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Concept Paper

Toward the Systemic Replacement of Mitochondrial DNA with Somatic Mutations

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Abstract: The accumulation of lipofuscin, i.e., indigestible intracellular debris, might be the main cause of age-related diseases that we see today. However, without being able to replace mutated mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) that has suffered epigenetic drift, damage, and mutations, we will still eventually succumb to aging. Thus, we must save copies of mitochondrial and nuclear DNA at as young an age as possible, or at least from cell types with the lowest rates of mutation. 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. A strategy for doing so is described herein.

Keywords: mitochondrial DNA mutations; nuclear DNA epigenetic drift; nuclear DNA damage; nuclear DNA mutations; tissue-resident macrophage replacement; secretory autophagy; arrestin domain containing protein 1 [ARRDC1]-mediated microvesicles (ARMMs); *Cryptococcus neoformans*

Introduction:

I recently described an approach for systemically removing lipofuscin [1]. It relies on tissue-resident macrophage (TRM) replacement from edited HSCs that have been transplanted using non-genotoxic conditioning [2]. 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 [3], and mutations [4] using TRM replacement in combination with whole-body induced cell turnover (WICT) [5]. 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. The only cells that will definitely require mtDNA replacement, if it is necessary before the implementation of the TRM/WICT approach, are stem cells and long-lived, post-mitotic cells. However, a number of non-stem cells in renewing tissues may also have become reversibly senescent as a result of mtDNA mutations. Thus, a non-specific treatment may be of use.

We must sequence our mitochondrial and nuclear genomes at as early an age as possible. For those who are currently elderly, harvesting DNA from tissues with low mutation rates, e.g., germline stem cells, might be necessary. Mitochondrial DNA heteroplasmy may need to be taken into account, as well.

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 [6] may help stave off these issues.

Strategies for Replacing Somatic Mutated mtDNA:

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) [7,8]. Self- or trans-replicating RNA [9,10] can also be exported, which would encode TFAM to increase mtDNA copy number in target cells [11], 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 [12,13].

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 [14], 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-1 α , 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 mitochondria⁷; fusion with recipient cell plasma membranes may occur⁸. 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 [15,16]. 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 [17] can be enacted, wherein it is taken up into the nucleus of target cells [18], 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 [19]. 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 [20,21] could also generate one effector progeny cell that overexpresses Drp1 and then forms tunneling nanotubes with the cells around it, allowing for the transfer of multiple mitochondria and replicating RNA [22–24].

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 [25,26]. LLO would be activated at endosomal pH and could potentially help facilitate escape of the mitochondria from their encapsulating endosomes [27]. Replicating RNA vectors in viral capsids, e.g., a picornaviral capsid, would also be released after lysis.

Partial 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 [28]. A strategy called Mr PB [29] 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 [30]. *C. neoformans* cells have around 35 mitochondria [31]. 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 [32]. 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 [33]. 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 listeriolysin O could allow it to invade a wide variety of cell types [25,26]. 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.

Conclusion:

At some point, mtDNA mutations will need to be addressed. Discussed in this paper 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 [34]. Also, an artificial vector called MITO-Porter can fuse with human mitochondria [35]. There are immunogenicity concerns that will need to be addressed, but this is likely not an insurmountable issue.

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 Yamanaka factor expression may be enough to deal with that possible issue [36]. In addition to resetting the epigenetic signature of a cell, partial reprogramming can also enhance DNA damage repair. Edited TRMs could secrete Yamanaka factor RNA or package a cytoplasmic DNA plasmid into a transfer vector for long-term, small molecule-inducible expression in the relevant cell types [17,18]. Otherwise, the TRM/WICT strategy would be necessary for this potential issue as well.

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