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The road to rack and ruin: selecting deleterious mitochondrial DNA variants

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Mitochondria constitute the major energy-producing compartment of the eukaryotic cell. These organelles contain many molecules of DNA that contribute only a handful of proteins required for energy production. Mutations in the DNA of mitochondria were identified as a cause of human disease a quarter of a century ago, and they have subsequently been implicated in ageing. The process whereby deleterious variants come to dominate a cell, tissue or human is the subject of debate. It is likely to involve multiple, often competing, factors, as selection pressures on mitochondrial DNA can be both indirect and intermittent, and are subjected to rapid change. Here, we assess the different models and the prospects for preventing the accumulation of deleterious mitochondrial DNA variants with time.

1. Introduction

Mitochondria were once free-living organisms (α-proteobacteria) that were successfully co-opted by a host cell, which thereby gained the capacity to produce energy more efficiently. Over the course of evolution, the original 'mitochondrial' genome has been reduced in size owing to redundancy and the transfer of many genes to the nucleus. In humans and other mammals, mitochondrial DNA (mtDNA) now encodes only 13 proteins and 24 RNA elements (two ribosomal and 22 transfer RNAs) necessary for their translation. Mutations in any of these genes can lead to loss of function and disease owing to energy insufficiency and possibly increased oxygen radical formation.

Mitochondrial DNA is inherited through the maternal line in most plants and animals, including all mammals. Mitochondrial DNA is also quite unlike nuclear DNA in that there are typically a thousand or more copies present per cell. Thus, mutant mtDNA abundance can range from a level below one in 1000 molecules to greater than 99% of the total. With so many copies of mtDNA dispersed across many organelles, one might naively expect deleterious mutations to be selected against continuously, as would be the case for a mutant bacterium growing among a population of fully functional microbes. However, the discovery of deletions of substantial portions of the mitochondrial genome in humans with mitochondrial diseases in 1988 demonstrated that such simple 'purification' of the intracellular mitochondrial population was not always the case [1]. Moreover, it was shown later that mutant mtDNA molecules accumulate during the process of ageing [2].

At the heart of understanding the complex ways in which mutant mtDNAs arise, multiply and play roles in ageing and disease, it is important to recognize the processes of mutation and selection operating on mtDNAs. Mutant mtDNAs comprise not only deletions but also point mutations, with most of the disease-causing mutations being located in the 22 transfer RNA genes scattered throughout the mitochondrial genome. Although the entity on which selection ultimately acts is the individual mtDNA molecule, the dynamics of selection are complicated by the facts that: (i) there are typically thousands of mtDNA molecules per cell; (ii) these mtDNA molecules are distributed among many organelles, which undergo processes of duplication and removal

that operate independently of cellular mitosis; and (iii) the mitochondria are dynamic entities owing to organelle fusion and fission. Hence, the equivalence between the individual present-day mitochondrion and its distant microbial ancestor is not clear cut. How defective mtDNA variants become fixed in somatic cells is the focus of this article.

When satellite cells of muscle derived from patients with deleted mtDNAs are cultured in the laboratory the level of mutant mtDNA declines rapidly [3]. Thus, it has been known since 1989 that whereas deleted mtDNAs accumulate in post-mitotic cells and tissues, they are strongly selected against in dividing cells. In contrast, pathological mtDNA point mutations persist in cell culture. This distinction is recapitulated during ageing where deletions accumulate in post-mitotic tissues such as muscle, heart and brain, whereas it is point mutations that accumulate with age in dividing somatic cells (e.g. colonic crypts) [4]. These findings suggest deletions of mtDNA compromise cellular proliferation to some degree, whereas those point mutations that accumulate in dividing cells clearly cannot restrict the host cell's growth rate to any great extent (although some functional changes have been described) [5]. As dividing cells need to double their mitochondrial mass during every cell cycle this might be the process that is restrained when the organelles harbour partially deleted mtDNAs, although, as elaborated below, this need not be wholly related to energy insufficiency.

The primary purpose of mitochondria is energy production, and there is no question that mutant mtDNAs cause respiratory insufficiency and thereby disease; indeed, mtDNA loss is lethal to multicellular organisms [6]. Notwithstanding the vital role of respiration, it is possible for cells to grow, indeed, proliferate rapidly, without this function. This is true not only of yeasts, which helpfully produce alcohol for human consumption in the absence of oxygen, but also human cells (lacking mtDNA) grown in the laboratory [7]. Moreover, a well-known property of cancer cells is the so-called Warburg effect, with proliferating cells shifting ATP production from mitochondrial oxidative processes to glycolysis. These extreme situations illustrate how selection pressures on mtDNA vary from unforgiving to relaxed. Therefore, we should be wary of models of mtDNA selection in dividing cells that rely solely on energy capacity as the driver of selection. Selection is crucially measured in terms of quantitative effects on the relative numbers of the different mtDNA genotypes. Thus, it is noteworthy that mathematical models of various hypotheses to explain the dynamics of mtDNA mutations, especially deletions, revealed some serious challenges in explaining how mtDNA mutations might accumulate [8,9].

2. Hitch-hiking deletions of mitochondrial DNA

Any loss-of-function mutation in mtDNA, no matter how severe, can persist in a cell or tissue if it coexists with wild-type mtDNA copies (hitch-hiking). This in essence is why deleterious mtDNA mutants can cause disease, as they would be lost early in embryogenesis in the absence of accompanying wild-type molecules. Deletions of mtDNA are unusual in that they have a unique additional method of hitch-hiking: tandem partial duplications [10]. That is, they comprise one complete wild-type genome and a second partially deleted genome in a single molecule of mtDNA [11]. In a sense, these stowaways are preferable to deletion monomers, because even if they were to reach 100% of the total, then the cell would retain the equivalent of 50% wild-type molecules (in terms of DNA content). However, partial duplications are more dangerous than deletions in that deletions can (at least in theory) be sorted into mitochondria separate from those with wild-type mtDNA. Partial duplications also present problems in the laboratory where judicious selection of restriction enzymes is needed to distinguish them from partial deletions of mtDNA, and polymerase chain reaction- (PCR-) based methods cannot discriminate between the two; hence, the reports of mtDNA 'deletions' in aged tissue samples [12,13] might instead be about partial duplications of mtDNA. PCR-based methods have another weakness, they are not quantitative for partially deleted mtDNAs, as the products are smaller than those of the wild-type mtDNA, and PCR markedly favours short over long products [14]. Thus, the dramatic examples of partially deleted (or duplicated) mtDNAs in aged and diseased tissues should be viewed with this fact in mind. Notwithstanding this caveat, the detection of mutant mtDNAs correlates well with cytochrome oxidase negative fibres, demonstrating that they are sufficiently abundant to cause loss of mitochondrial function. Moreover, different cell types and tissues clearly carry different types of mutant mtDNA, not only rearrangements, for which estimating the mutant load is straightforward [4,12,13].

3. Selection of mitochondrial DNAs via a direct replicative advantage

Partial duplications and other tandem iterations of human mtDNA are the clearest example of selection based on replicative advantage known. To understand this concept, it is helpful to consider first the stark evidence from yeasts.

Because yeasts grow in the presence or absence of oxygen, mitochondrial energy production and mtDNA are readily dispensable in these organisms. Accordingly, yeast mutants lacking the ability to respire occur spontaneously at relatively high frequency. One of the mutant forms is the rho minus (ρ^-) strain that has rearranged mtDNAs. It is found that ρ^- mtDNAs comprise a tandemly repeated fragment of the mitochondrial genome that can be as few as tens of nucleotides. The residual piece of yeast mtDNA must of course contain a sequence that can permit the initiation of replication, i.e. act as a replication origin, and because the mass of ρ^- mtDNA is similar to that found in cells containing wild-type mtDNA, these origins are much more prevalent in ρ^- strains, reflecting the enormous selective advantage of the mutant mtDNA, termed hypersuppressiveness [15]. Crucially, the equivalent phenomenon was demonstrated for human mtDNA when it was observed that not only can partially duplicated mtDNAs with additional origins of replication be selected in patients, but also in cell culture such mtDNAs can mutate further to give partially triplicated mtDNA. This in turn has a selective advantage over partially duplicated mtDNA, even though it is associated with a much more marked impairment of respiratory capacity [16]. These findings provide compelling evidence for the hypothesis that deleterious variants of human, as well as yeast, mtDNAs can be actively selected by a 'selfish' mechanism based on DNA replication efficiency. Recently, moreover, recurrent point mutations of mtDNA have been detected in specific tissues of healthy human subjects that are likely the result of replication advantage [17].

This is all very well; additional or enhanced origins of replication are an obvious means of conferring a selective advantage, but what of the many pathological point mutations of mtDNA dispersed throughout the genome? Surely the 100–200 pathological mtDNA mutations (http://www.mitomap.org) cannot all create origins of replication? Well, this is not as fanciful as one might at first imagine. Although there is a long-standing designated origin of replication (O_H) in the major non-coding region (NCR)[1], there is also evidence of initiation of DNA replication across a region of several kilobases adjacent to the NCR in mammals [18], and this initiation zone seems to peter out, rather than have a strict boundary. Indeed, in birds, initiation has been detected to occur in fragments throughout the mitochondrial genome [19]. Thus, it appears, replication can initiate almost anywhere in the vertebrate mitochondrial genome, although the NCR continues to play at least one essential role in the process of replication, namely termination [20]. Therefore, it follows that a mutation anywhere in the genome might increase the probability of initiation of replication and thereby confer a direct replicative advantage. That said, origin-firing is only the most obvious means of conferring a replicative advantage on a DNA molecule and subtler mechanisms might be more important numerically for mitochondrial DNA disorders. One such, that of reduced replication pausing, is discussed below in connection with protein factors that might influence the selection of deleterious mtDNA variants.

Partial deletions of mtDNA, unlike duplications, have no additional material to expand the number of origins. The most parsimonious idea is that their replication cycle is shorter than their full-length counterparts, allowing both faster and more subsequent rounds of replication [21]. However, this might be only part of the explanation (see also [8]). If mtDNA is either always membrane attached, as suggested by its resistance to alkaline stripping [22], or solely during replication [23], then DNA architecture and topology for an 11 kb molecule will be different from a 16 kb molecule. In particular, the amount of twisting is likely to be greater for the shorter mtDNA. This might, for example, cause an increase in single-stranded DNA extrusion enabling 'licensing factors' to bind and promote origin firing. Alternatively, a mutation might alter the balance between the different mechanisms of mtDNA replication [24] to its advantage. Another intriguing possibility is the recent suggestion by Kowald & Kirkwood [25] that there is a feedback mechanism regulating mtDNA replication, which is compromised in those mtDNA deletions that have been reported to undergo clonal expansion in aged animal tissues. Finally, although almost nothing is known about the regulation of mtDNA degradation, it should be recognized that a mtDNA variant could confer an advantage by decreasing the probability of degradation, as opposed to enhancing replication.

4. Forces counteracting selfish selection of deleterious mtDNA variants

Clearly, if there were no mechanisms to recognize the quality of the products of particular mtDNAs then the organelles would be rapidly overrun with selfish mutations that increased mtDNA numbers at the expense of the function of its products. Originally, it was thought that (macro) autophagy randomly selected part of the cytoplasm including organelles for recycling under conditions of acute energy need. However, it was later discovered that preferential targeting of defective mitochondria (mitophagy) can occur [26]. This observation has further been incorporated into an evolutionary explanation for the role of the fusion–fission cycle in animal mitochondria, whereby fusion aids metabolic control but creates vulnerability to clonal expansion of mutant mtDNA, whereas fission creates the context for selective destruction through mitophagy of the defective mtDNAs [27]. Targeting whole mitochondria to lysosomes (mitophagy) has been proposed as a key component of the mitochondrial quality control (MQC) system. More recently, it has been recognized that the range of half-lives of mitochondrial proteins spans two orders of magnitude, indicating a hitherto unrecognized level of discrimination of mitochondrial protein turnover [28]. Furthermore, it has been shown that respiratory complex I can be degraded via mitophagy, in neuronal stem cells carrying pathological (m.3243A > G) mtDNAs, while sparing other components of the respiratory chain, yet this selectivity did not rid the cells of mutant mtDNA [29]. Hence, the extent to which mtDNA turnover is regulated by mitophagy is not known. Nevertheless, several reports hint that mitochondrial dynamics and quality control pathways can impact mtDNA segregation. Decreased expression of the mitochondrial fission protein, Drp1, advantaged mutant mtDNA in one study [30], whereas rapamycin (an activator of autophagy) accelerated segregation to wild-type mtDNA [31]. Finally, greatly elevated Parkin (an activator of mitophagy) expression selected against one of two types of deleterious mutant mtDNA [32].

Whatever the physiological mechanism(s), there is striking evidence for purifying selection of mtDNA in the mouse germline [33]. That is, deleterious variants are less prevalent than expected were mutations inherited without any form of phenotypic selection. This is also likely to be the case in humans, based on the fact that a sizeable majority of pathological point mutations are found in the transfer RNA genes whose products are much more diffusible than (membrane) proteins and therefore more difficult to link to their respective mtDNA of origin upon organelle fusion [27].

5. Stable heteroplasmy: the persistence of a fixed proportion of mutant and wild-type mtDNA

One of the most remarkable findings arising from the study of human cells carrying mixtures of mutant and wild-type mtDNA is the long-term persistence of fixed proportions of two genotypes (stable heteroplasmy) [34]. What is more, two sister cells cloned at the same time may contain quite different mutant loads, and severe respiratory incapacity is no obstacle to the maintenance of high levels of mutant mtDNA [35]. These observations suggest that the mitochondrial genotype might be 'locked' in some way and all variants are excluded from selection—whether good or bad. This implies interconnections between nucleoids, which might for example be ATAD3-dependent (see [36] and the following section). Nevertheless, physically, nucleoids rarely interact [37], perhaps to prevent defective mtDNA variants piggybacking on nucleoids containing wild-type mtDNA. The system maintaining stable heteroplasmy may fail, or be suspended in some circumstances [38], and while the consequences can be devastating for the affected individuals, it does so only rarely, and so is advantageous to the human population as a whole. An imperfect

system that allows some deleterious variants to escape and accumulate during ageing is also only of concern to the individual and not to the survival of the species.

6. The machinery of mtDNA selection

If we are to intervene and prevent or slow the accumulation of mutant mtDNA molecules, then it would be helpful to elucidate the factors that impinge on this process. Currently, there are few hard data on the proteins that influence the segregation of mutant and wild-type mtDNA. We know from work in yeasts that hypersuppressiveness depends on the ability of the organism to resolve recombination (Holliday) junctions. The key protein is a Holliday junction resolvase (HJR), CCE1 [39], as it processes the highly interconnected mtDNAs that form in ρ^- strains [40]. In the absence of the enzyme, the network of mtDNA cannot be untangled and distributed evenly to daughter cells. A human protein homologous to CCE1 (TEFM) has been identified; however, it lacks a key catalytic residue of HJRs and instead appears to be involved primarily in mitochondrial transcription [41]. Nevertheless, a partner protein, post-translational modification or cofactor might enable TEFM to resolve recombination junctions. Intramolecular DNA recombination in mitochondria depends on the host cell or tissue expressing the relevant apparatus and targeting it to the organelles, but intermolecular mtDNA recombination requires the physical interaction of discrete molecules, and this occurred only rarely in one human cell line studied [37]. Moreover, in a laboratory strain of mice there was no evidence of intermolecular mtDNA recombination in the germ-line over the course of 50 generations [42].

An ability to resolve recombination junctions might be required at the end of the replication cycle as found for bacteria [43], and be necessary to regulate the networks of mtDNA molecules that form in adult human heart [44]. It is unlikely that mitochondria can create such DNA structures and yet be unable to resolve or otherwise process them. Whatever means they use constitutes a DNA recombination machinery in mitochondria. There is some empirical evidence for DNA recombination occurring in mitochondria from cell culture studies. Cells containing exclusively partially duplicated mtDNA (at the limits of detection) were replaced by wild-type mtDNA, presumably by intramolecular recombination in one study [16]. Moreover, artificially introducing two mitochondrial genotypes into one human cell line resulted in recombination between the two types of mtDNA, albeit at low frequency [45]. It has long been noted that some patients contain mixtures of deleted, wild-type and partial duplicated mtDNA and that these are inter-convertible via homologous recombination [46]. Yeast HJR CCE1, expressed in human cells, is capable of converting tandem partial triplications and duplications to wild-type mtDNA molecules [47], thereby demonstrating that this capacity would help combat this type of mtDNA rearrangement. Tracking down the endogenous proteins that form the apparatus of mitochondrial DNA recombination might prove far from straightforward. For example, animal mitochondria contain an RNase P that has been cobbled together from three proteins that have other functions [48]. Nevertheless, two members of the mTERF family have been linked to termination of mtDNA replication [49], so they represent a good place to start.

A potential link to DNA replication has been found for the most common point mutation of mtDNA (m.3243G > A) via the founding member of the mTERF family. mTERF binds to mtDNA and can cause replication pausing at a tridecamer sequence that includes nucleotide position 3243. The pathological mutation decreases mTERF binding *in vitro* [50], and so molecules with the mutation will not be subjected to replication pausing, thereby conferring a replicative advantage on the molecules carrying the mutation [51]. Even if many mtDNA mutations turn out to be fixed by random processes, this mechanism might explain why m.3243A > G is the most common pathological point mutation.

The machinery that mediates the physical segregation of mtDNA might well influence the selection of different mitochondrial genotypes. Two proteins have been implicated in this process, but it remains a largely unexplored area of mitochondrial biology. One of the proteins, ATAD3, is particularly intriguing, as it was found in enriched preparations of mtDNA and its amino-terminal part has a remarkable preference for binding to DNA structures with a triple-stranded section (D-loop) [22]. Furthermore, gene silencing of ATAD3 induces topological changes in mtDNA, and there appears to be an inverse correlation between nucleoid size and ATAD3 abundance [52]. ATAD3 has its fingers in several pies, as it also physically interacts with mitochondrial ribosomes, based on affinity purification studies [36], and so could play a pivotal role in maintaining a link between mitochondrial translation products and the parent mtDNA molecule [53]. The accessory subunit of mitochondrial DNA polymerase gamma, POLG2, has long been known to have DNA binding properties that are superfluous to its role as a processivity factor [54] and modulating its expression in human cultured cells indicates that it can alter the DNA content of mitochondrial nucleoids [55].

7. Alternative selection mechanisms for pathological variants and clonal expansion during ageing

Other explanations have been advanced to explain the fixation of mutant mtDNA. The simplest of all, and yet one of the most powerful, is random drift, and this model is compatible with much of the behaviour of mtDNA variants [56]. However, random drift does not explain results for short-lived animals (e.g. rats) [9]. Nor can it be readily reconciled to the different clinical features associated with m.3243A > G in different families [57,58]. Furthermore, it is flatly contradicted by the systematic biases in mtDNA segregation reported in human cultured cells [35], and in mouse solid tissues [59].

Free radical production is concentrated in the mitochondrial compartment of the cell, as illustrated by the severe pathologies resulting from ablation of the mitochondrial [60], but not the cytosolic [61], form of superoxide dismutase. However, to what extent a 'vicious cycle' of defective mtDNA, impaired mitochondrial respiration with increased oxygen radical formation, followed by further DNA mutation, contributes to loss of mitochondrial function is debated, as is the role of mitophagy in clearing deleterious mtDNAs [62,63]. Neither appears wholly satisfactory, as a vicious cycle cannot explain clonal expansion of specific mutant variants [4,12,13], and mitophagy clearly cannot meet the challenge of specifically disposing of disease-causing mutant mtDNAs, or again clonal expansion during ageing, although one can of course posit that it is an age-related decline in mitophagy that accounts for the latter phenomenon.

The contribution of reactive oxygen species (ROS) tends to focus on its potential for damage, but its role in mtDNA segregation might reflect its ability to act as a signal transducer [64]. A direct link between ROS and mtDNA replication has been established in yeasts [65], although it involves another protein that lacks an obvious homologue in humans.

Another idea, called 'survival of the slowest', notes that the fate of a mutant depends on its rate of degradation as well as replication, and proposes that defective mitochondria which are metabolically less active generate less oxidative damage in their membranes and for this reason are degraded less frequently than wild-type [66]. Although this hypothesis appears hard to reconcile with mitochondrial dynamics, because evidence shows that dysfunctional mitochondria are preferentially degraded [26,67], instead of being spared, compromised MQC might permit it.

8. Concluding remarks

Here, we have concentrated on the properties of mtDNA itself and how proteins that interact with it directly might favour the selection of deleterious variants. However, a comprehensive answer to this question will require this information to be combined with the other models. Thus, the explanation for the fixation of mutant mtDNAs in disease and ageing is expected to feature replicative advantage, mitochondrial dynamics and protein turnover, free radical production and signalling, random genetic drift and its alter ego stable heteroplasmy. Current research is providing us with a fuller understanding of the contributors to each of these processes. The overall balance between these often competing forces ultimately determines the burden of mutant mtDNA that each of us carries. The challenge outstanding is to clarify the extent to which it determines our fitness and longevity.

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Endnote

[1]Whose precise location is disputed; in humans, nucleotide positions 55, 111, 168, 191 and 300 are all candidate initiation sites.

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