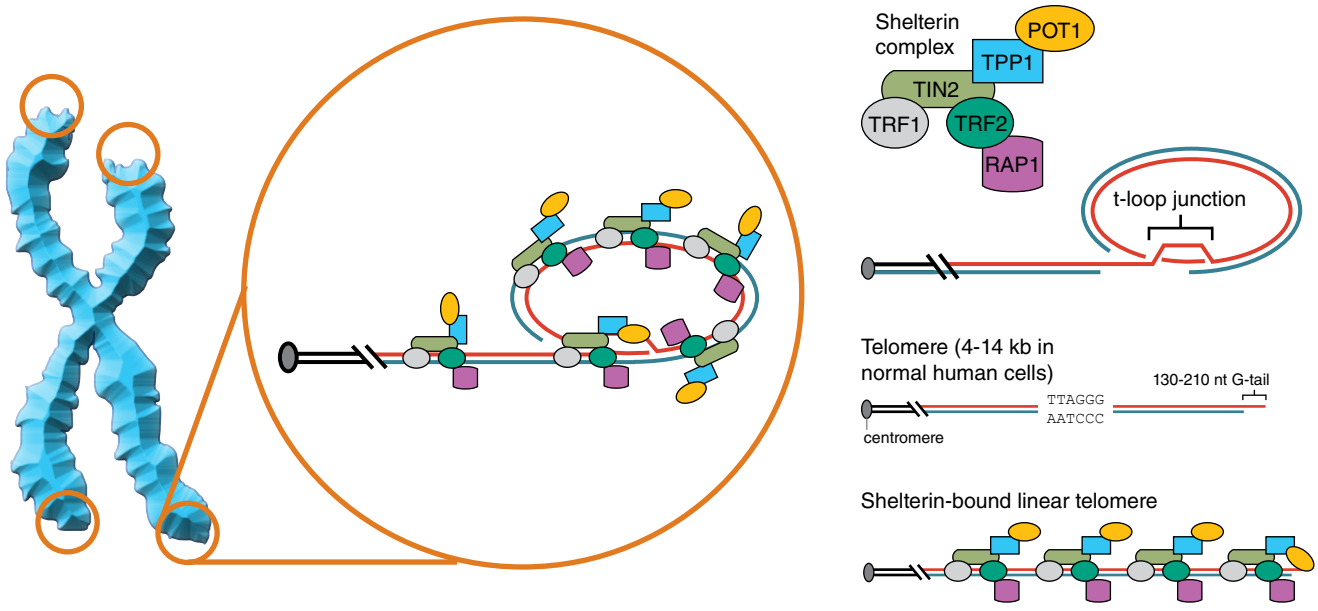


Figure 1: Telomere forming a loop. *Detail redrawn from* Figure 1: Structure of Telomeres, *pg. 320, Nature Reviews, Volume 11, May 2010, Alternative lengthening of telomeres: models, mechanisms and implications, Anthony J. Cesare and Roger R. Reddel.*

The most effective way to identify cells that are exploiting the ALT mechanism is that they express extrachromosomal C-rich circular telomeric DNA (*C-circles*). Existing assays for C-circles use a radioactively-labeled telomeric DNA probe; but at the SRF-RC, we have recently developed an innovative version of this assay that instead uses a non-radioactive digoxigenin (DIG)-labeled probe, allowing us to identify cells generating C-circles using a chemiluminescence signal.

We are also working to establish fluorescence microscopy protocols for the detection of another key marker of ALT activity, termed "*ALT-associated PML nuclear bodies*" (APBs) (***Figure 2***). Establishing these two assays for assessing ALT activity is an essential step towards deciphering the molecular mechanism behind ALT and finding ways to shut it down.

In 2013, the RC team is working to streamline the novel C-circle assay, bringing the turnaround time closer in line with the rapid assays available for telomerase activity. This will put tests for the two maintenance systems on a similar footing for researchers and would allow more rapid testing of candidate ALT genes. The novel assay could also potentially enable a faster classification of patient cancers into telomerase-exploiting and ALT-exploiting tumors. Knowing whether a given person's cancer uses one mechanism or the other would allow clinicians to give patients more accurate prognostic information and would facilitate personalized medicine using emerging telomerase-inhibiting therapies and future ALT-inhibiting ones.

The ultimate goal remains to use what we learn about the genetic basis of ALT to develop the first therapies able to shut it down, eradicating ALT-based cancers. Such a therapeutic approach could also be combined with telomerase-based strategies to create powerful combination therapies against cancer.

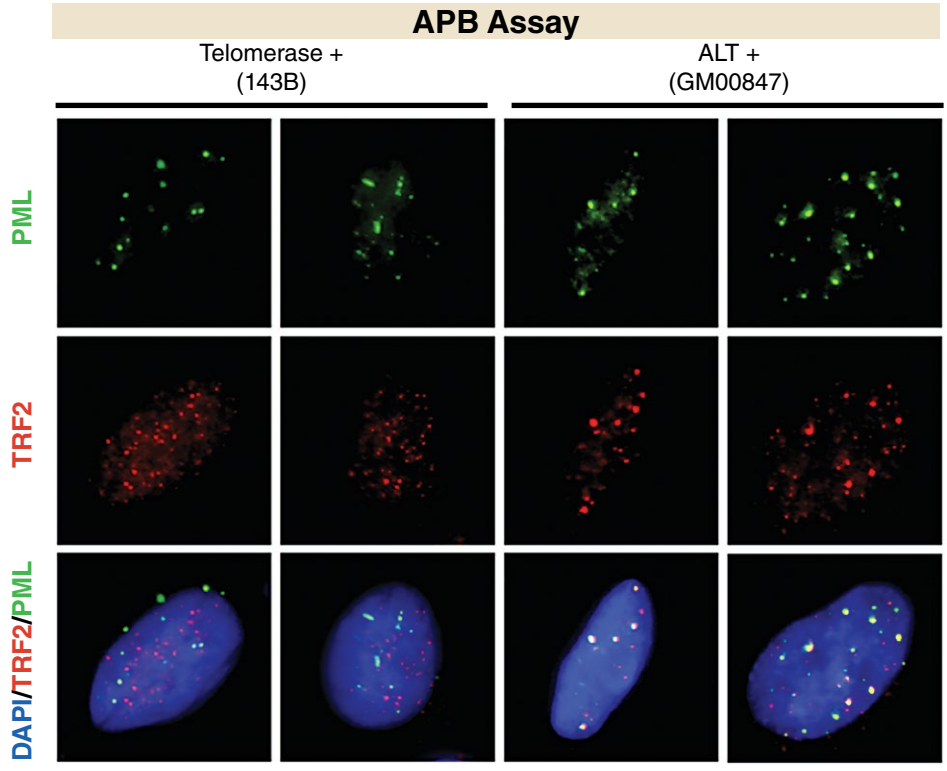


Figure 2: Fluorescence imaging of ALT-associated PML nuclear bodies (APBs).

Extracellular Matrix Stiffening

Yale University, New Haven CT, and SENS Research Foundation Laboratory, Institute of Biotechnology, University of Cambridge, Cambridge UK

Researchers: David Spiegel (Yale); Chris Lowe, William Bains, Rhian Grainger, Graziella El Khoury (SENS Research Foundation/ Cambridge)

Our arteries slowly stiffen with age, in substantial part because of chemical *crosslinking* of their structural proteins by blood sugar and other fuels in the circulation. Like the crosslinking that causes rubber windshield wipers to become stiff and brittle over time, the crosslinking of arterial proteins with age leaves us with increasingly rigid blood vessels. This ongoing stiffening of the blood vessels makes them progressively less effective at cushioning organs like the kidneys and the brain from the relentless pounding of the pulse, and it also leads to an insidious rise in systolic blood pressure with age. Together, these effects contribute to the slow loss of the ability of the kidneys to filter toxins from our blood with age and a rising risk of disabling stroke and dementia.

In aging and diabetic rats, dogs, and even monkeys, prototype drugs that break crosslinks have been shown to reverse the stiffening of the arteries and the heart, improving arterial health and preventing worse pathology. Unfortunately, these first-generation crosslink drugs proved to be much less effective in humans, most likely because they target a particular form of crosslink that is less common in humans.

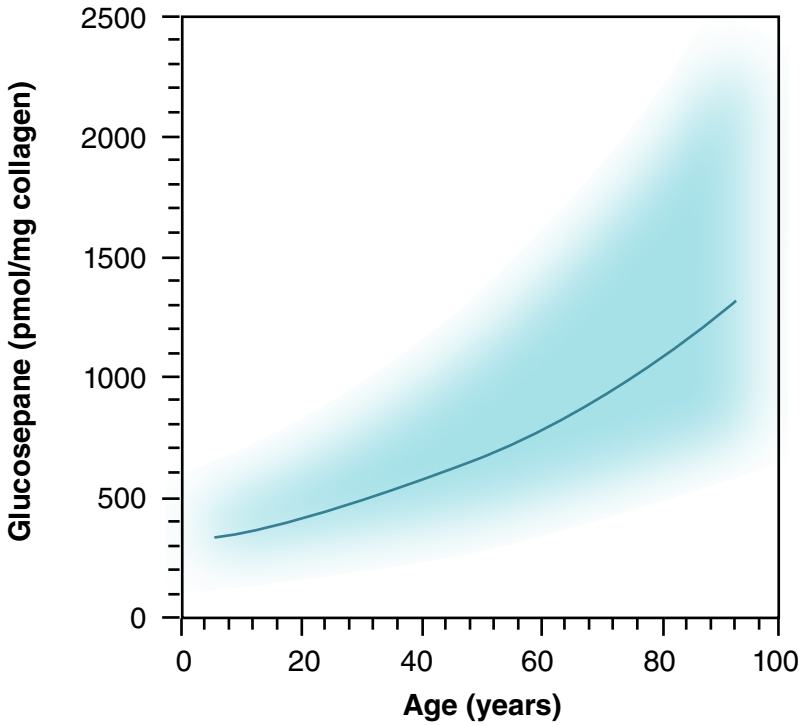


Figure 1: Glucosepane levels in human skin collagen rise with age.
Redrawn from Sell et al, J Biol Chem. 2005 Apr 1;280(13):12310-5. PMID: 15677467.

To make a better crosslink-breaking therapy, we must target the main crosslink that actually builds up in *human* tissues such as arteries and skin collagen (see ***Figure 1***): a complex chemical shackle called *glucosepane*. A glucosepane-cleaving drug would allow the proteins of aging arteries to move freely again, returning their flexibility and cushioning capacity to youthful health and functionality. As a result, damage to the kidneys would be prevented and strokes averted.

Late in 2011, SENS Research Foundation and the Cambridge University Institute of Biotechnology announced the establishment of a new SENS Research Foundation Laboratory at Cambridge. With a generous targeted donation from software entrepreneur Jason Hope, scientists in the Cambridge SENS center and Dr. Spiegel's Yale lab initiated work on biomedical solutions to glucosepane crosslinks, starting from the ground up. Since then, Dr. Spiegel has now developed a way to synthesize glucosepane in the lab (***Figure 2***); this artificially-produced glucosepane can now be used to develop reagents that can rapidly and specifically detect proteins that have been crosslinked by it. The development of such reagents is, in turn, a significant enabling technology for the development and testing of candidate glucosepane-breaking drugs.

The Cambridge group has been working on methods of extracting intact crosslinked proteins from the tissues of dogs and marmoset monkeys in order to assay their abundance and to test the effects of potential crosslink-breaking agents. They have also been working on finding ways to measure glucosepane *cleavage* — first in the test tube, and then in real animal and human tissues. One conclusion has already emerged from this research: none of the commercially-available monoclonal antibodies against related crosslink molecules are able to cleave glucosepane to any significant degree, and many do not even bind it.

Dr. Spiegel's group has also recently published a report clarifying how the first generation crosslink-breaking drug worked. Both of these findings further emphasize the need for novel crosslink-breaking therapies.

The GlycoSENS collaboration expects to begin publishing the results of this research in peer-reviewed scientific papers in 2013. These results lay the groundwork for our efforts in developing new anti-crosslink therapies, and may be expected to attract more researchers to enter (or return to) biomedical research on crosslinks and new therapies to remove them.

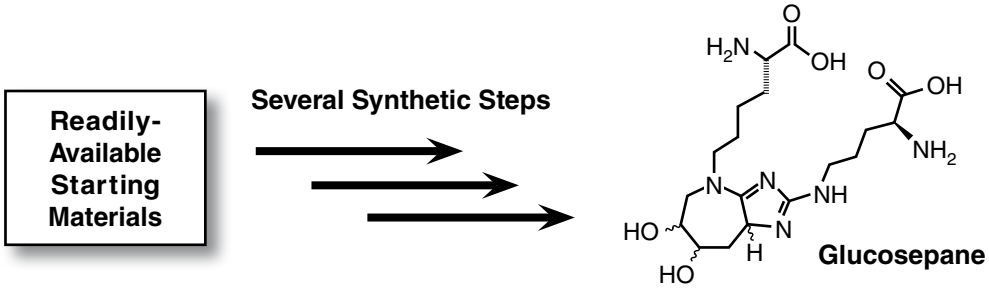


Figure 2: Making Glucosepane. *The Spiegel group's approach to making glucosepane. Two low-cost, common laboratory chemicals are combined in several steps to give the precursor to glucosepane, which can easily be used to make glucosepane-linked amino acids or peptides. Image via William Bains.*

Lysosomal Aggregates

Rice University, Houston TX

Researchers: Pedro Alvarez, Jacques Mathieu, Jason Gaspar

Atherosclerosis, like macular degeneration, is ultimately a disease of accumulating, undegraded waste inside the lysosome. As LDL ("bad") cholesterol circulates through the body, it is prone to become oxidized and form toxic byproducts. Notably, *7-ketocholesterol* (7KC) is the major oxidized cholesterol product found associated with oxidized LDL and with atherosclerotic plaques that narrow arteries, impairing function and endangering circulatory health. These toxic byproducts attract scavenger cells (*macrophages*) from the immune system, which engulf the toxins in an attempt to protect the blood vessel wall from injury.

Macrophages' lysosomes, however, have only a limited capacity to process such modified cholesterol, and they can be severely disabled by 7KC. Dysfunctional and dying macrophages become the "foam cells" that are the basis of atherosclerotic plaques.

The Rice University team is working to develop novel enzymes to clear 7KC and other oxidized cholesterol products out of macrophage/foam cell lysosomes, restoring them to normal function and beginning the reversal of the atherosclerotic process.

Dr. Mathieu and colleagues performed a series of experiments in 2012 testing the effects of boosting several components of the macrophage's cholesterol-transport and -efflux machinery on 7KC toxicity. Their most important advance was demonstrating for the first time that a novel cholesterol-metabolizing enzyme could protect human cells against 7KC toxicity (see ***Figure 1***). Called *DS1 cholesterol oxidase* (DS1-ChOx), this enzyme was almost unique in its ability to degrade both cholesterol and 7KC in preliminary testing *in vitro.*

To test its ability to detoxify 7KC in human cells' lysosomes, the Rice team linked DS1 to the signal sequence and transmembrane domain of *LAMP-1*, a protein that assembles into the membranes of the shuttling vesicles that carry cargo to the lysosomes.

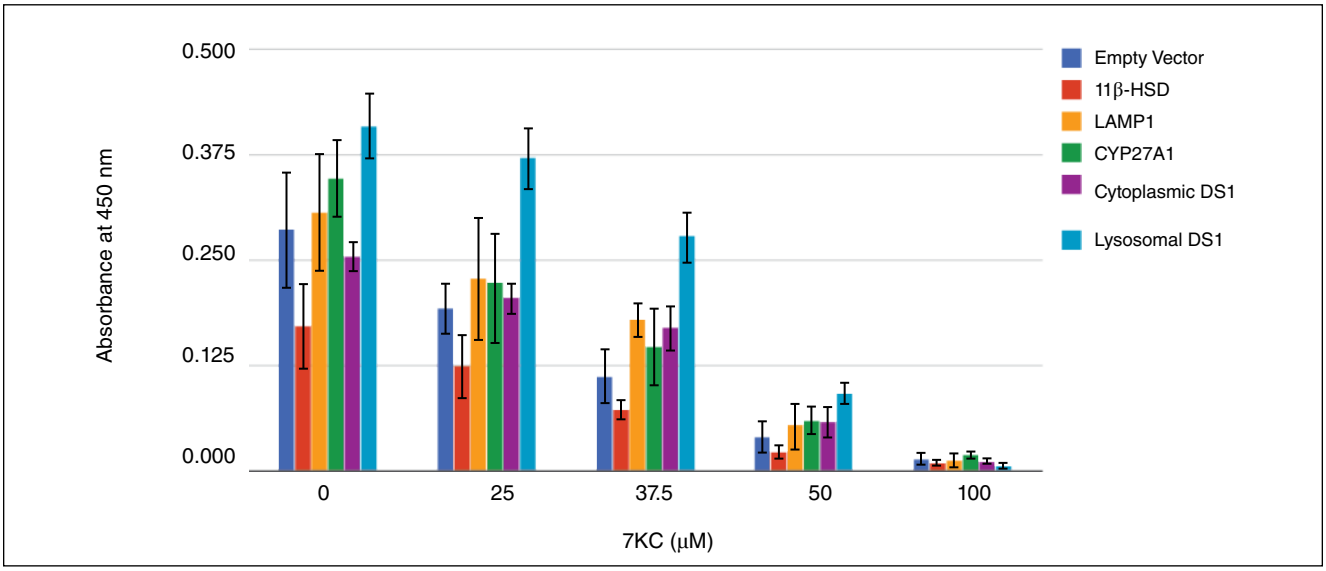


Figure 1: Lysosomally-Targeted DS1 Cholesterol Oxidase Protects Against 7-Ketocholesterol Cytotoxicity in Human Fibroblasts. *Higher absorbance at 450 nm indicates greater numbers of viable cells. Image via* Increased resistance to oxysterol cytotoxicity in fibroblasts transfected with a lysosomally targeted Chromobacterium oxidase., *Biotechnology and Bioengineering, Sept. 2012, Mathieu JM, Wang F, Segatori L, Alvarez PJ. Redrawn by SENS Research Foundation volunteer Alex Foster.*

This linking would cause the cell to insert the engineered DS1-ChOx into the membranes of such vesicles, which would then drop the enzyme off at the cell's lysosome with their regular cargo.

After confirming that their construct could deliver DS1 to the lysosome of human fibroblasts, Dr. Mathieu *et al* showed that their lysosomally-targeted DS1 is very effective at protecting human fibroblast cells from the toxic effects of 7KC (see ***Figure 1***). In fact, this particular DS1 variant is substantially more powerful than either alternative cholesterol-metabolizing enzymes or DS1 targeted to the main cellular compartment instead of to the lysosome specifically (see ***Figure 1***).

This result constitutes the first demonstration of the potential use of SRF's approach to managing lysosomal aggregates to form the basis of a truly regenerative therapy for atherosclerosis. If applied clinically, this approach could potentially return sickened macrophages to health, allowing them to mobilize out of the atherosclerotic lesion and open the way for the body's normal healing response to repair the injured vessel wall.

In ongoing work, Dr. Mathieu *et al* are now performing studies involving tweaking the structure of many existing cholesterol-degrading enzymes to increase their activity towards 7KC. They are also testing the effects of novel enzymes that would remain active in the lysosome even in the event of acidity loss due to 7KC poisoning, and the ability of each of the proteins known to be involved in effluxing cholesterol-related molecules out of the lysosome to reduce the toxicity of oxidized cholesterol products.

Beyond that, the team is continuing to develop a new model of classical lipofuscin (see ***Figure 2***) accumulation, which is anticipated to more accurately reflect its chemical structure, the physiological process by which it is generated, and the harm that it inflicts on the cell. This new model will initially be tested using fibroblasts but the researchers anticipate extending this work to heart muscle cells, where classical lipofuscin was first identified. Going forward, they will screen numerous enzymes with broad specificity toward protein-based or lipid-based molecules, individually and in combination, to determine their capacity to break classical lipofuscin down.

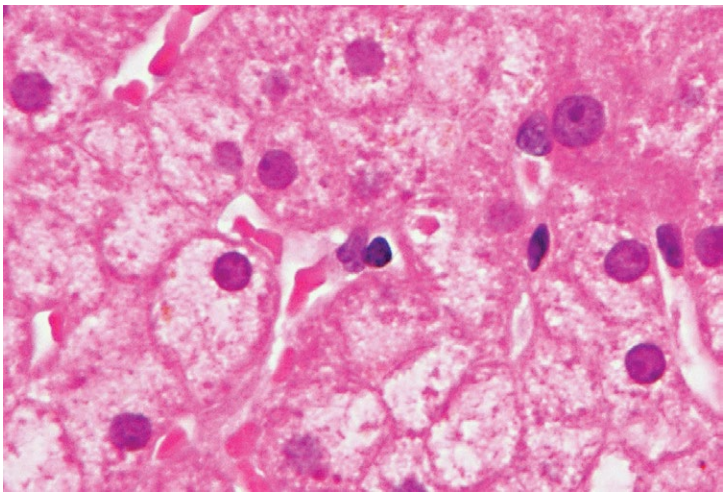


Figure 2: Micrograph showing lipofuscin in a liver biopsy with ground glass hepatocytes; H&E stain. *Image by user Nephron, From Wikimedia Commons. This file is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license.*

Epimutations

Albert Einstein College of Medicine (AECOM), Bronx NY

Researchers: Jan Vijg, Silvia Gravina

Over time, cells suffer permanent damage (mutations) to the DNA code that holds the instructions for making all of the proteins needed by our cells, tissues, and organs.

We know that mutations accumulate with age, at different rates and in different patterns depending on the tissue. Dr. Vijg's group, via thorough testing, has shown that *mutations* don't accumulate during adulthood in most mouse tissues. But the picture is much less clear when it comes to *epimutations:* permanent damage to the "scaffolding" of DNA, which helps the cell to control which genes are turned on or off at any time and in any given cell type. While it is certain that mutations and epimutations play a major role in cancer, it remains to be determined with certainty whether they also contribute to other age-related diseases and tissue dysfunction.

It is conceivable that an accumulation of epimutation-laden cells could contribute to age-related functional decline by causing cells to engage in abnormal gene expression, leading to cell death or dysfunction. To confirm or refute this hypothesis, we need reliable ways to quantify the rate of accumulation of epimutations in single cells. Single-cell assays are necessary because many cells in a tissue will "intentionally" change their epigenetic state at once, in a *systematic* response to a changing environment, whereas truly random (*stochastic*) changes in the epigenetic state of cells will occur in each cell independently of its neighbors (see ***Figure 1***).

Because random damage to any *one* of the many *specific* epigenetic targets in a given cell will be *individually* rare, even a high load of such epimutations — occurring independently of each other, and to different epigenetic sites in different individual cells across a whole tissue — would simply be drowned out in the 'noise' of a whole-tissue analysis. This would be true in this situation even though these unique epimutations would collectively disable, derange, or destroy many cells in the tissue. Only single-cell analysis will uncover the real load of epimutations, and allow us to see if they are really abundant enough to harm us.

One of the most important epigenetic modifications is *DNA methylation:* the reversible addition of methyl groups at special control points in DNA (see ***Figure 2***). With SENS Research Foundation funding, Dr. Gravina and coworkers in Vijg's group have now successfully adapted the existing "bisulfite sequencing" method for evaluating DNA methylation status at the base pair level for use in single-cell analysis. In doing so, they have in effect developed a novel method for DNA methylation epimutation detection that can be applied in a genome-wide or locus-specific fashion.

To validate their single-cell analysis protocol, the AECOM Epimutations team evaluated the state of DNA methylation in thousands of individual liver cells, each isolated from a different mouse. They also looked at locations that control the expression of genes that are either *always* expressed in normal liver cells (and should therefore have no methylation), or *never* repressed in normal liver (and so firmly turned off with methyl groups).

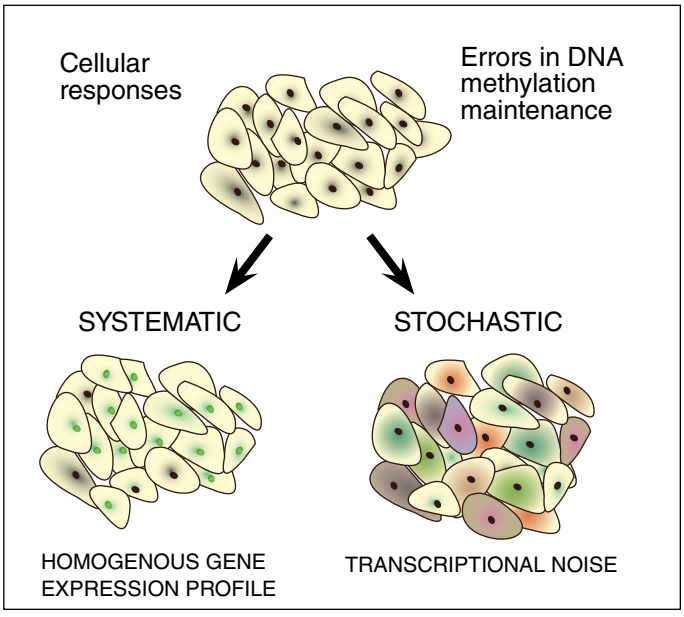


Figure 1: Possible DNA methylation changes during aging. *Image via Silvia Gravina.*

Epimutations were therefore identifiable by comparing the methylating and demethylating of these sites in individual cells with what was characteristic of thousands of other liver cells. Then, to validate their genome-wide methylation sequencing approach, the team compared the DNA methylation pattern of single liver cells with the pattern in the rest of the same animal's organ.

Due to the complexity of the analysis and the large amount of data processing required, the AECOM team established a collaboration with Dr. Achim Tresch and Mr. Kemal Akman at the University of Munich to perform a sophisticated mathematical model for genome-wide DNA methyl-typing. A patent disclosure of some of the novel techniques is pending.

The new methods developed by Dr. Gravina could become the basis of sophisticated new diagnostic technologies for the "epityping" of cancers (and potentially other diseased tissues) using minimally-invasive tissue biopsies. One promising first target for such diagnostics is assessing the DNA methylation patterns in the promoter regions of tumor suppressor genes in circulating tumor cells.

From a wider perspective, the AECOM team is now positioned to characterize and establish the magnitude of epigenetic drift in different organs and tissues during aging, beginning with the brain. Success in this endeavor would resolve the critical question of how much random epimutations contribute to the degenerative decline in somatic cells and tissues, and in the diseases and disabilities of aging.

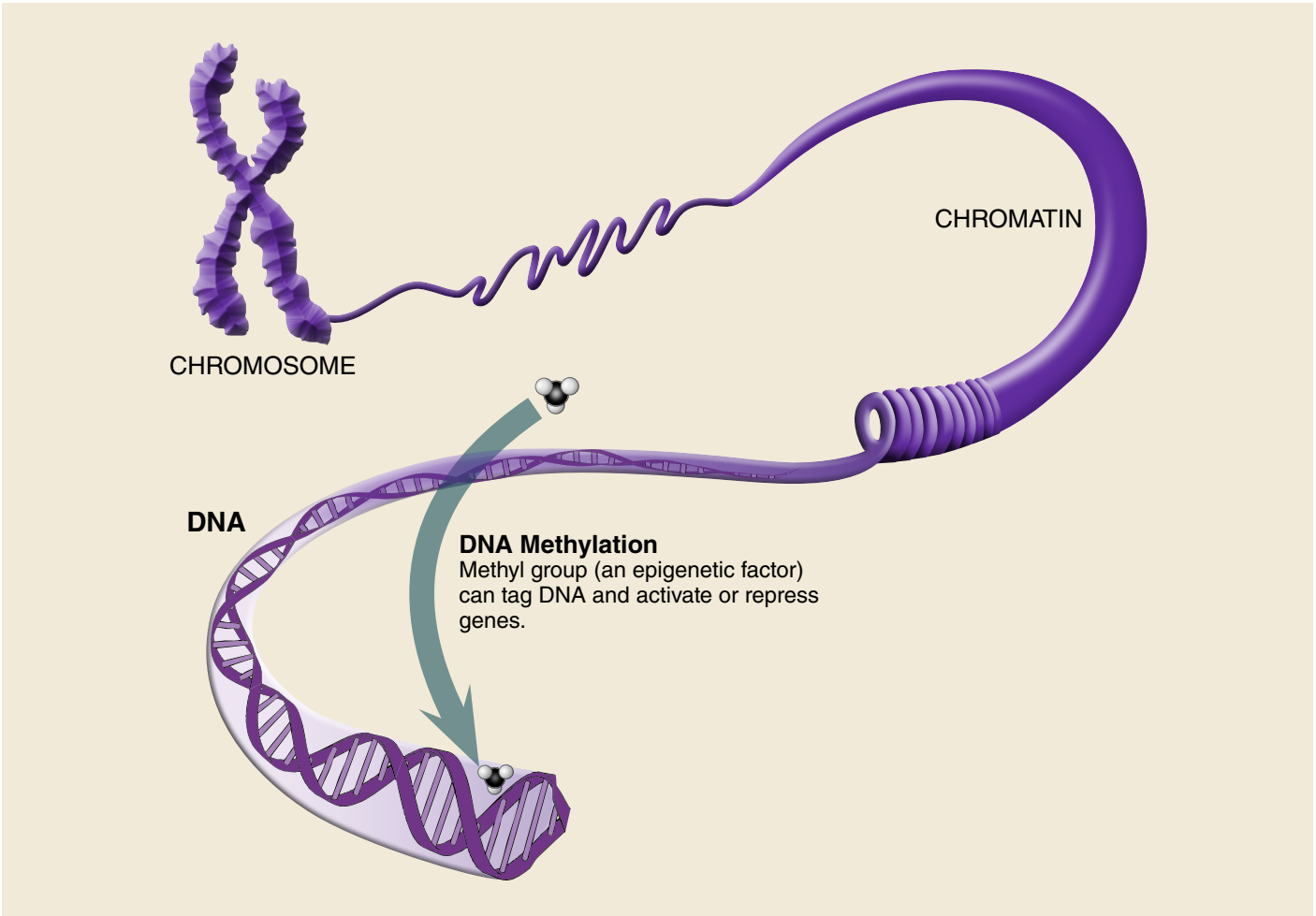


Figure 2: DNA Methylation. *Image adapted and redrawn by Anne Corwin from epigenomics_NIH_FULL.jpg, provided by NIH Common Fund.*

Extracellular Aggregates

University of Texas, Houston TX, and Brigham and Women's Hospital, Harvard University, Boston MA

Researchers: Sudhir Paul, Yasuhiro Nishiyama, Stephanie Planque (University of Texas); Brian O'Nuallain (Brigham and Women's Hospital, Harvard Medical School)

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. Amyloid clumps are toxic and hard for the body to break down. They accumulate as deposits in various organs with advancing age, disrupting organ structure and impairing function. One important amyloid disease of aging is caused by the aggregation of the transporter protein transthyretin (TTR) into amyloid that deposits in many organs, including the heart. The insidious effects of TTR amyloid can start appearing in middle age, progressively becoming sufficient to impair the function of the lungs, the kidneys, and other organs — most particularly the heart. Over 10% of individuals over the age of 70 are seriously affected by TTR amyloid, and the condition becomes near-universal with further aging. TTR amyloid appears to be a major contributory factor to the death of "supercentenarian" — those among us who achieve 110 years or more of life.

Additionally, mutations in the TTR gene produce a protein form that is more easily twisted into amyloid, including a mutation affecting 3-4% of African Americans that causes heart failure. Patients carrying some of these mutations can develop early-onset familial amyloidosis prior to age 30. There is no approved treatment for TTR amyloidosis, and replacement of the failed organ is the only option. The SENS Research Foundation-funded TTR Extracellular Aggregates collaboration is developing antibodies that recognize and remove TTR amyloid deposits from tissues safely. The antibodies could be used for diagnosis and treatment of patients with both age-induced and genetic forms of TTR amyloid.

To generate antibodies that bind TTR, Dr. O'Nuallain is exploiting the acquired immunity paradigm. Such antibodies might be used to identify people with undiagnosed cardiac amyloidosis and possibly also as therapeutic agents. Dr. O'Nuallain immunized three strains of mice with three different immunogens containing TTR aggregates. One group was immunized with either fibrils of the non-mutant protein's amyloid, or with a mutant TTR rendered into a soluble state. Three other groups of mice, each of a different strain, were immunized with a mixture of both non-mutant TTR fibrils and the resolubilized mutant protein. Exposure to these foreign proteins triggered the mouse immune system to generate novel antibodies that targeted aggregated TTR and not the physiologically-functional form of TTR. B-cells from the immunized mice were then fused with cancerous mouse B-cells to generate twenty 'immortal' mouse cell lines, each secreting a unique TTR-reactive monoclonal antibody.

Seven of the resulting antibodies demonstrate diagnostic and therapeutic potential. They bind strongly to patient-derived TTR amyloid without reacting with the physiological TTR form, and they retain their binding activity in the presence of plasma from normal humans. These and additional antibodies from the immunized mice are also being tested for the ability to catalyze the degradation of TTR amyloid in collaboration with Dr. Paul.

Meanwhile, Dr. Paul has been developing a class of catalytic antibodies (catabodies) that break peptide bonds in TTR amyloid, based on innate immunity principles. In previous research, Dr. Paul identified catabodies naturally produced by young and old humans that specifically degrade the beta-amyloid peptide clumps found in the brain of patients with Alzheimer's disease. With funding from SENS Research Foundation, he has identified catabodies that target TTR amyloid. These newly-discovered molecules are members of the immunoglobulin M (IgM) class of antibodies that are synthesized as part of the first-line, innate defense against dangerous substances detected by the immune system (see ***Figure 1***).

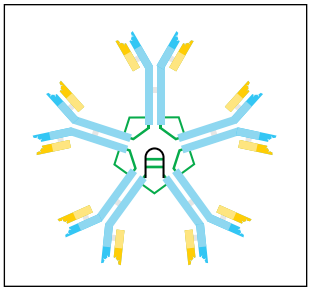


Figure 1: Immunoglobulin M (IgM) Structure. *Image by Artur Jan Fijałkowski, licensed under the Creative Commons Attribution-Share Alike 2.5 Generic license.*

These catabodies completely dissolved TTR amyloid in the test-tube without degrading the physiological TTR form or off-target proteins. Probing the catabodies showed that their degrading power derived from a serine protease type of enzyme mechanism, with no dependence on other blood enzymes or phagocytic cells required by conventional antibodies for effective target removal. Moreover, the catabodies did not form stable immune complexes that exert harmful side-effects. Immunoglobulins G (IgGs), the class of antibodies usually associated with acquired immunity, were unable to degrade TTR amyloid. From these findings, Dr. Paul's group theorizes that production of catabodies to TTR amyloid is an ancestral immune function that humans use as a first-line surveillance strategy to delay age-associated amyloidosis.

Supplementing the natural defense function of catabodies with an externally-provided catabody that is specific for the TTR amyloid form holds potential for a safe and efficacious treatment. The catabody can be delivered intravenously or by gene therapy. Both delivery methods have been validated for the Alzheimer beta-amyloid target in animal models by Dr. Paul and his collaborators.

Catabodies offer several potential advantages over the conventional amyloid binding antibodies that have been the focus until now in developing immunotherapies for amyloid disease. The catabodies actually destroy their targets, rather than merely binding to them. A single catabody molecule can be reused thousands of times to degrade molecule after molecule of the amyloid. In contrast, conventional IgG antibodies offer only a "one-shot" capability, and some of them may rely on phagocytic cells that ingest the IgG-target protein immune complex.

Because catabodies remove the amyloid independent of phagocytic cells, they do not carry the risk of inflammation and vascular damage caused by conventional antibodies. By minimizing the amount of catabody needed to remove large amyloid quantities, the cost of catabody therapy should be lower than immunotherapy regimens that rely on conventional monoclonal antibodies. Delivering catabodies via a gene therapy approach could offer the additional advantage of making repeat catabody administrations unnecessary.

SENS Research Foundation is now funding further work to isolate and optimize a cell line producing a specific catabody that clears TTR amyloid, thus providing a renewable source of the candidate therapeutic catabody. Dr. Paul's team is employing mechanism-based TTR amyloid analogs to selectively trap the best catabody from a large antibody library (see ***Figure 2***). Various genetic engineering technologies to optimize the catabody activity are available to the team, including combinatorial pairing of the light and heavy chain IgM variable domains, and use of alternate constant domains to maximize catalytic activity and catabody half-life in the blood.

In 2013, the collaborative team hopes to identify lead antibodies and catabodies suitable for diagnosis and therapy of TTR amyloidosis. Further evaluation will entail testing in a transgenic mouse model of TTR amyloidosis. If these antibodies and catabodies prove themselves to be efficacious and safe in animal models, then the ground is laid for clinical trials to treat age-associated and familial amyloidosis in humans.

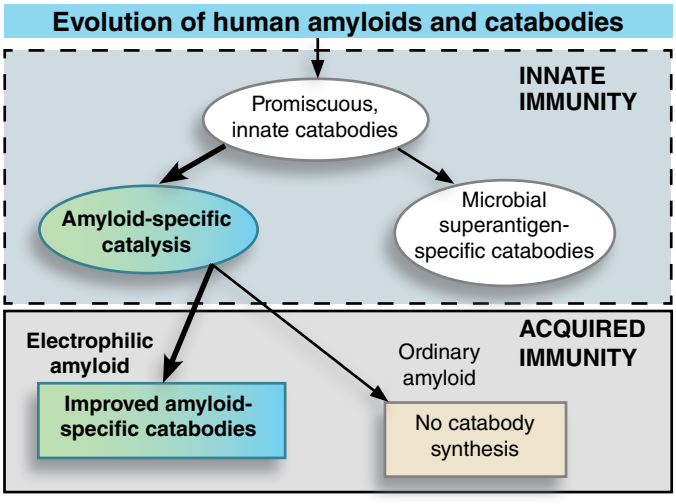


Figure 2: Native catabodies appear to have evolved as an innate, physiological defense mechanism against the development of amyloidosis in humans. *Dr. Paul and colleagues are working to harness the power of acquired immunity by immunization with mechanism-based electrophilic amyloids that stimulate the cells responsible for producing the catabodies. Image via Sudhir Paul, redrawn by Anne Corwin.*

Cancerous Cells / Cell Loss and Atrophy

Wake Forest Institute for Regenerative Medicine (WFIRM), Winston-Salem NC

Researchers: Graça Almeida-Porada, Christopher Porada

At Wake Forest, SENS Research Foundation is funding Dr. Graça Almeida-Porada's group in a project to restore intestinal structure and function. Dr. Almeida-Porada's central goal in this project is the development of a regenerative medicine approach to treating *inflammatory bowel disease* (IBD), an autoimmune disorder that devastates the cells lining the intestine (see ***Figure 1***).

Though IBD is not itself a disease of old age, SENS Research Foundation is supporting this work because therapies that repopulate the cells of the gut are critical to the development of the superior cancer therapies that we are pursuing.

Radically more effective therapies to prevent and cure cancer are likely to depopulate the stem cell reserves of several tissues and will therefore require future clinicians to replace the missing cells with fresh, cancer-protected stem cells. This is loosely analogous to many of today's cruder cancer therapies, which involve clearing out a patient's bone marrow and then replacing it with new, healthy cells. Moreover, regenerative therapies for the gut would be of enormous value to people receiving many existing cancer therapies, such as radiation therapy during treatment for pelvic or abdominal cancer.

The WFIRM researchers are developing a combination cell therapy based on the transplantation of modified mesenchymal stem cells (MSC), which have potent anti-inflammatory/immunomodulatory effects to protect them from attack by the body's immune system. These are being modified to transiently express molecules that will enhance their homing and anti-inflammatory potential, boosting their ability to engraft within the injured/inflamed intestine and to attract and support endothelial progenitor cells (EPC), which will further contribute to the repair of the tissue and the restoration of its blood supply.

In 2012, Dr. Almeida-Porada and colleagues worked to enhance techniques whose effectiveness they demonstrated in earlier studies in sheep (see ***Figure 2***) by engineering regenerative MSC with genes expressing the immunomodulatory molecule *HLA-G1* (which protects them from attack by the body's *natural killer cells*) and *Ephb2* (a molecule that enhances the homing of cells to the intestine).

In addition, Dr.Almeida-Porada's group is now using an alternative engineered gene expression system to deliver the HLA-G1: *minicircles*, a form of supercoiled DNA molecule.

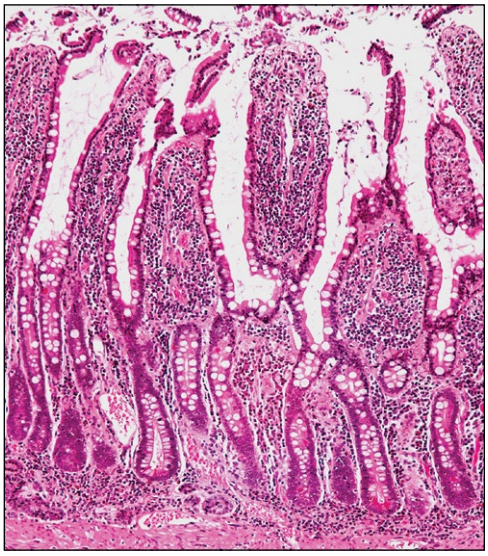


Figure 1: Low magnification micrograph of small intestinal mucosa. *Image by user Nephron, from Wikimedia Commons. This file is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license.*

Minicircles have two advantages over conventional gene-delivery systems. First, unlike conventional virus-based vectors, minicircles do not insert themselves into (and therefore, do not risk mutagenic disruption of) the host cell's DNA, enhancing the safety of the engineered gene. Second, minicircles only temporarily express their therapeutic gene.

This will allow the transgene to help the engineered MSC survive and home during the specific window in which they are needed to enhance the active recolonization of the gut, without having the genes continue to be expressed after they have become redundant and potentially inappropriate.

The team have now shown that MSC modified to express HLA-G using minicircles are just as effective as their original system at protecting the cells against natural killer cells.

In 2013, the WFIRM lab is testing a new approach to cloning the gene for Ephb2 in order to generate a novel construct that will yield superior expression of the gene. They will then begin testing the power of the engineered cells – alone and in combination – to restore the absorptive and blood vasculature cells in the intestinal walls of a mouse model of IBD.

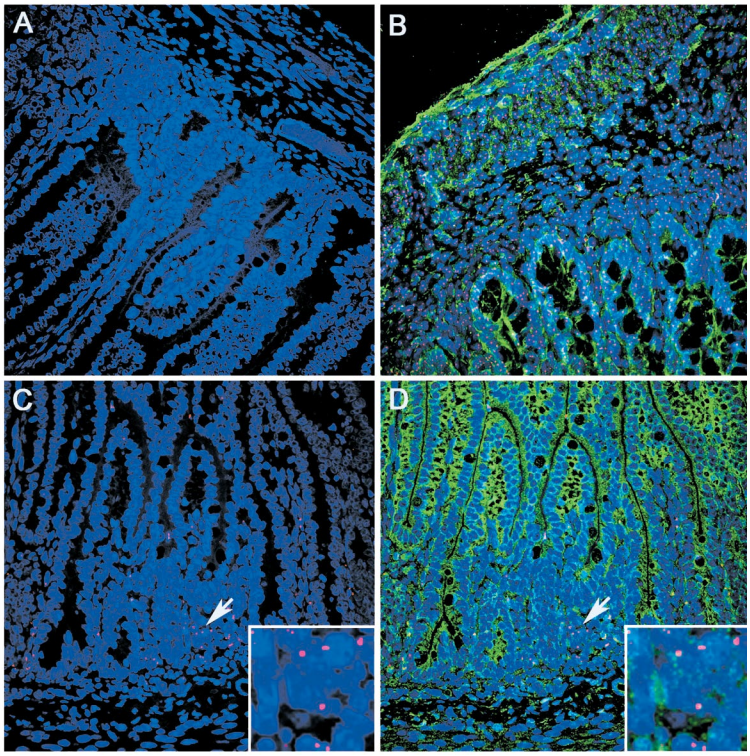


Figure 2: Human BMSC contribute to the fetal sheep intestine. *Human-specific FISH probe (red) does not detect labeled nuclei in normal fetal sheep intestine (A), but labels every nucleus in fetal human intestine (B). It can thus be used to identify human cells that have been successfully transplanted into chimeric fetal sheep intestine (C). Additional labeling with pan-cytokeratin supports the conclusion that transplanted cells engraft in the sheep intestine and adopt an appropriate epithelial cell fate (D). Image via Dr. Graça Almeida-Porada.*

Cell Loss and Atrophy

Wake Forest Institute for Regenerative Medicine, Winston-Salem NC

Researchers: John Jackson, Shay Soker, James Yoo

The *thymus* is a gland located at the top of the breastbone, where a class of immune cells called *T-cells* mature. As part of the degenerative aging process, the thymus shrinks in size. Some of the functional tissues needed to complete the maturation of T-cells are replaced by fat cells; and the structure of the remaining functional tissue decays.

Put together, this atrophy of the thymic gland robs the body of the ability to generate so-called "naïve" T-cells, which exit the thymus ready to fight off never-before-encountered infections. Along with accumulations of age-damaged, defective T-cells, thymic atrophy is one of the reasons why we become increasingly vulnerable to influenza, pneumonia, and other infectious diseases as we age. Engineering healthy, youthful thymic tissue would thus help to restore the vigorous immune response of youth.

With SENS Research Foundation funding, the WFIRM Cell Loss and Atrophy group is now extending an exciting tissue engineering platform to the thymus: the decelluarized scaffold technique. In this method, donor organs are purified of their original cells using detergents and of their DNA using specialized enzymes (see ***Figure 1***).

What remains is the amazingly complex non-living protein structure that gives the organ its shape and that permits the organ's cells to take hold, grow, and function in proper relationship. This natural scaffold is then repopulated with cells taken from the recipient, resulting in an implantable engineered neo-organ, which eliminates the risk of immune rejection (and the dependence on dangerous immune-suppressing drugs to prevent it).

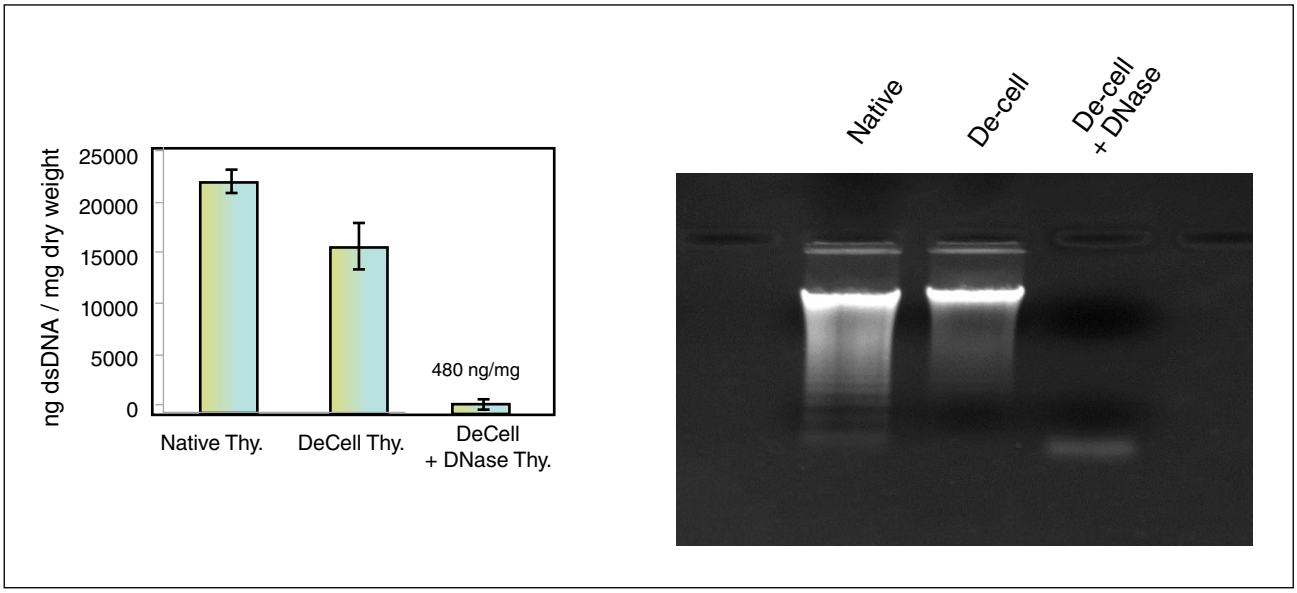


Figure 1: Removal of residual DNA from thymic scaffold.
Images via Wake Forest Institute for Regenerative Medicine.

Already, the WFIRM team has used the decellularization technique to produce thymus scaffolds from mice and pigs (refer to ***Figure 2***, upper right). Epithelial cells have been seeded onto these scaffolds, leading to stromal cell proliferation and partial coverage of the scaffolding (***Figure 2***, lower), which the researchers are now characterizing.

To complete the reseeding procedure, bone marrow stem cells depleted of T-cells will be added to the epithelial-cell-seeded scaffold, and the production of new T-cells will be observed.

Dr. Jackson and coworkers are now further optimizing their decellularization protocols to minimize damage to the fine structures of the scaffolding. Once the initial characterization of the scaffolds has been performed, the engineered thymus will be transplanted into mice lacking one of their own. Researchers will then evaluate their T-cell production capacity and the functional properties of the resulting cells.

Once the mouse studies are complete, a larger (pig) animal model will be used to begin preclinical studies. The production of a bioengineered thymus has the potential to supplement or replace thymuses degenerated by aging or damaged by disease or trauma, and to rejuvenate the aging immune system.

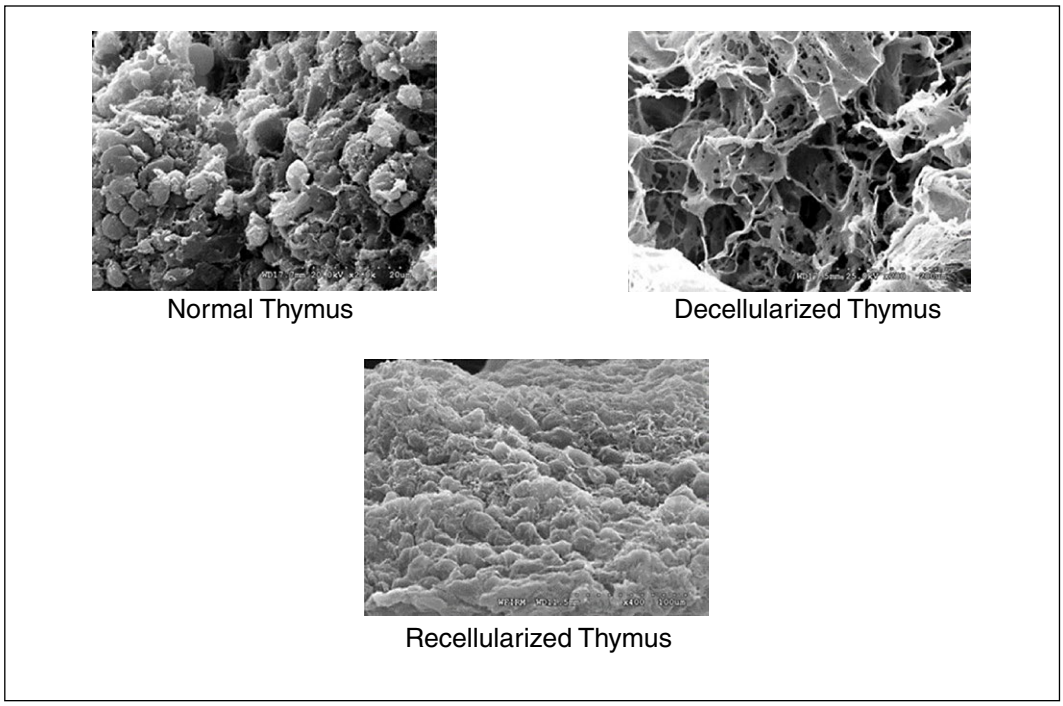


Figure 2: Decellularization of mouse thymus and reseeding with thymic epithelial cells. *Images via Wake Forest Institute for Regenerative Medicine.*

Death-Resistant Cells

Buck Institute for Research on Aging, Novato CA

Researchers: Judith Campisi, Kevin Perrott, Sam Curran, Nick Schaum

Cellular senescence is a metabolic state into which cells sometimes enter in response to stressors such as DNA damage, the activation of specific genes that may predispose the cell to becoming cancerous, or the loss of tissue integrity that occurs in in trauma and wounding. In each of these conditions, there is a danger of excessive cell growth; and cellular senescence is first and foremost a state in which cells irreversibly stop dividing.

However, senescence also affords these cells resistance to signals for apoptosis (cellular suicide) and induces the adoption of the senescence-associated secretory phenotype (SASP), in which they secrete numerous inflammatory signaling molecules and protein-degrading enzymes into their local environment. This ongoing inflammatory and tissue-degrading activity is thought to play a role in the chronic inflammation that is widespread in aging tissues, which in turn promotes the progression and propagation of multiple discrete diseases of aging, as well as the more generalized rise in frailty that limits mobility and independence with age.

With SENS Research Foundation funding, scientists at the Buck Institute have been screening small molecules for their effects on fibroblasts (a kind of skin cell) rendered senescent by ionizing radiation *in vitro*, with the aim of identifying agents that could interrupt the SASP and its downstream effects or selectively kill senescent cells. Such agents could potentially ameliorate diseases and disabilities of aging that are promoted by the SASP.

Previous screening work at the Buck (by post-doctoral researcher Remi Martin-Laberge in collaboration with the Hughes Lab) had identified the natural phenolic compound apigenin as an effective SASP inhibitor in these cells. This past year, the Buck team performed several studies characterizing the mechanisms underlying this effect. Mr. Perrott confirmed that the reduced secretion of these proteins from senescent cells was the result of downregulated expression of the genes for the various SASP factors, and not due to post translational changes in protein stability.

In prior research, the Campisi lab had discovered that the stress-inducible protein p38 induces the SASP, largely by increasing the activity of nuclear factor kappa-B (NFκB), a transcription factor regulating many SASP components. Apigenin was able to suppress the secretion of a representative member of the SASP in cells which had undergone senescence as a result of p38 induction and was also able to cut NFκB activity in half in cells rendered senescent via radiation, thereby reducing the secretion of the SASP in turn. The p38-NFκB pathway to SASP induction appears to work independently of the DNA damage response, and consistent with this, apigenin did not affect representative markers of that response.

Early in the activation of the SASP, senescent cells produce higher levels of an inflammatory regulator known as *interleukin-1 alpha*, which activates the receptor on their surfaces that is designed to respond to this signaling molecule. The Campisi lab has discovered that activation of this receptor is critical to the release of the full complement of SASP factors, including both interleukin-6 (IL-6) and also interleukin-1 alpha itself. Thus, the SASP generates a vicious cycle, in which production of interleukin-1 alpha early in the process tends to lock the cell into a self-perpetuating SASP response (see ***Figure 1***). Importantly, apigenin was shown to interrupt this vicious cycle.

Separately, the Buck Institute is continuting to screen a novel library of proprietary bioactive polyketides for compounds that selectively target senescent cells including mTOR (mammalian target of rapamycin). Investigation of these compounds' effects on SASP and on senescent cells is underway.

They have also begun investigating the causes of senescence in mesenchymal stem cell (MSC) populations – a phenomenon that can limit the therapeutic utility of these cells, which are of interest to many investigators. Originally initiated by Kevin Perrott, this MSC work is now being led by Sam Curran. Understanding the causes and effects of MSC senescence will help researchers optimize protocols for any potential clinical use.

SENS Research Foundation funding also supported research by Nick Schaum in the Campisi lab, who discovered a previously-unknown role of a protein called *HMGB1* in supplying a "missing link" between the inflammatory SASP secretions and the arrest of cell division that, together, are the hallmarks of cellular senescence. HMGB1 is known to play a role in inflammation caused by cell or tissue damage, leaking out of necrotic cells and secreted actively by macrophages when stimulated by pathogens. Nick and colleagues showed that one of p53's first actions to drive the senescence response in response to "risky" signaling in the cell is to relocate HMGB1 out of the cell's nucleus and release oxidized HMGB1 into its local environment. This action regulates both of the hallmark features of senescence. On the one hand, the loss of HMGB1 from the nucleus pushes the cell to stop dividing. On the other hand, the release of oxidized HMGB1 out of the cell is an early event in the chain of signals that drives the release of IL-6 and other elements of the SASP.

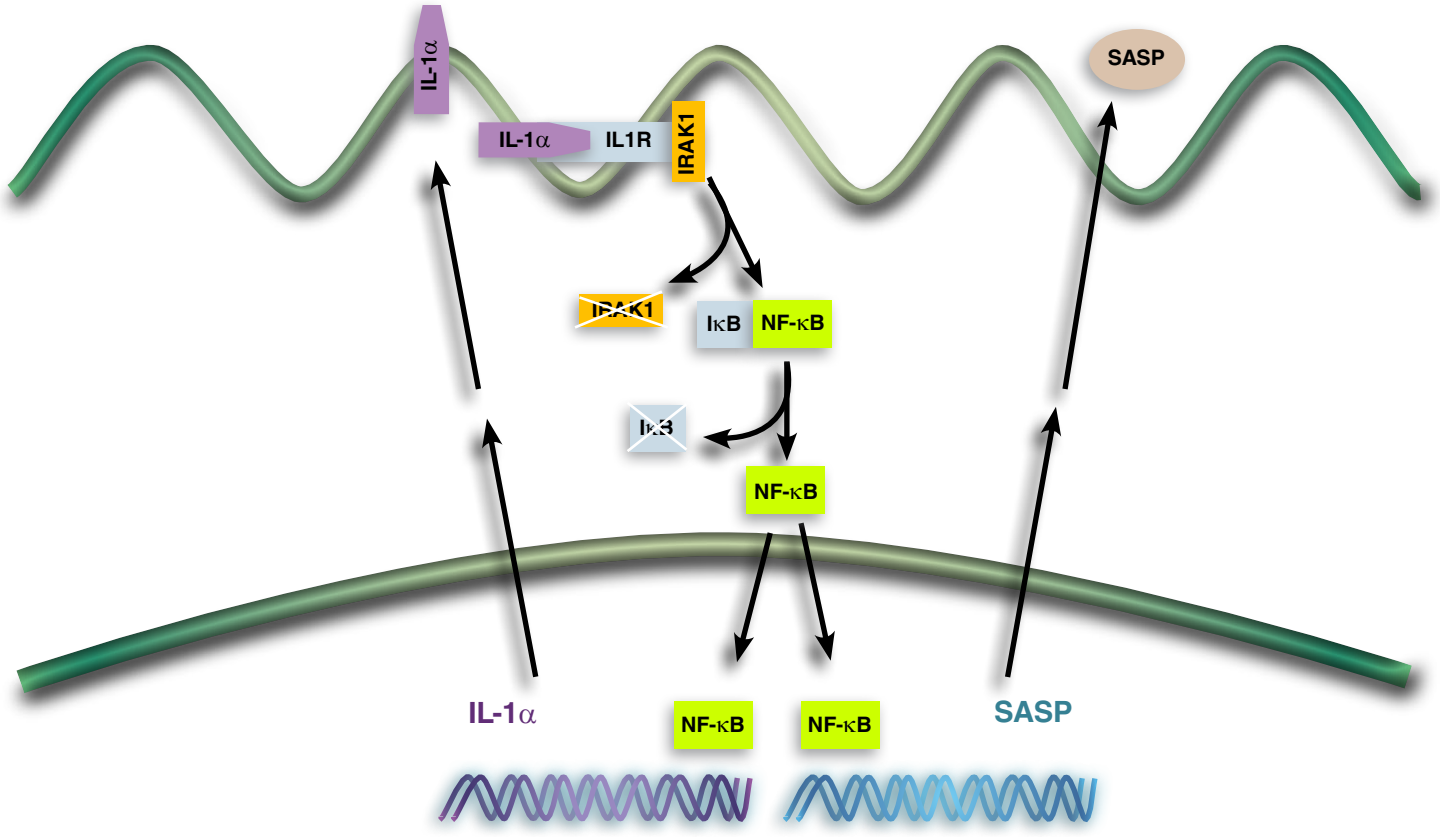


Figure 1: Interleukin-1 alpha enforces a vicious cycle that perpetuates the SASP.
Redrawn from an image provided by Remi Martin-Laberge.

Death-Resistant Cells / Cell Loss and Atrophy

University of Arizona College of Medicine and Arizona Center on Aging, Tucson, AZ

Researchers: Janko Nikolich-Žugich, Megan Smithey

As part of the degenerative aging process, the immune system becomes progressively weaker over time, and we become terribly vulnerable to infectious disease. One suspected reason for this increasing vulnerability is the expanding pool of old T-cells that will only target a long-fought foe crowding out T-cells that would otherwise fight other pathogens. Another factor is the gradual shrinkage and decay of the thymus gland, which slows the production of new T-cells from precursors arriving from the bone marrow.

Funding from SENS Research Foundation enabled Dr. Janko Nikolich-Žugich to test of several possible approaches to reducing or reversing this age-related sapping of the immune system.

Working in Dr. Nikolich-Žugich's lab, Dr. Megan Smithey first infected a group of pubescent mice with one of two of persistent viral infections known to induce large accumulation of virus-specific CD8+ (killer) T-cell clones: herpes simplex virus (HSV) or murine cytomegalovirus (MCMV). These viruses (and their human equivalents) are very clever at evading eradication by the immune system and occasionally reactivate throughout life, driving the gradual expansion of a CD8 clone on patrol for them – at the expense of alternative populations of T-cells.

Dr. Smithey then tested multiple possible strategies to preserve or restore the immune system in different subgroups of these mice. One group of HSV-infected mice were put on the antiviral drug famcyclovir (FAM) as soon as they first became infected, in an effort to prevent the multiple rounds of viral reactivation that drive T-cell clone expansion. Indeed it did so. When the animals were old, less than half as much of the immune "space" in FAM-treated mice was occupied by HSV-specific T-cell clones as in untreated mice; and those T-cells showed less of the dominance of the "effector" T-cell type that is typical of such inflations.

Another group of animals infected with MCMV instead received therapies later in life, at age 20 months (roughly early retirement age in humans). One group had their old T-cell population depleted; two other groups received the same T-cell depletion, followed up by infusion of young, naïve T-cells at either two or six weeks prior to an immune challenge.

Uninfected control animals and infected mice from each treatment subgroup, were then administered a vaccine against West Nile Virus (WNV), which tends to be deadly in old mice. Others were left unvaccinated. Finally, at age 22 months, the mice were actually infected with live WNV.

No intervention seemed to give test animals a better response to WNV vaccination as compared with old, untreated animals, whether on CD8 T-cell response or in antibody production. It is unclear just what to make of the latter finding, however, because of a surprise finding. In this study, even untreated controls in the old group produced more antibodies in response to vaccination than younger animals did. In mice, as in humans, this is exactly the opposite of what normally happens with age. In uninfected or HSV-infected animals, the strongest predictor of a specific vaccine response was the number of remaining naïve T-cells they had. This relationship was, however, badly disrupted in MCMV-infected mice, whether given an intervention or not.

Nonetheless, there was some suggestion that several of the anti-immunosenescence interventions may have helped vaccinated animals survive WNV infection compared to aged mice that had not also had the virus-driven T-cell expansions (see ***Table 1***). Although none of the results were statistically significant, the raw numbers seem especially to suggest that the most comprehensive late-life immunorestoration protocol saved a substantial number of mice from dying of WNV. While an ambiguous outcome, the trends seen in these relatively small groups of animals suggest that a more realistic version of the therapies we would actually deliver to aging humans could indeed prove effective in making their immune systems young again.

Table 1: Mortality after WNV Challenge

Group	Vaccination	Survival (to d50)	n =
Adult	None	66.67%	12
Old	None	25.00%	12
MCMV	None	0.00%	8
Adult	WNV	100.00%	16
Old	WNV	66.67%	12
MCMV	WNV	56.25%	16
MCMV + T cell depletion	WNV	68.75%	16
MCMV + T cell depletion + naïve T cell transfer 6 weeks prior to WNV Vaccination	WNV	84.62%	13
MCMV + T cell depletion + naïve T cell transfer 2 weeks prior to WNV Vaccination	WNV	61.54%	15
HSV	WNV	54.55%	11
HSV + Famcyclovir	WNV	61.54%	13

Enabling Technology: Maximally Modifiable Mouse

Applied StemCell, Inc, Menlo Park CA

Researchers: Ruby Yanru Chen-Tsai, Jiabin Qiu, Qi Zheng, Ivy Zhang

A rising number of the candidate rejuvenation biotechnologies currently in early stages of development at SENS Research Foundation's RC and at extramural facilities we fund are approaching readiness for testing in mouse models.

Projects involving germline genetic modification of mice are by their nature lengthy efforts, more so when the modification must be tested for its ability to rejuvenate aging mice. First, researchers must establish a line of mice with the transgene established in their genomes. Then the mice must be born, weaned, and raised into adulthood for two years or more before any therapy aimed at bona fide rejuvenation can be tested. Finally, the therapeutic genes must be activated, and researchers must wait for at least another year to see the full effects of the therapy play out.

A mouse engineered to readily take up significant genetic modification at any point during its life would substantially contract the timeline needed to gather critical data regarding the effectiveness of applied interventions.

A promising alternative to conventional mammalian-virus-based somatic gene therapy is the use of gene-insertion enzymes from bacteriophages (or "phages,"), a class of virus whose hosts in nature are bacteria. These phage integrases catalyze precisely-targeted, one-way recombination between paired DNA recognition sequences: one ("attP") in the phage genome and another ("attB") at a specific site in the bacterial host, where the phage DNA is inserted. Moreover, phage integrases can be used to insert arbitrary amounts of DNA into the host genome.

The phage integrase from the mycobacteriophage *Bxb1*, in particular, is extremely precise: it will only insert its genetic payload at very specific attB sites. The Bxb1 integrase has already been demonstrated to be a highly effective tool for somatic gene therapy in fruit flies and has been shown to allow repeated, high-titer delivery of novel genes. Unfortunately, mammals lack attP sites in their genomes, and thus the Bxb1 integrase cannot be used to insert new genes into mammalian model organisms such as the mouse.

This limitation could be overcome by a one-time germline insertion of the Bxb1 insertion sequence into a site in the mouse genome that was transcriptionally-active but safe for such insertions. Once engineered with this novel attP site, the Bxb1 integrase system could be used at any time during the mice's lifespan to insert therapeutic genes of any size into their genomes, with no risk of mutational disruption of their own genes even after repeated rounds of treatment. The effects of such genes on age-related disease could then be rapidly evaluated, and if improvements needed to be made, a new transgene could be constructed and tested immediately in mice of the same age.

With funding from SENS Research Foundation, such "Maximally Modifiable Mice" are now being generated by Dr. Yanru Chen-Tsai, the former head of Stanford's transgenic mouse research center, who has extensive experience working with bacteriophages for genetic engineering. Dr. Chen-Tsai's group is generating two mouse models, each containing the Bxb1 attP site in a different transcriptionally-active locus (see ***Figure 1***). One of these loci (Rosa26) has been used for over 20 years and has proved itself to be a good site for bacteriophage transgene expression, while the other (H11) was identified in a genome scan study as yielding the best gene expression level both *in vitro* and *in vivo* among all the tested loci.

The Applied StemCell group has already achieved the first step in generating such mice, which is to make DNA targeting vectors that will allow the insertion of Bxb1 attP sites at the preselected H11 or Rosa26 locus in the mouse genome.

They will next use these vectors to knock the Bxb1 attP sequence into the appropriate locus in the genomes of mouse embryonic stem cells. Cells that successfully 'take' the attP site into their genomes at the intended location will then be microinjected into mouse blastocysts, and the resulting "chimeric" mice will then be bred to establish the new gene in the germline and in both of the twinned sides of the paired helix of the mice's chromosomes.

At the end of the project, the MMM team will establish mouse lines from these mice, which will then be available for site-specific integration of any gene of interest.

In addition to their direct use in accelerating the testing of rejuvenation biotechnologies, Maximally Modifiable Mice will also be of great use to biological science and biomedicine more broadly; and their generation will generate substantial intellectual property (IP) of great value to the Foundation.

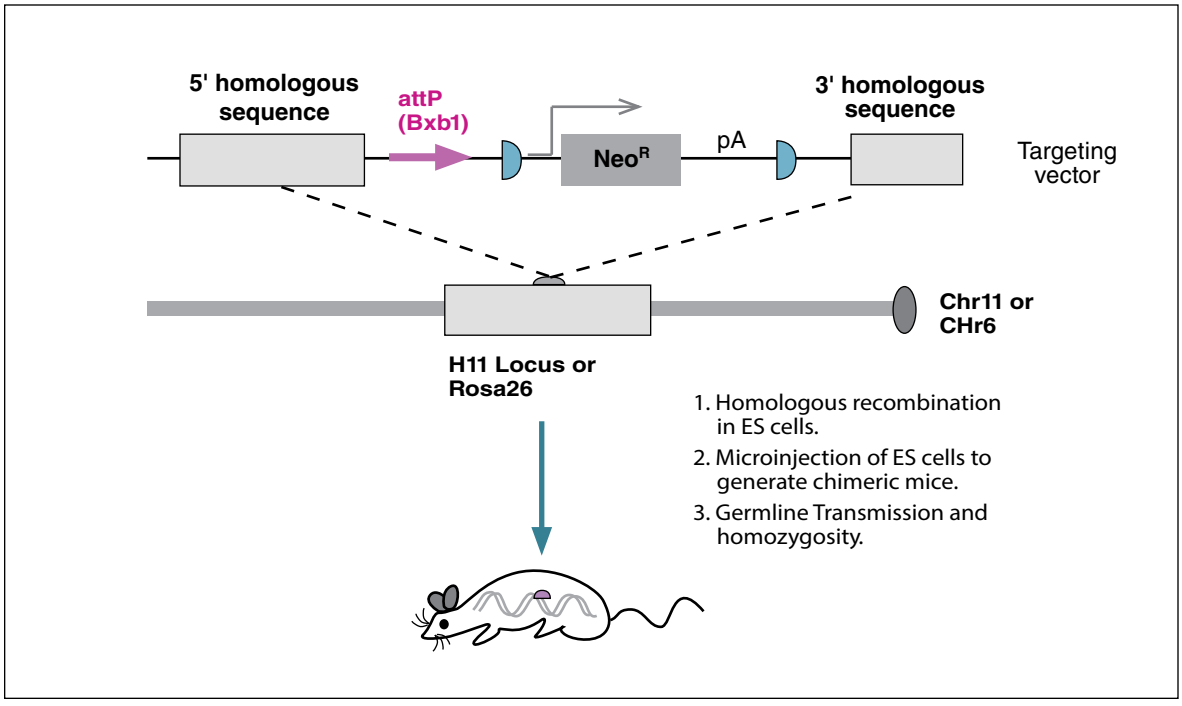


Figure 1: Schematic summary for generating Bxb1 attP mice for site-specific Bxb1 integrase-mediated transgenesis.

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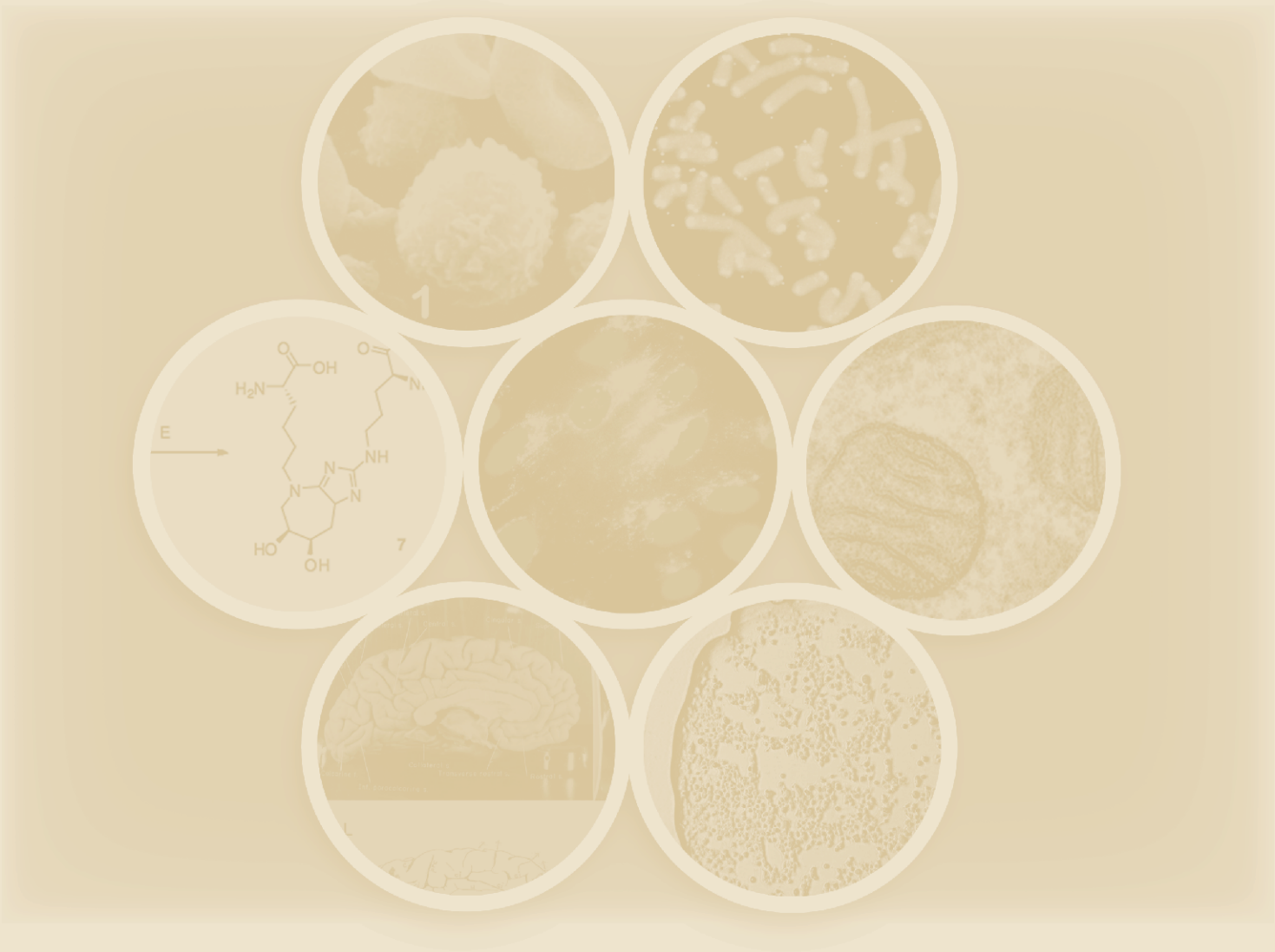

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