Review

A Blueprint for Curing Aging

Michael Renteln *

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A Blueprint for Curing Aging

Michael Renteln

* Correspondence: mrenteln@gmail.com

Abstract:Aging kills ~100,000 people a day - more than any other cause of death combined. The exact causes of aging have been much discussed, but the most pressing issue with regard to aging appears to be lipofuscin accumulation. That is, the accumulation of indigestible cellular garbage that needs to be removed from our cells, then the body. In this piece, I will explain why I think "getting rid of the garbage" should be at least one of our main goals with regard to longevity research for now. Of course, if cancer strikes before then, it will need to be addressed. Curative cancer therapy approaches are discussed herein. I also list the other causes of aging aside from lipofuscin accumulation - and how we can treat them. The same foundation on which the systemic lipofuscin removal approach is built can be used for treating genetic disorders and acquired illnesses as well.

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Introduction:

Over the last century or so, there have been many theories about why we age. However, one stands out as being the most plausible based on the evolutionary and mechanistic evidence. It is the "garbage catastrophe theory of aging." Drs. Brunk and Terman posited years ago that the problem of aging can essentially be summed up as a "garbage disposal issue[i]." The main idea of their proposal is essentially that old molecules are sometimes damaged in ways that prevent the lysosomes from breaking them down properly, and over time these damaged, old molecules accumulate inside the lysosomes. Eventually, the lysosomes become full of this indigestible garbage, i.e., "lipofuscin", and cannot function normally; then there is a garbage back-up and the cell starts to decline in terms of health.

In 2006, Dr. Aubrey de Grey and Ben Zealley proposed "Strategies for Engineered Negligible Senescence" (SENS) as a means of ending aging[ii]. Initially, it was met with skepticism, but longevity-promoting therapies related to SENS have been the focus of much research of late. SENS encompasses seven types of age-related damage: cancerous nuclear DNA (nDNA) mutations, senescent cells, mitochondrial DNA (mtDNA) mutations, intracellular indigestible garbage, extracellular aggregates, cell depletion, and extracellular cross-links.

Over time, lipofuscin occupies a large portion of the cytoplasm in certain cell types. It is mainly a problem for non-dividing or slowly/infrequently dividing cells. Whether or not lipofuscin itself can exert negative effects on the cell that contains it, simply accumulating to a critical level would logically cause a garbage backup. The cells may try to produce more lysosomes - but will eventually reach capacity. Decreasing autophagy efficiency would also potentially accelerate lipofuscin accumulation, as undigested material may sit around in the cytoplasm for longer and become oxidatively damaged. It also makes sense that reaching a critical threshold of lipofuscin in non-dividing cells throughout the body could accelerate the accumulation of the other six categories of age-related damage as defined by Dr. de Grey and Zealley. This could subsequently accelerate lipofuscin accumulation. Lipofuscin accumulation leading to decreased autophagy and the accumulation of the other six categories of damage could thus cause a downward spiral in terms of functionality[iii].

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Dysfunctional mitochondria are a hallmark of aging[iv]. With lipofuscin accumulation, damaged mitochondria - sometimes with mtDNA mutations, sometimes just with damage to their membranes, proteins, lipids, and DNA - will not be recycled as rapidly and may then start to accumulate. MtDNA mutation accumulation is one of Dr. de Grey's categories of age-related damage; mtDNA mutations accrue over time. MtDNA damage and even mutations can be selected against in a cell by virtue of mitophagy. MtDNA mutation accumulation can be staved off by overexpressing mtDNA repair proteins. However, I will address mtDNA mutations on a fundamental level later in this piece.

Due to a garbage back-up, tau may begin to build up inside neurons, eventually becoming hyper-phosphorylated and forming neurofibrillary tangles[v]. Similarly, β-amyloid that is normally degraded may continue to persist, building to levels that lead to plaque formation. Hopefully, if lipofuscin is removed, plaques that have already formed could regress - in line with Le Chatelier's Principle. In other words, if lipofuscin is cleared and β-amyloid in solution is taken up and autophagocytosed, insoluble plaques may start to dissipate back into solution as well - at which point the β-amyloid in solution would again be taken up and degraded. Transthyretin amyloid could also be another amyloid that forms in the extracellular spaces of our bodies as a result of lipofuscin accumulation in various tissues[vi].

Furthermore, it is clear that with the extracellular matrix (ECM) not being properly cared for by its resident cells, other damage such as fragmentation, glycation, elastocalcinosis[vii], and cross-linking could rapidly reach pathological levels. After extensive lipofuscin removal, hopefully much of this will damage prove to be reversible. If healthy proteostasis could be restored within our parenchymal cells, ECM turnover efficiency should be restored to youthful levels - although it may not be able to reverse all the damage that has accumulated in elderly individuals. Certain cross-links may be cleavable by endogenous, secreted enzymes; after treatment, said cross-link–degrading enzymes could be secreted at the appropriate levels.

Glucosepane is a cross-link that becomes prevalent with age in human tissues that is uncleavable - or at least not cleaved very efficiently/often. Exogenously administered cross-link–breaking molecules or enzymes could be helpful[viii,ix]. However, there are almost certainly a multitude of indigestible cross-links that accumulate with age. As with lipofuscin xenocatabolism[x], an unfeasible number of enzymes might be required to address the problem in this way. Fortunately, even if there are some cross-links that can't be degraded by endogenous enzymes - if the ECM turnover efficiency is restored through extensive lipofuscin removal - molecules bound together by uncleavable cross-links could be excised and phagocytosed by tissue-resident macrophages or endocytosed by tissue-resident cells. Some may reach the lysosome and reside there as lipofuscin[xi]. But if lipofuscin can be periodically cleared, that is alright. Tissues can have forced ECM turnover[xii] periodically. Also, tissue-resident macrophages (TRMs) could be replaced with edited versions via replacement by small molecule CSF1R inhibitor-based depletion and autologous, edited hematopoietic stem cell (HSC) transplant. These edited TRMs could patrol tissues and help repair ECM damage.

ECM damage doesn't seem to be an issue with the "immortal" Hydra vulgaris. H. vulgaris has a body column with cells that continuously divide - driving cells out towards its extremities. The cells at its extremities slough off[xiii,xiv]. Thus, it has a built-in mechanism for ridding itself of lipofuscin. While most of its ECM, i.e., mesoglea, is shed along with the cells at its extremities, the mesoglea in the head region is turned over, and relatively slowly at that[xv]. Even with stationary mesoglea in the head region, this organism appears not to age - or at least age very slowly. Of course, the human ECM is much more complex than that of H. vulgaris. However, the longevity of its mesoglea may indicate that if lipofuscin removal is done periodically (perhaps every decade) in humans starting at a young age, ECM damage may not really be a problem for a

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long time at least. If started at an older age, a large portion of the damage may be reversed, and then subsequent ECM damage accumulation would hopefully be very slow.

Additionally, cancer is more likely to initiate or progress if many microenvironments throughout the body are corrupted by lipofuscin accumulation. Along those lines, stem cell niche corruption may prevent them from replicating efficiently to replenish tissues. Subsequently, they may start to accumulate lipofuscin as well (if their replication is slowed).

Senescent cells may start to accumulate if tissue-resident immune cells are rendered inert by lipofuscin accumulation and the non-functionality of the parenchymal cells around them also caused by lipofuscin accumulation, leading to dilapidation of the ECM. Here I refer to irreversibly senescent cells, which have suffered DNA damage and can no longer function properly. However, many cells that show signs of senescence like the senescence-associated secretory phenotype[xvi] may be reversibly senescent. They may have entered into that state due to epigenetic damage brought on by lipofuscin accumulation. I have much hope that this epigenetic state can revert back to a youthful state once lipofuscin is removed, however.

A recent article suggests that epigenetic damage is mostly reversible at least[xvii]. The hallmarks of aging that it related to were mitochondrial dysfunction, nutrient sensing, and stem cell composition. If lipofuscin is removed, damaged mitochondria will be recycled and nutrient sensing processes should go back to normal. Stem cells in this study likely show a younger epigenetic age because they divide frequently and thus dilute out their lipofuscin. That relates to my hypothesis about why Yamanaka factors rejuvenate tissues[xviii]; I believe it may be because they transiently induce a pluripotent stem cell state. Thus, cells that normally wouldn't divide start to divide transiently, and thereby dilute out their lipofuscin. Sox2, one of the four Yamanaka factors, also initially stimulates autophagy[xix] - which decreases "false" lipofuscin. I'm defining false lipofuscin as intracellular garbage that a cell could potentially recycle if it were encouraged in some way. "Real" lipofuscin is intracellular garbage that cannot be digested no matter how we manipulate a cell's metabolism - namely the junk that must be removed.

We would sequence our mitochondrial genomes and ideally nuclear genomes with the best sequencing technology available - to have pristine reference sequences for the future[xx]. It was determined that high-level mitochondrial DNA heteroplasmy is rare in humans[xxi]; low-level heteroplasmy is rather prevalent, however. Hopefully, low-level heteroplasmy or situations where an inherited variant becomes dominant through expansion is not required in any of our tissues for healthy functionality. If not, we will only need to worry about sequencing our dominant, inherited mitochondrial genome.

We probably also need to freeze cells from our bodies with pristine DNA, so that we can turn them into edited HSCs, deplete our TRMs, and transplant the edited HSCs, allowing the TRMs that result from the edited HSCs to indirectly replace our old stem cells and enact whole-body induced cell turnover[xxii] (before medical, computerized microrobots and/or nanorobots can be developed). We could mobilize and harvest HSCs, or just harvest circulating monocytes. These circulating monocytes could be reprogrammed into iPSCs and then we could differentiate them into HSCs. Printing the mitochondrial genome is possible for replacement purposes, but the nuclear genome is much, much larger, and also has complex epigenetic considerations.

Lipofuscin Removal:

Recently, I described a method of removing lipofuscin from the whole-body that is based on TRM replacement with edited versions using a small molecule to deplete them and autologous HSC transplant with edited cells to replace them[xxiii]. These edited TRMs would then transiently be made hyper-motile via small molecule administration - and they would secrete an RNA vector to instruct nearby cells to export their lipofuscin (via secretory autophagy of lysosomes). They would also be instructed to make more - via TFEB overepxression. The edited TRMs could then potentially

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phagocytose LAMP-1 positive vesicles, as secretory autophagy of mitochondria lead to their expulsion without an encapsulating membrane. The phagosomes encapsulating the lysosomes would be prevented from fusing with TRM lysosomes. After the TRMs phagocytose an experimentally determined number of lysosomes, a synthetic gene circuit for counting would trigger asymmetric division of the TRMs - wherein all the phagosomes containing lysosomes would be inherited by one of the progeny cells. That progeny cell would then migrate to the gastrointestinal tract lumen; they would be chemoattracted there by gut microbes that have been modified to overproduce an inert, orthogonal small molecule. Alternatively, a device placed in the peritoneal cavity could slowly secrete such a small molecule, and egress could be effected manually.

This treatment should perhaps be effected once a decade starting at ~30 years of age. A more extensive treatment would likely be required for those who are currently elderly. That is in large part because there may be a lot of cytosolic lipofuscin in post-mitotic or slowly/infrequently dividing cells that will subsequently be delivered to new lysosomes after the old, lipofuscin-laden lysosomes have been secreted from the cells (and excreted from the body).

Cancer:

If systemic lipofuscin removal is an extremely potent rejuvenation therapy, our risk of getting cancer would greatly decrease. If one first receives (an extensive) lipofuscin removal treatment at an advanced age - one's risk of getting cancer might be somewhat higher than it would be for a younger individual, but would still be much lower after treatment. Spontaneous cancer in younger individuals is possible, although it's much more rare than in the elderly (e.g., through essentially random metabolic errors or excessive sunlight exposure). Notably, cancer is especially unlikely in young people who are unstressed, eat healthy, exercise regularly, and do not get excessive sun exposure. Early detection of cancer is also extremely beneficial; we should all frequently have an ensemble of blood tests, as well as full body scans. Full body scans may not be too helpful for very early screening, but larger growths can be identified.

I have written three articles on the subject of solid tumor therapy about a novel approach that I call Oncolytic Vector Efficient Replication Contingent on Omnipresent Mutation Engagement (OVERCOME)[xxiv,xxv,xxvi]. OVERCOME involves multiregion sequencing of a patient's tumor or tumors and using an intracellular microbe to detect one or more of their clonal mutations; it would respond after detection by replicating within/destroying any cancer cell containing the targeted mutations. Clonal mutations are those that are contained in all of a patient's cancer cells. A variation of OVERCOME it that I mention in my second and third articles involves targeting a small set of subclonal mutations that together are present in all of the patient's cancer cells. OVERCOME or its variation may be enough to cure cancer or at least effectively treat it without side effects in a way that can be repeated indefinitely.

Blood cancers can be treated via HSC transplantation potentially[xxvii,xxviii,xxix] - but it would be better to use autologous HSCs. Purging autologous HSCs is possible through clonal mutation detection by an intracellular microbe[xxx].

Overexpressing DNA repair and tumor suppressor proteins[xxxi,xxxii,xxxiii] in our cells could help stave off cancer until we develop computerized microrobots and/or nanorobots that can eliminate/prevent cancer without fail. As I mention later in terms of overexpressing longevity proteins or underexpressing proteins that accelerate aging, it is not really advisable to alter gene dosage or introduce new genes into our cells, but it may be a necessary risk here if OVERCOME or the autologous HSC blood cancer therapy I mentioned are not always curative or cannot even stave off death from cancer in all cases.

Lipofuscin removal could potentially help treat or even cure slow-growing cancer in the elderly in some cases - at least in part by re-establishing a healthy immune system.

The best type of anti-cancer strategy aside from computerized microrobots and/or nanorobots would be to pre-empt it entirely in a biological manner. Such an approach was conceived of by Dr.

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de Grey. He calls it "Whole-body Interdiction of Lengthening of Telomeres" (WILT)[xxxiv]. It involves deleting the machinery required to lengthen telomeres in all cells in our body and periodically reseeding our stem cells with similarly bioengineered cells. This would essentially completely eliminate the problem of cancer. Unfortunately, aside from telomerase there is another mechanism cancer cells can use to extend their telomeres, called "alternative lengthening of telomeres" (ALT). It has been estimated that ALT is active in 10-20% of all cancers[xxxv]. One or more of the proteins involved in ALT may be required for other critical cellular processes - so knocking them out of our cells may not be possible. Another crucial issue is that reseeding stem cells in certain tissues at least may not be particularly facile. Dr. de Grey, however, has pointed out that the more rapidly dividing stem cells like those of the skin, gut, and immune system seem easier to reseed than stem cells that divide more slowly, like those in the brain and heart.

Perhaps we could knock out hTERT in all of our cells and install an intracellular bacterium that replicates up to a small number inside our stem cells; they could dispense telomerase periodically (when induced via exogenous small molecule). They could have many mechanisms in place to ensure that their telomerase cannot be hijacked by the host cell. Maybe they could have many redundant, inducible kill switches. But if they are cleared, we would have to re-"infect" all of a patient's stem cells again. The intracellular bacteria could use potentially use molecular switches to detect mRNA or protein that indicate that certain ALT proteins are required at that moment. That seems a bit difficult, but perhaps less so than reseeding stem cells in areas like the brain. These bacteria would have to keep pace with stem cell division, although this is not necessarily impossible[xxxvi,xxxvii].

Senescent Cells:

Senescent cells can be destroyed using a small molecule[xxxviii,xxxix], peptide[xl,xli], or senolytic CAR T-cells[xlii]. The T-cells could be chemoattracted to a peptide secreted by senescent cells[xliii,xliv,xlv]. Edited TRMs could also secrete the peptides or a nucleic acid vector[xlvi,xlvii] that destroys senescent cells.

If we can slow down aging via interventions such as periodic senescent cell destruction, Klotho administration, blood transfusions, and anti-aging gene therapy, it would give those who are elderly right now more time to live so that they might benefit from the lipofuscin removal therapy, which might take a bit of time to develop. Apart from potentially decreasing things such as neurological deterioration, sarcopenia, and vascular degeneration, these interventions gene may also help to stave off cancer.

Telomere Shortening:

It has been proposed that not enough telomerase is made in our stem cells, and that over time their telomeres shorten so much they become senescent and we age. (Shorter telomeres are also associated with an increased risk of cancer.) It is well-known that telomere length decreases with age. However, at a very advanced age, telomere length actually stops decreasing and even begins to increase before leveling off. This is in agreement with the Gompertz curve[xlviii].

Importantly, HSCs from aged individuals can still function normally if they are "rejuvenated" ex vivo and then transplanted into a young niche[xlix]. It appears as though every compound that is used to "rejuvenate" aged stem cells (e.g., CASIN and rapamycin) actually decreases false lipofuscin in the cells through the stimulation of autophagy[l,li]. From a theoretical perspective, one could imagine that if a stem cell is slowly-dividing, it could build up lipofuscin over time even with some replication. Alternatively, a rapidly-dividing stem cell could be restrained by a niche full of lipofuscin, and then start to accumulate lipofuscin itself. Lipofuscin removal from the stem cells/their niches may make them better able to degrade damaged telomerase components and generate new ones, but it is still possible that a gene vector encoding telomerase will be needed to lengthen the telomeres of our stem cells - preventing them from becoming cancerous or senescent. To negate this potential issue, edited TRMs could use COURIER to periodically secrete

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hTERT mRNA to surrounding stem cells - or secrete a gene vector that integrates into a safe harbor locus in the stem cell genomes or utilizes an S/MAR sequence.

Constitutive telomerase expression in many tissues from birth leads to oncogenic effects[lii]. However, periodic overexpression of telomerase in all our adult stem cells could be safe for a long period of time if (DNA repair proteins and) tumor suppressor proteins are also overexpressed[liii].

Lifelong overexpression in non-cancer resistant mice appears to be safe and beneficial, but mouse telomere biology is not the same as human telomere biology[liv].

An adeno-associated virus serotype 9 (AAV9) vector encoding hTERT has been shown to extend the lifespan of adult/old mice when injected intravenously[lv]. Additionally, a cytomegaloviral (CMV) vector encoding hTERT generated even more impressive results - when administered intranasally and intraperitoneally in old mice[lvi].

Whether telomerase overexpression in humans is safe is still up for debate, although one individual, Liz Parrish, elected to undergo injections with an AAV9 vector encoding hTERT[lvii]. Thus far, no oncogenic effects have occurred. Additionally, her leukocyte telomeres have increased in length. It would be safer, however, to also overexpress DNA repair and tumor suppressor proteins in one's cells[lviii]. Hopefully this individual stays cancer-free, and receives a therapy that allows for the overexpression of said proteins as soon as possible - via AAV9 or another vector. However, AAV vectors generally do not integrate, so the vector may be lost at this point.

Memory B and T cells:

Another issue is whether we should be concerned about memory B and T cells. Specifically, should we be concerned that they will eventually build up in our bodies over time through exposure to different pathogens to the point where there is no more space for naïve B and T cells? Dr. de Grey mentioned this as being an issue, especially for CMV. If the thymus and other lymphoid tissues are free of lipofuscin and still functioning properly, perhaps this will not be a problem - as the clonal expansion of memory B and T cells may be a compensatory action to counter the marked drop in the output of naïve B and T cells due to bone marrow/spleen aging and thymic involution, respectively[lix].

However, perhaps for the elderly who already suffer from a clonal expansion of memory B and T cells, removing lipofuscin from lymphoid tissues may not be enough. We may also need to eliminate the memory B and T cells that have clonally expanded and massively skewed their pathogen resistance profiles. It may be relatively simple to do that with immunotoxins against memory B- and T-cells in general or CAR T-cells against them. Instead, however, we may be able to specifically target clones that are most prevalent using a gene vector-type approach wherein CD45-positive cells are transduced. Encoded molecular switches would search for particular DNA or RNA sequences and eliminate the cell if they are detected. Notably, if memory B and T cells sit around for long periods of time without replicating - i.e., when the body is not exposed to their corresponding pathogen, they could fill with lipofuscin over time and be unable to function properly (e.g., re-enter a replicative state when necessary) after that.

hTERT overexpression in patient HSCs may be helpful during and/or after general memory B and T cell clearance, at least. hTERT mRNA could be delivered via lipid nanoparticles[lx].

Other interventions (including anti-aging gene therapy other than for hTERT):

Altering endogenous gene expression or adding foreign genes to our cells when it is not necessary may not be prudent. Introducing DNA in general into our nuclei through gene therapy could be problematic; it could integrate at sites of double-strand breaks, for example. Also, if one meddles with finely-tuned genetic circuits, it may result in cancer. If one meddles with said circuits in many of our cells at once, it also may have other very harmful effects potentially. Dr. de Grey has mentioned these concepts. However, perhaps there are some good, safe targets that can be underexpressed or overexpressed to increase longevity.

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One great potential target is the DREAM complex, which when inhibited increases the DNA repair capacities of somatic cells to that of germline cells[lxi]. Mitochondrial DNA repair enzymes could also be overexpressed potentially. Tumor suppressor protein overexpression may also be helpful[31,32,33]. PTEN overexpression in mice leads to less cancer as well as a healthier metabolism[33].

TFEB, a protein that is considered the "master regulator of autophagy", might also be a target that would be safe to periodically overexpress throughout the body. Notably, gene vectors may not be necessary for altering TFEB gene dosage. A company called Generian is developing a small molecule inhibitor of the E3 ligase that degrades TFEB, which would thereby boost its levels in our tissues[lxii]. Perhaps overexpression of TFEB from a gene vector would be more potent, however. In mice, TFEB can safely be overexpressed in the ventral midbrain at least[lxiii]. It should probably be periodically activated rather than constitutively activated, as the latter may have detrimental effects.

FOXN1 may also be a good target. FOXN1 overexpression has been shown to counter-act thymic involution[lxiv]. I argue that this is because it increases cell division[lxv] as well as proteasome activity[lxvi], which can partially compensate for declining autophagy efficiency due to lipofuscin accumulation. Maybe TFEB overexpression in the thymus would yield similar results. Perhaps the thymus accumulates lipofuscin much more rapidly than other organs because it is so metabolically active and thus its constituent cells need to divide much more frequently to dilute out the lipofuscin.

One could inject TRMs into the thymic area and secrete an immunotoxin for thymus-specific macrophages. They could then repopulate the limited area, and secrete FOXN1 with a cell-penetrating peptide to help rejuvenate the organ.

Klotho is also a pro-longevity protein. Gene therapy may be unnecessary here; injections of the protein could be utilized[lxvii].

Platelet factor 4 may also be helpful as a therapy; a recombinant version could potentially be administered intravenously[lxviii].

While edited TRMs could secrete mRNA via COURIER to surrounding cells, it would be more effective to transfer a gene vector to target cells. In stem cells, it would either integrate or utilize an S/MAR sequence. Targeted integration into a safe harbor locus can be effected with CRISPR transposases or dual prime editing combined with a large serine recombinase.

Blood transfusions can be utilized to introduce young blood factors and/or dilute out old blood factors[lxix].

Mitochondrial DNA mutations:

MtDNA has a 10-100x higher rate of mutation than nDNA, as mitochondria are sites of free radical production. We may need to replace mtDNA before nDNA that has suffered epigenetic drift, damage, and mutations. If so, we will need a strategy to deliver pristine mtDNA to cells around the body and destroy somatically mutated mtDNA.

I recently described an approach for systemically removing lipofuscin[23]. It relies on tissue-resident macrophage (TRM) replacement from edited HSCs that have been transplanted using non-genotoxic conditioning[lxx]. A similar strategy may enable us to replace mutated mitochondrial DNA (mtDNA) throughout the body. This would be important if mtDNA replacement is necessary prior to the replacement of cells with nDNA that has suffered epigenetic drift, damage[lxxi], and mutations[lxxii] using TRM replacement in combination with whole-body induced cell turnover (WICT)[lxxiii]. As mtDNA mutations accumulate at a 10-100x faster rate than nDNA mutations due to free radical production in the mitochondria, they may have to be addressed before nDNA that has suffered epigenetic drift, damage, and mutations.

Even if mtDNA mutations will be an issue before nDNA epigenetic drift, damage, and mutations, and the TRM/WICT strategy is developed in time, it would still be ideal if it did not need to be applied solely for mtDNA mutations, as it very involved - and neuronal replacement would ideally be effected as infrequently as possible.

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We must sequence our mtDNA and freeze some cells at as young an age as possible from cell types with the lowest rates of mutation. We should also sequence our nDNA if possible. Skeletal muscle samples may be a good source of at least relatively pristine mitochondrial DNA. Germline stem cells would be ideal for nuclear DNA mutations, but skeletal muscle may be a good source for that as well[lxxiv].

Mitochondrial DNA heteroplasmy may need to be taken into account in this context. High-level heteroplasmy in some tissues at least is detrimental, but some tissues could possibly require it or be negatively affected in terms of functionality if they are made homoplasmic.

Systemic lipofuscin removal should help to keep mtDNA mutation and nDNA epigenetic drift, nDNA damage, and nDNA mutation rates to a minimum. Additionally, overexpression of mtDNA repair genes and inhibition of the DREAM complex[61] may help stave off these issues.

When/how often we need to replace our mtDNA can be informed by prior human cases of mitochondrial genetic disorders in which mtDNA hyper-mutation occurs.

One way to replace mtDNA throughout the body would be to secrete mitochondria from edited TRMs after systemic TRM replacement using arrestin domain containing protein 1 [ARRDC1]-mediated microvesicles (ARMMs)[lxxv,lxxvi]. Self-amplifying RNA (saRNA)[lxxvii] or trans-amplifying RNA (taRNA)[lxxviii] can also be exported, which would encode TFAM to increase mtDNA copy number in target cells[lxxix], as well as nucleases that target old mtDNA. The new mtDNA that is being exported would have mutations in non-coding regions or synonymous or even non-synonymous mutations in coding regions. It was shown that certain SNPs in the mitochondrial DNA are actually beneficial, so this should be possible[lxxx,lxxxi].

Importantly, the entire mitochondrial genome based on a digital sequence can be "printed" and utilized therapeutically, as the genome is only 16,569 base pairs and mitochondrial epigenetics would likely not be a concern.

Monocytes appear to frequently secrete extracellular vesicles[lxxxii], and TRMs likely can do so as well. However, the secretion of larger vesicles such as microvesicles may be more rare due to plasma membrane size constraints - even when stimulated. The rate of microvesicle secretion may not be rapid enough to make a sizable dent in the overall number of mtDNA copies in target cells. It is also unclear how to substantially increase the availability of donor cell mitochondria for secretion purposes. PGC-1α, TFAM, and Drp1 overexpression could help to maximize mitochondrial biogenesis, mtDNA replication, and fission, but having a fragmented mitochondrial network could be detrimental for TRM functionality or cause inflammation. Endosomal escape may also be an issue with ARMMs, although mitochondria donated by mesenchymal stem cells via ARMMs were able to fuse with recipient cell mitochondria[75]; fusion with recipient cell plasma membranes may occur[76]. Furthermore, mitophagy could destroy at least some of the pristine genomes before the mitochondria that contain them fuse with target cell mitochondria and the genomes can be replicated. It was demonstrated that a mitochondrial targeted meganuclease can produce shifts in heteroplasmy over time[lxxxiii,lxxxiv]. However, there probably must be a large enough initial quantity of the genome that is to be selected via replicating RNA, which has a limited lifetime. Thus, although each mitochondrion may contain multiple mitochondrial genomes, if only a small number of mitochondria are delivered to a target cell with hundreds or thousands of mitochondria, this may not be enough - i.e., selection for the unmutated mitochondrial genome may take too long to be feasible via replicating RNA. If transfer of a cytoplasmic DNA plasmid[lxxxv] can be enacted, wherein it is taken up into the nucleus of target cells[lxxxvi], low-level selection could potentially be effected for as long as necessary.

Exopher secretion is another possibility, but much less is known about its molecular biology[lxxxvii]. They would also be even more difficult for TRMs to secrete. Additionally, uptake by non-phagocytic target cells may be problematic. Endosomal escape may not be efficient here either. If fusion of large vesicles with the plasma membrane of target cells is the goal, that could be an issue, as well.

A synthetic gene circuit that causes periodic asymmetric division of the TRMs[lxxxviii,lxxxix] could also generate one effector progeny cell that overexpresses Drp1 and then forms tunneling nanotubes

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with the cells around it, allowing for the transfer of multiple mitochondria and replicating RNA[xc,xci,xcii].

The effector cell could simply lyse, releasing all of its punctuate mitochondria. An invasin could be expressed on the outer membrane of the mitochondria, to facilitate their uptake by neighboring cells, but endosomal escape may be an issue. Listeriolysin O (LLO) could be overexpressed by the effector cell prior to lysis, and may be taken up in endosomes with at least some of the mitochondria[xciii,xciv]. LLO would be activated at endosomal pH and could potentially help facilitate escape of the mitochondria from their encapsulating endosomes[xcv]. Replicating RNA vectors in viral capsids, e.g., a picornaviral capsid, would also be released after lysis.

Partial cell fusion would also be a possibility to deliver large quantities of new mitochondria (and a replicating RNA vector) as well.

Bacteria can conjugate with mammalian mitochondria, although whether second strand synthesis occurs afterwards is unknown[xcvi]. A strategy called Mr PB[xcvii] could be employed to replace TRMs systemically with new versions that contain intracellular bacteria that replicate up a tolerable copy number, at which point they would be restrained via quorum sensing. Mr PB just involves elimination of patient TRMs and repopulation via repeated infusions of immune cells - instead of HSC transplantation. Even if a few copies of a bacterial vector can be delivered to target cells, there is a range of mitochondria morphology that is possible in terms of fusion and fission. If the bacteria conjugate with a large mitochondrion and inject a multitude of mitochondrial genomes, this would be helpful, but if only a few, distinct mitochondria are targeted, this might not be sufficient. Even with genetic modifications, xenophagy or dysfunction and lysis of the vector would likely occur at some point, so it does not have an indefinite amount of time.

Instead of bacteria, an intracellular yeast like Cryptococcus neoformans could be used as the mtDNA delivery vector. This may be possible because the yeast Saccharomyces cerevisiae can maintain a mammalian mitochondrial genome in its mitochondria[xcviii]. C. neoformans cells have around 35 mitochondria[xcix]. Additionally, each yeast mitochondrion may generally contain multiple mitochondrial genomes that are compacted in nucleoid structures, as with mammalian mitochondria. To reduce its size and probably increase its invasiveness, an acapsular version can be utilized. Typically, C. neoformans is 4-6 μm in diameter. Smaller cells may be required to invade certain adult stem and post-mitotic cell types. Fortunately, C. neoformans cells as small as 1.1 μm in diameter have been found[c]. It may take more research to figure out how to limit the size of C. neoformans. Of course, the maximum size of the yeast that can be utilized would be important to transfer the maximum number of mitochondria. Perhaps a diameter of around 2.5 μm would be ideal.

If the yeast are maintained at a low copy number, wherein yeast vacuole fusion is prevented, they could keep pace with the host cell TRM division if that occurs[ci]. They could also be secreted continuously at low level via expulsion or vacuole lysis and secretory autophagy. It would be retargeted to a cell surface protein that is ubiquitously expressed by adherent cells. Expression of an invasin and listerolysin O could allow it to invade a wide variety of cell types[94,95]. The yeast could enter target cells, lyse their vacuoles, and then lyse to release their mitochondria and replicating RNA encoding TFAM and nucleases. Xenophagy of the yeast mitochondria in target cells could be avoided via the inclusion of deubiquitylases in the mitochondrial outer membrane. Human mitofusins could be expressed to promote fusion with the target cell mitochondria.

At some point, mtDNA mutations will need to be addressed. Discussed in this section is a method of replacing somatically mutated mtDNA throughout the body. While fusion of yeast mitochondria with human mitochondria may not ensue naturally, synthetic biology should be able to rectify that situation if necessary. Interestingly, mouse mitochondria can fuse with human mitochondria[cii]. Also, an artificial vector called MITO-Porter can fuse with human mitochondria[ciii]. There are immunogenicity concerns that will need to be addressed, but this is likely not an insurmountable issue.

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Nuclear DNA Epigenetic Drift, Damage, and Mutations:

Periodic lipofuscin removal should help to reverse/stave off nuclear DNA epigenetic drift, damage[civ], and mutations.

Lipofuscin removal, DNA repair protein overexpression, tumor suppressor protein overexpression, cycles of fasting, vigorous exercise, and hot/cold shocking the body may be enough to address the potential issue of pathological levels of epigenetic drift in stem cells and long-lived post-mitotic cells.

If not, multiple cycles of transient Yamanaka factor expression may be enough to deal with that possible issue[cv]. In addition to resetting the epigenetic signature of a cell, partial reprogramming can also enhance DNA damage repair. Edited TRMs could potentially secrete saRNA or taRNA encoding Yamanaka factors or package a cytoplasmic DNA plasmid into a transfer vector for long-term, small molecule-inducible expression in the relevant cell types[cvi,85,8]. Otherwise, the TRM/WICT strategy would be necessary for this potential issue as well.

If our stem cells acquire mutations less quickly than our long-lived post-mitotic cells, we could potentially increase long-lived post-mitotic cell turnover - maybe at least in part by setting a lower threshold of DNA damage that stimulates apoptosis. Overexpressing tumor suppressor proteins may be one way to increase the turnover of generally-mutated cells. Senescence is another possible consequence, but could be cleared by the immune system or other means.

Whole-body induced cell turnover after HSC transplant with edited, pristine HSCs and TRM replacement is also possible - wherein adult stem cell reseeding is not effected. Of course, with dramatically increased cellular turnover, we would have to induce telomerase more frequently in our stem cells.

However, it looks as though the mutation rates are relatively similar[cvii,cviii]. Thus, we may need to replace our adult stem cells at the same time as our long-lived, post-mitotic cells. At a certain point at least, adult stem cells will need to be replaced as well.

General Dilapidation of Body Structures:

Periodic lipofuscin removal should help to reverse/stave off general dilapidation of body structures like atherosclerotic plaques and aneurysm formation. Repair biotechnologies is doing great work in the field of atherosclerosis treatments[cix,cx,cxi].

Other ways to stave off these issues would be to promote extensive ECM turnover more frequently than it occurs endogenously[12], in combination with more even more frequent lipofuscin removal treatments than once a decade - to help clear out any ECM material that is phagocytosed or endocytosed and contributes to lipofuscin. Structural dilapidation may also be mitigated if we bioengineer tissue-resident macrophages to patrol tissues more extensively and more actively assist with the maintenance of our bodies.

Eventually, we may need lab-grown autologous tissues to fix general dilapidation of our body structures. For example, we may need to replace blood vessels in certain regions. Frequent scans should help us identify problematic areas.

Discussion:

It is important that we sequence our mitochondrial and nuclear genomes as soon as possible after birth, as well as harvest some cells at as early an age as possible and freeze them. These cells should be of the type or types with the lowest rates of mitochondrial and nuclear DNA mutation. For the nuclear genome at least, this would likely be germline stem cells. That would hold true for males throughout their lives, whereas germline stem cells in females may disappear or at least acquire a large number of mutations by around middle-age. Germline stem cells for males may also have the most pristine mitochondrial DNA. While mitochondria from sperm are generally not passed on to the next generation, mtDNA mutations lead to male infertility[cxii]. Spermatogonial stem cells may

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require pristine mtDNA to produce healthy sperm, and thus mtDNA repair or selection in the former cells may be increased over somatic cells.

Although somatic mutations do build up in the mitochondrial genomes of humans with age, the overall tissue burden of mtDNA mutations may be low[cxiii]. Mutations were once estimated to affect only ~32% of mtDNA molecules in 77-99-year-old humans[cxiv]. One's mutation-free, or at least relatively mutation-free, dominant mitochondrial genome sequence can perhaps be determined through sequencing many mitochondrial genomes from circulating leukocytes, buccal cells, skeletal myocytes, and cardiomyocytes, and selecting the most common sequence. (If germline stem cells for males have the most pristine mitochondrial DNA, they could be sequenced for men; either such cells would be sequenced alone, or the other aforementioned cell types would be sequenced, too.)

Some of the patient's HSCs may have one or more homoplasmic mutations, which would be passed to all of their progeny cells. Also, using multiple tissue types would give clinicians different mutational spectra in general that could help to remove other mutant sequences from consideration. However, this may be too unreliable. Preceding extensive lipofuscin removal may improve the results, as it would allow mitophagy to ensue, destroying mtDNA with deleterious mutations. Unfortunately, this still may not be good enough; most mutations may already be neutral or beneficial. Any mutations that have not been previously documented as beneficial SNPs cannot be assumed to be so, of course. If an elderly patient has a young maternal relative or a tissue sample from a maternal relative from when that relative was young, that relative's mitochondrial genome sequence could potentially be used instead.

Conplastic mice appear to be healthy, and can even be healthier than their wild-type counterparts in terms of aging[cxv]. With periodic lipofuscin removal, the latter aspect of conplasticity is unlikely to be important, but the aforementioned study does illustrate that the exact mitochondrial DNA sequence may not be important, as long as it is a similar, healthy variant. Of course, this was from development; replacing all the mtDNA in adults with a different genome could possibly cause issues, including a severe immune response. That would be manageable, but severe deficits in terms of functionality would not. This is something that may need to be tested first in a non-human primate model. Even if one's reference sequence is determined from a tissue sample taken when one is young, systemic mtDNA replacement in this manner may need to be tested in an aged non-human primate model. If both of those things must be tested in non-human primates, in both cases, replacement would be tested in chronologically older animals, some that have first undergone preventative lipofuscin removal therapy multiple times, and some that have undergone extensive lipofuscin removal at what was originally a late age. In terms of personal identity with neuronal cellular biology, that is a little less clear - and cannot be perfectly studied in non-human primate models. However, systemic mtDNA replacement with a healthy variant probably would not have any overt cognitive effects, and may be necessary for elderly patients without a suitable maternal relative or maternal relative tissue sample. The study referenced here indicated that there are cognitive differences with mtDNA variation in mice, but this was in the context of development[cxvi].

Conclusion:

Presented in this paper are ostensibly all the forms of age-related damage that must be addressed for us to achieve biological immortality. Also presented are therapeutic approaches that could be used to address them.

Eventually we will have computerized microrobots and/or nanorobots that can eliminate/preclude cancer invariably, mechanically destroy lipofuscin or use a laser to do so, repair any damaged and mutated DNA (as well as correct any epigenetic drift), and repair structural dilapidation.

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