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Cause or casualty: The role of mitochondrial DNA in aging and age-associated disease

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ABSTRACT

The mitochondrial genome (mtDNA) represents a tiny fraction of the whole genome, comprising just 16.6 kilobases encoding 37 genes involved in oxidative phosphorylation and the mitochondrial translation machinery. Despite its small size, much interest has developed in recent years regarding the role of mtDNA as a determinant of both aging and age-associated diseases. A number of studies have presented compelling evidence for key roles of mtDNA in age-related pathology, although many are correlative rather than demonstrating cause. In this review we will evaluate the evidence supporting and opposing a role for mtDNA in age-associated functional declines and diseases. We provide an overview of mtDNA biology, damage and repair as well as the influence of mitochondrial haplogroups, epigenetics and maternal inheritance in aging and longevity.

1. Introduction

Complex multicellular life owes its origin to the endosymbiosis of two primordial prokaryotes [1,2]. The endosymbiont became the mitochondrion, providing the eukaryotic cell with abundant ATP through oxidative phosphorylation. While most mitochondrial proteins are now encoded in the nuclear genome, mitochondria have retained a portion of their own genome, the mitochondrial DNA (mtDNA). This is not only a legacy of their independent origin but seems to be essential for coupling oxidative phosphorylation to ATP synthesis [3]. In most metazoans, the mitochondrial genome has been compacted to a double-stranded circular molecule 11–28 kilobases with few intergenomic regions, no introns and overlap between some of the coding regions [4,5]. Mammalian mitochondrial genomes are quite typical and consist of 16.6 kilobases which encode thirteen respiratory complex subunits as well as two ribosomal and twenty-two transfer RNA [6]. The thirteen protein-coding genes retained in the mtDNA encode subunits of complexes I, III, IV and V, which are the four complexes which pump protons and are partially imbedded in the inner mitochondrial membrane (Fig. 1). High GC content of the genes, hydrophobicity of the proteins and the ability to modulate mitochondria individually seem to be the driving forces behind retention of these genes in the mtDNA [7].

The past century has seen increasing understanding of how mitochondria not only enable our existence but also how disruption of their function may play an important role in age-related decline. Mitochondrial dysfunction has long been recognized as one of the hallmarks of aging [8] and theorized to play a causative role in aging pathology [9,10]. It is well established that mitochondrial function deteriorates with age in different tissues such as heart [11], muscle [12] and liver [13]. However, the proximal cause of the decline in mitochondrial function remains elusive.

There are several roles which mtDNA may play in the pathology of aging as well as several age-related diseases which will be discussed in this review. These roles include changes in mtDNA copy number, epigenetic modifications and mtDNA mutations which have been linked with the age-associated functional decline of both the mitochondria and organism as a whole. In addition, alterations in the mtDNA genome have been linked with altered susceptibility to a wide range of age-associated diseases including Alzheimer's disease, Parkinson's disease, sarcopenia, heart failure and cancer. Variations in the mtDNA genome may also contribute to differences in lifespan between individuals as well as between different animal species. However, while there is a large body of correlative data linking mtDNA to aging and age-associated disease, demonstration of a direct role is lacking. This review will provide a breakdown of the relative strengths and weaknesses of the data supporting and opposing a role of mtDNA in the aging process.

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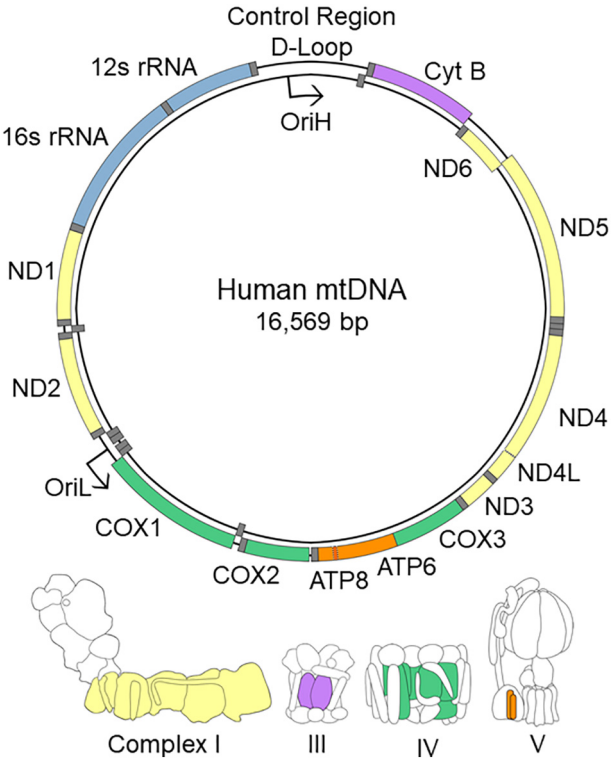

Fig. 1. The human mitochondrial genome consists of 16,569 bp encoding 13 respiratory complex subunits, 2 ribosomal RNAs and 22 transfer RNAs. The encoded proteins are all localized in the inner mitochondrial membrane and are some of the core subunits of respiratory complexes I, III, IV and V.

2. Mitochondrial DNA structure and organization

The mammalian mitochondrial genome is a double-stranded supercoiled circular molecule of about 16.6 kb. It is composed of a heavy and light strand (based on the proportion of higher molecular weight nucleotides). The heavy strand contains the 2 rRNA sequences, 14 of the 22 mitochondrial tRNAs and all the protein-encoding genes with the exception of ND6 (Fig. 1). There is also a large non-coding region of about 1 kb that contains regulatory elements for the initiation and termination of transcription of both strands. The D-Loop (displacement loop) or control region is within this non-coding region and contains the OriH (heavy strand origin of replication), where replication is initiated. There is a second origin of replication in the light strand, the OriL [14] (Fig. 1).

The mtDNA genome is packaged into nucleoids, which are dynamic structures that are normally formed by the mtDNA genome and the proteins involved in its replication and transcription. Although this structure provides a level of organization, it is not as tightly packed or organized as nuclear DNA (nDNA). The nucleoid proteins include polymerase-γ (PolG) (the mtDNA replicative polymerase), mtSSB (single stranded binding protein), twinkle (DNA helicase), Phb (prohibitin) and TFAM (transcription factor regulates mtDNA) [15]. The structure and organization of the mtDNA nucleoid is an emerging area of research with new proteins frequently being identified. The nucleoids are located on the matrix (interior) side of the inner mitochondrial membrane in close proximity to the oxidative phosphorylation system (Fig. 2).

3. Mitochondrial DNA age-associated changes

Until recently, the study of mtDNA damage had been challenging due to the difficulty of detecting variations present in low abundance in any particular cell or tissue. In contrast to nuclear DNA, which is present in only two copies per cell, mtDNA can be present in hundreds to thousands of copies, and these do not always contain the same sequence. Mutated mtDNA can coexist with wild-type mtDNA in a condition called heteroplasmy. The development of next generation sequencing or ultra-deep sequencing has helped to characterize both mtDNA heteroplasmicity [16,17] and the different types of damage and mutations that can be present in disease and aging. Some recent studies have suggested that heteroplasmy may serve a protective role since the ratio of mutant to wild-type DNA is key to the development of disease. This ratio is the determinant of the so called “Threshold Effect”; pathogenic mutations need to reach at least 70 to 90% of heteroplasmy to have a detrimental effect on mitochondrial function [18]. This threshold effect has been mimicked in a *Drosophila* model by manipulating a mitochondrial ribosomal gene, achieving different pathological phenotypic manifestations depending on the mutation dosage [19]. Therefore it is suggested that for mtDNA damage to produce detrimental effects, the mutation levels must reach high frequency within each cell, a process that would take many years to occur [20].

To examine this process in human aging, Christiansen and colleagues [21] along with Lee and colleagues [22] found declines in mtDNA copy number in whole blood to occur with age and lower mtDNA copy number to be associated with poorer health outcomes. This work was followed up Gu and colleagues who used available whole-genome sequencing data from peripheral blood mononuclear cells from more than 1500 women from two British cohorts. They found that aged individuals accumulated higher levels of mutant mtDNA than younger women and that these were associated with the appearance of age-related blood physiological markers [23]. A very recent study has found a positive correlation in elderly subjects between the abundance of the pathological A3243G mtDNA mutation and reduced strength, cognition, metabolism and cardiovascular fitness [24]. They also reported that the patients with the highest mutation burden presented a higher risk of dementia and stroke mortality [24].

The D-Loop is the region of mtDNA most prone to genome instability [25]. This is likely because it is the most exposed region as the primary replication initiation site. Studies have shown this region accrues high levels of DNA point mutations with age in a range of tissues including the brain, blood, skin, muscle and heart [26–29]. One of the most common mutations is a T414G transversion which has been found in up to 50% of mtDNA molecules of half of the individuals tested above 65 years of age [27]. A high frequency of mtDNA deletions in the D-Loop has also been observed in the central nervous system of 27-month-old rats and mice when compared to 7-month-old animals [30,31].

Large mtDNA deletions have also been detected in human mtDNA during aging. A 5 kb deletion of mtDNA, which is usually associated with mitochondrial disease, has been reported to accumulate in the heart, brain and muscle of aging humans [32,33]. There is evidence that mtDNA defects can clonally expand and create mosaic phenotypes in some tissues. Ragged red fibers (RRF) are muscles fibers with mitochondria deficient for cytochrome *c* oxidase (respiratory complex IV) activity, that can result from mutations or deletions in either mtDNA or nDNA. These fibers accumulate with age and are thought to be a contributing factor for sarcopenia (discussed in Section 8.3) [33]. Further supporting the role of clonal expansion, Khrapko and colleagues found clonal mutant mtDNA that had expanded from an initial single mtDNA molecule in human buccal epithelium and cardiac muscle [29]. Similarly, Turnbull and colleagues reported a clonal expansion of mtDNA mutations in the colorectal epithelium with age, while the frequency of de novo mtDNA mutations did not increase, suggesting that clonal expansion is the driving force behind mitochondrial dysfunction with age [34]. Similar clonal expansion events have been observed in skin fibroblasts and blood of elderly subjects, producing an elevated load of heteroplasmic or even homoplasmic mutations [35]. In some cases, it is thought that the origin of clonal expansion could arise from stem-cell aging. It was shown within normal gastric epithelium that mtDNA mutations are present in its stem-cells which are then passed on to the

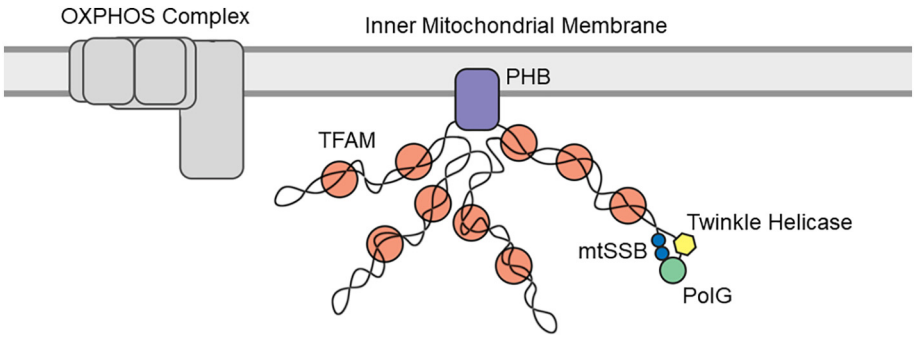

Fig. 2. The mitochondrial genome is located on the matrix side of the inner mitochondrial membrane and is packaged with numerous proteins which are still being discovered. These proteins are involved both in its replication, transcription and stability.

differentiated progeny [36].

4. Mitochondria DNA damage and repair

The mechanism of mtDNA replication is controversial and new theories have emerged in recent years. The 'Strand-Displacement model' originally proposed in 1982 argues that replication displaces the lagging stand, leaving it unprotected. This could result in low stability and highly error prone replication that could increase the risk of single-strand breaks and misreads [37]. A newer theory proposes that mtDNA replication occurs through a bootlace model which involves the formation of RNA-DNA duplexes, with RNA hybridizing to the displaced lagging DNA strand and thereby preserving genome stability [38].

Mutations in mtDNA can originate from different causes; they can be maternally inherited or result from defects in the maintenance and repair systems. Mutations can also be generated by genotoxic agents such as ROS or UV irradiation. The mtDNA repair system plays an essential role in the repair of such mutations [39] (Fig. 3). More and more similarities are being found between the mtDNA and nDNA repair systems. Proofreading activity by the PolG 3′-5′ exonuclease is one of the most important systems in the maintenance of mtDNA integrity and correction of replication errors. Until recently, PolG was thought to be the sole DNA polymerase present in mitochondria, but the protein PrimPol has been identified as possessing roles in mtDNA translesion synthesis [40]. This enzyme is not only able to elongate DNA and to synthesize new DNA primers for PolG; it is also capable of skipping and copying ahead of DNA lesions [41]. PrimPol therefore provides a new translesion synthesis mechanism that was further confirmed by Pohjoismaki and colleagues [42]. While PrimPol-deficient mice showed a metabolic disease phenotype that appeared by middle-age and was associated with mitochondrial dysfunction (Chocron, Blanco, unpublished findings), the impact of this gene on aging has not yet been evaluated.

Base excision repair (BER) is the best characterized DNA repair process in mitochondria and is the main process involved in the removal of oxidized bases such as 8-OHdG (8-hydroxydeoxyguanine). Very recently, Polymerase B (Pol Beta) has been found to be localized in mitochondria and involved in the BER mechanism [43,44]. Mismatch repair, single strand breaks and double strand breaks repair have each been reported in mitochondria DNA [14] and Polymerase Q (Pol Theta) has been suggested to have recently a role in these processes [45,46]. Some studies have shown age-related declines in BER efficiency in mouse skeletal muscle but at the same time, increases in efficiency were seen in liver and heart [47]. In rat brains, BER activity was also reported to decrease with age with a concomitant decrease in expression of both PolG and 8-OHdG glycosylase, which is one of the BER enzymes [48]. However, it is unclear whether these changes contribute to age-related changes in functionality of these tissues. In addition, such observations have been limited to rodents and more work is needed to assess whether these changes are relevant to human aging.

In addition to proof-reading errors, deficits in mitophagy have been posited as a strong contributor to the accumulation of damaged mtDNA with age. Mitophagy is the process by which damaged mitochondria are engulfed by double-membrane vesicles called autophagosomes and destroyed [49]. In the best known mitophagic pathway, dysfunctional mitochondria cannot maintain membrane potential, resulting in the stabilization of PINK1 (PTEN-induced putative kinase 1), which then recruits Parkin to initiate mitophagy dependent on ATG (autophagy-related) proteins [49]. Mitophagy plays an important role in control and removal of mtDNA damage and in dealing with non-functional mitochondria which may contain excessive mtDNA damage [50]. It has been observed that mitophagy is disrupted in pathologies such as Alzheimer's disease and other neurodegenerative disorders [51,52] and is therefore being investigated as a potential therapeutic target [53]. In a fly Huntington disease model, PINK-1 overexpression has been reported to be neuroprotective [53]. Additionally, patients with early onset parkinsonism who carry a mutated PINK-1 protein harbor an increased level of mtDNA mutations [54]. It is worth noting that lifespan extending interventions such as caloric restriction and mTOR inhibition both lead to increased autophagy and mitophagy [55]. This supports the argument that the accumulation of mtDNA mutations with aging is due primarily to failure in the mitophagic processes to clear defective mtDNA rather than changes in rates of damage accrual [56].

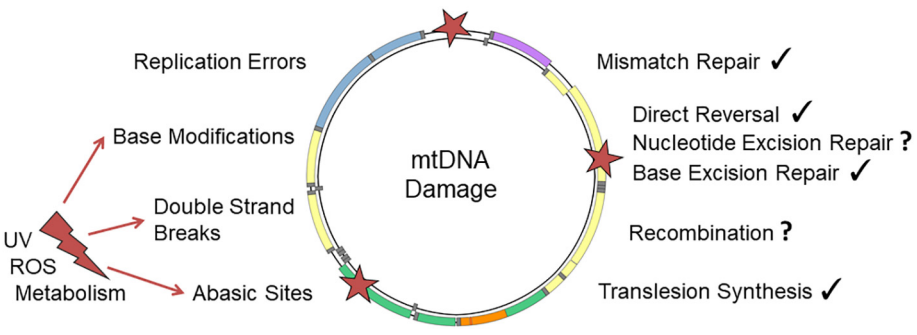

Fig. 3. Different types of damage-causing agents (ROS/UV/Metabolism) originating either from the external environment or from within the cell generate different types of damage that can each be repaired by a corresponding repair mechanism. Some of these mechanisms are unconfirmed in mitochondria and are thus depicted with a question mark (?).

Table 1
The effects on lifespan of knockdown (null), knockout (KO) and transgenic overexpression (OE) of mitochondrial oxidant scavenging and redox enzymes in *C. elegans, D. melanogaster* and *M. musculus* are summarized. In the two cases where a transgenic model was created but lifespan was not assayed, these studies are referenced.

Effects of mitochondrial redox enzyme manipulation on lifespan

	C. elegans	*D. melanogaster*	*M. musculus*
SOD2 null	Life extension [68]	Early lethality [64]	Lethal [65,66]
SOD2 KD	No effect [210]	Life reduction [211]	No effect [70]
SOD2 OE	Life extension [212]	Life extension [72]	No effect [71]
GPx4 null	–	–	Lethal [213]
GPx4 KD	–	–	Life extension [76]
GPx4 OE	–	–	–
PRX3 null	–	–	Not reported [214]
PRX3 KD	–	–	–
PRX3 OE	–	–	–
TRX2 null	No effect [215]	–	Lethal [216]
TRX2 KD	–	–	Life reduction [60]
TRX2 OE	–	–	–
TXNRD2 null	No effect [215]	Lethal [217]	–
TXNRD2 KD	–	–	–
TXNRD2 OE	Not reported [215]	Life extension [84]	–

5. The Mitochondrial Free Radical Theory of Aging (MFRTA)

Mitochondria are the major source of ROS production within the cell [57]. This led Denham Harman to propose the Mitochondrial Free Radical Theory of Aging (MFRTA) [9,58], which posits that ROS produced by oxidative phosphorylation damages mitochondrial proteins, membranes and DNA [9]. This was proposed to cascade into a vicious cycle where mutations result in further mitochondrial dysfunction, that in turn lead to more ROS production, and eventually result in cell senescence or death [59].

Models of gain and lack of function of mitochondrial ROS scavenging enzymes have been generated in an attempt to test this theory. The results from these models have been contradictory, especially when evaluating their effects on lifespan (Table 1). Superoxide dismutases (SODs), catalase, thioredoxins (TRX), peroxiredoxins (PRX) and glutathione peroxidases (GPx) are the major antioxidant defense mechanisms within different cellular compartments. The effects of homozygous and heterozygous knockout or transgenic overexpression of various antioxidant enzymes in mice have been reviewed by Pérez and colleagues [60]. Mitochondrial superoxide dismutase (SOD2), also known as manganese-dependent superoxide dismutase (MnSOD), reduces the highly reactive superoxide radical to hydrogen peroxide. SOD2 has been described to associate directly with respiratory complexes [61] to neutralize superoxide radicals where they are generated and is essential to the viability of many organisms, including yeast [62,63], flies [64] and mice [65,66]. In conflict with the critical role of SOD2 in removal of ROS from the mitochondria, many reports have shown limited effects of this protein on lifespan. In the nematode *Caenorhabditis elegans* deletion of any or even all of the SOD genes caused no reduction in lifespan under normal conditions (survival under oxidative stress was decreased) [67–69] and null mutations in SOD2 actually extended lifespan in these animals [68]. Mice heterozygous for a null SOD2 mutation did show increased levels of oxidative damage to both mtDNA and nDNA and had a much higher incidence of cancer. Significantly, however, both the mean and maximum lifespan of these mice was indistinguishable from wild-type and they showed no evidence of accelerated aging [70]. Conversely, overexpression of SOD2 either by itself or in combination with other antioxidant enzymes repeatedly showed no effect on lifespan in mice [60,71]. Reports on the effect of SOD2 overexpression in *Drosophila melanogaster* have been conflicting [72–74]. Manipulations of other mitochondrial antioxidants have produced similar results and argue against a causative role of mitochondrial ROS in aging [60,75,76].

An interesting exception is a study wherein catalase was ectopically expressed in the mouse mitochondria (catalase is normally found only in peroxisomes). These mice, known as mCAT mice, have been reported to live longer [77], maintain mitochondrial function into old age [78] and show a reduction in multiple age-related pathologies [79–81]. Recent studies have demonstrated that the benefits of mCAT overexpression in lifespan were related to both improved mitochondrial function and consequent attenuation of age-associated insulin resistance in muscle [78]. The ability of mitochondrial-targeted catalase to prevent age-related functional declines is curious, especially when manipulation of the mitochondria's native H_2O_2 scavenger, GPx4, has had little to no effect on lifespan [60]. In contrast, mitochondrial-targeted catalase has shown no effect on lifespan in flies [73,82]. The contribution of the mitochondrial thioredoxin system in this matter has also recently been evaluated. This system is involved in reducing the mitochondrial peroxirodoxin as well as both reducing oxidized proteins and signaling to apoptosis [83]. The rate limiting enzyme for this system is mitochondrial thioredoxin reductase (TXNRD2), which has been recently shown to be more abundant in longed-lived primate and rodent species [84]. While its role in mammalian aging is still under investigation, overexpression of TXNRD2 in flies increased median lifespan, suggesting a potential causative role in longevity [84].

While many studies focus on lifespan and pathology as endpoints, several have looked at the mtDNA for evidence as to whether or not ROS plays a major role in generating mutations. Oxidative damage can modify guanine nucleotides to 8-OHdG, which can then mis-pair with adenine and in one round of replication, lead to a TA transversion. Cytosine can also be oxidized and it is thought that this can lead to GC to AT transitions. Therefore GC to TA transversions and GC to AT transitions should be the most prevalent mutations resulting from oxidative damage [85]. Vermulst and colleagues studied the mutagenic contribution of ROS by comparing hearts derived from 26-month-old mice expressing ectopic mitochondria-targeted catalase (mCAT) and non-transgenic littermates. They found a 4-fold reduction in the mutation burden of mice expressing mCAT. In addition, they observed that GC to AT transitions were responsible for 81% of the mutation events in the aged control mice [86]. In contrast, oxidative stress is not thought to contribute greatly to age-related mtDNA mutations in *Drosophila* since only a small proportion of mutations were due to GC to TA transversions [87]. Other studies in aged human brains did not find significant changes on the mutations related to oxidative damage [88].

6. Mitochondrial DNA and progeria models

The majority of data linking mtDNA to aging is correlative. The closest evidence to a direct link between aging and mtDNA damage may be from progeria models. Here mutations which accelerate accumulation of mtDNA damage have been reported to produce aging-like phenotypes.

Goran-Larsson and colleagues designed the well-known mtDNA "mutator" mouse which contains a defect in the proof reading mtDNA Polymerase gamma exonuclease ($PolG^{mut/mut}$) [89]. Prolla and colleagues also designed a mouse with a mutation in the same gene which produced deficits in proof reading capacity [90]. These mice possess a number of phenotypes which mimic aging, including rapid mortality; $PolG^{mut/mut}$ mice have a median lifespan of approximately 13 months. They also exhibit hair graying, hair loss, weight loss, reduced subcutaneous fat and kyphosis (curvature of the spine), all by approximately 9 months of age [89,90]. However, other researchers have noted that $PolG^{mut/mut}$ mice harbor somatic point mutations an order of magnitude higher than what has been observed in aged human tissues [91]. In addition, the age-like phenotypes displayed by the $PolG^{mut/mut}$ mice include many disorders and traits which are not seen during 'normal aging' in mice, which calls into question whether the $PolG^{mut/mut}$ mouse does indeed represent a model of accelerated aging [92].

Other findings which argue against mtDNA damage causing accelerated aging include a study reporting that mice which are heterozygous for the PolG proofreading mutation continue to have over one order of magnitude higher levels of mitochondria DNA mutations than wild-type mice and yet exhibit a phenotype and lifespan little different than wild-type [86]. Moreover, aging wild-type mice also present a higher level of mutations than aged humans. This suggests that the multi-organ failure of the homozygous mutator mice at the early age of 5 months may be caused not by premature aging but by energetic deficiency. Evidence for energetic deficiency includes heart hypertrophy and mosaic deficiencies for activity of complexes II and IV in skeletal muscle [89].

The PolG mutator mouse also presents evidence against the vicious cycle proposed by MFRTA. While they have an increased load of mtDNA mutations and deletions, they do not show higher levels of oxidative damage in proteins, lipids or DNA [93]. Heart-specific expression of the PolG mutation produced similar results that did not increase with age [94]. In addition, very recent data from the same group has reported that BER deficiency alone or combined with oxidative stress did not increase mtDNA mutation load in mice [95].

In contrast to the findings in mice there is limited human study evidence that mutation of mtDNA proofreading can accelerate onset of aging or age-related disease. There are several reported human progeroid mutations associated with nDNA proofreading and structural maintenance however there are no known human progeroid syndromes which impact mtDNA. Some studies have reported alterations in mitochondrial morphology and function under Hutchinson-Gilford progeria but no changes in mtDNA have been reported in these patients [96]. Mutation of PolG in humans is rare, there is one report of a dominant familial mutation in this gene among humans. This mutation produces mitochondrial dysfunction and progressive muscle weakness in adulthood but is not reported to produce progeria [97].

7. Mitochondrial DNA sequence and longevity: haplogroups and comparative biology

In all animals, the mitochondrial genome is significantly smaller than the nuclear genome and consequently has been sequenced in many more species. The human mitochondrial genome was sequenced [4] more than two decades before the nuclear genome and many studies have been done looking for associations between sequence polymorphisms and both disease and lifespan.

7.1. Mitochondrial haplogroups

A haplotype is a group of alleles that are inherited from a single parent, such as the patrilineal inheritance of the Y-chromosome and the matrilineal inheritance of mtDNA. Neither chromosome is able to recombine and is thus passed along relatively unchanged for generations, with variations only introduced through mutations. Such mutations enable ancestry to be traced and haplogroups are defined as similar haplotypes which have diverged from a common ancestor. The identification of human mitochondrial haplogroups has revealed geographic associations and patterns of human migration and ancestry [98,99]. Several mtDNA haplogroups have been reported to be associated with longevity in different populations (Fig. 4). The D4 and D5 haplogroups have been associated with longevity in the Japanese population [100] and the D4a sub-haplogroup in particular is enriched among Japanese aged over 105 years [101].

The J haplogroup is one of several present in populations throughout Europe and the Middle East, yet it associates with longevity only in specific sub-populations. It is over-represented among older northern Italian [102], Irish [103] and Finnish [104] cohorts but shows no correlation with longevity among Ashkenazi Jewish [105], Spanish [106] or southern Italian [107] cohorts. If the J haplogroup does contribute to longevity in a context-specific manner, it may be from allowing greater penetrance or manifestation of other mutations which themselves are beneficial in aging [108]. This theory arises from the converse observation: The J haplogroup has been reported to allow greater penetrance of pathological mutations and is associated with Leber's hereditary optic neuropathy (LHON) [109–111] as well as optic neuritis in cases of multiple sclerosis [112]. Paradoxically, there is evidence to suggest that the same variations which allow for greater penetrance of LHON mutations may themselves be the variations which can lead to prolonged lifespan. Several of the variations associated with the J haplogroup would be expected to decrease functionality of complex I or III [113] and indeed, mitochondria with the J haplotype have significantly lower maximal oxygen consumption than those with the H haplotype [114]. Intriguingly, the H haplotype seems to be protective against LHON [111]. All this suggests that the J haplogroup produces a reduction in mitochondrial function that, by itself, promotes life extension. It is not a robust phenotype, however, and concurrent mutations affecting mitochondrial function lead to more severe pathology in this background. Another study supporting this hypothesis examined variations in human mitochondrial genes and found that mutations occurring in genes encoded complex I subunits can promote longevity, but not when they co-occur with impairment of complex III or V [115].

7.2. Single nucleotide polymorphisms in the mitochondrial genome

A mutation from C to T at position 150 of the mtDNA control region (C150T) (Fig. 4) has been found to associate with longer lifespan across multiple populations [103,116–118] although not without exception [119]. Analysis of haplogroup-matched fibroblasts with and without the C150T transition revealed no association with respiratory capacity nor mtDNA copy number but did correlate with reduced ROS generation as measured by CM-H_2DCFDA (which fluoresces upon reaction with thiol groups within the cell) [120]. Considering all the evidence against a causative role for ROS in aging (discussed previously), this result is surprising. However, as the authors of the study point out, there were various background mutations in the cell lines which may have contributed to the phenotypes observed and until methodology exists to make targeted point mutations in mtDNA, establishing the effects of such mutations may remain elusive [120]. It is also possible that the C150T mutation might lead to different phenotypes in other tissues under different metabolic requirements.

7.3. Insights from comparative mitochondrial biology

Several studies have gone beyond comparing mtDNA sequences among the human population to compare across species. This does add to the difficulty of finding differences that actually influence lifespan and are not merely an unrelated feature of a particular clade. With this caveat, however, comparative biology allows one to compare across a much broader range of lifespans and potentially discover novel adaptations that we have not evolved.

In the proteins encoded in mammalian mtDNA, methionine abundance was found to negatively correlate with lifespan [121]. This correlation extended to nuclear-encoded respiratory subunits residing in the inner mitochondrial membrane as well. Interestingly, encoding for methionine seems to have been enriched in the genes of shorter-lived mammals (which tend to produce more ROS [122]), rather than selectively reduced in longer-lived [121]. This is likely a strategy to combat the excess ROS produced in the mitochondria as methionine residues react readily and then can be repaired by methionine sulfoxide reductases [123–125]. As the authors point out, this argues against a causative role for ROS in aging but rather that species which produce high levels have adapted to deal with it [121].

A study of the mtDNA molecule itself revealed that, among mammals, the relative abundance of cytosine on the L-strand correlates with longer lifespan. Conversely, thymine evinced a negative correlation. The authors speculated that this could be due to a decreased incidence

A

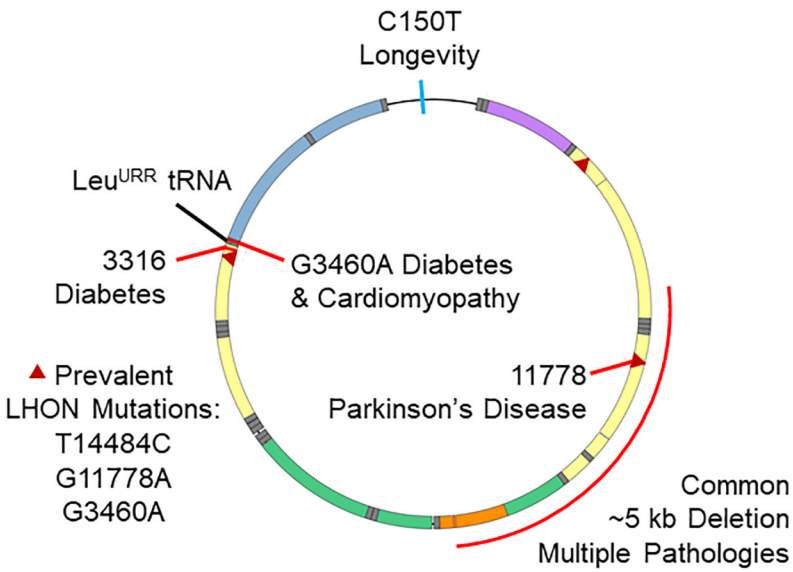

Fig. 4. Numerous associations have been found between specific mitochondrial mutations or haplogroups and longevity or disease. (A) In the control region, the C to T mutation at position 150 has been reported to correlate with longer lifespan. Mutations at positions 3243, 3316 and 11,778 are associated with diabetes and cardiomyopathy, diabetes and Parkinson's disease, respectively. Three common mutations that can cause Leber's hereditary optic neuropathy are indicated (triangles). A common deletion of nearly 5 kb can contribute to multiple pathologies. (B) Several mitochondrial haplogroups have been found to correlate with either longer lifespan or disease, or in the case of haplogroup J, with both.

B

Haplogroup	Reported Associations	References
D4a	Longevity	[99, 100]
D4b2b	Longevity	[99]
D5	Longevity	[99]
H5	Alzheimer's disease risk	[154]
J	Longevity; LHON disease risk	[101-103; 108-110]

of TT dimers which can be modified into cyclobutene pyrimidine dimers, which the mitochondria may not be able to repair [126].

More broadly, among metazoans, mitochondrially encoded cysteine has been found to negatively correlate with species lifespan [126,127], although this association was substantially diminished under correction for phylogenetic contrast [121]. This depletion is specific to components of respiratory complex I [128] and supports other studies that demonstrate a key role for complex I in determining lifespan [129]. Separate from lifespan determination, cysteine is also specifically depleted in complex IV of aerobic organisms relative to anaerobic [128].

Birds are of particular interest in the comparative biology study of aging because, relative to mammals, many of them live much longer than one would predict based on their size and metabolic rate [130,131]. Additionally, fibroblasts from multiple bird species have demonstrated longer survival under conditions of oxidative stress [132,133]. A feature of the mitochondrial genome of many bird species is a duplicated or bi-directional control region [134]. This duplication has apparently evolved independently several times in the relatively long-lived Psittaciformes (parrots and cuckatoos) [135] and Passeriformes (songbirds) [136] and has been associated with lifespan across several avian families [137]. It has been reported that the number and length of direct repeats in mtDNA negatively correlate with lifespan in mammals [138]. Initiating replication in multiple directions or from multiple locations along the mitochondrial genome may reduce the incidence of deletions that are theorized to result from these repeats [139]. In a study where human, mouse, quail and pigeon cells were all driven to replicative senescence, the avian cells not only underwent more doublings but, in contrast to the mammalian cells, did not show an accumulation of mutations in their mtDNA [140]. Further experiments pointed to a key role for autophagy in promoting replicative lifespan [140]. Whether the absence of mtDNA mutations resulted from characteristics of the genome itself or through elimination of mutated copies through mitophagy or some other mechanism requires further study, as indeed, does whether improved maintenance of mtDNA contributes to the lifespan of cells or birds.

8. The role of the mitochondrial genome in age-associated diseases

A number of studies have linked accumulation of mtDNA mutations and deletions with progression of a wide assortment of age-associated diseases (Table 2). In this section, the relative contribution of mtDNA to several of these diseases will be evaluated.

8.1. Parkinson's disease

Mitochondria dysfunction is strongly linked with Parkinson's disease. This connection was originally found upon observations that MPP + (an inhibitor of mitochondrial complex I) produced parkinsonian symptoms in drug users [141] and was replicated in rodent models [142,143]. Lower complex I activity was found in the substantia nigra of patients who had Parkinson's disease than controls [144].

Levels of mtDNA deletions have been reported in the striatum of patients with Parkinson's disease to be significantly higher than that seen under normal aging [145]. Similar increases in mtDNA deletions were reported in the substantia nigra under both aging and Parkinson's disease with greater prevalence under the latter. Many of the mutations appear linked with respiratory chain deficiencies [146]. The G11778A mtDNA mutation, which impairs a subunit of mitochondrial complex I, has been reported to produce parkinsonism although it also produces a range of other disorders as well [147] (Fig. 4). Mutations in the mtDNA polymerase PolG have also been reported to co-segregate with Parkinson's disease in some familial studies [148].

Table 2
Changes in mtDNA and mitochondrial function are summarized in the age-related diseases discussed in Section 8.

mtDNA in age-related diseases		
Disease	Rodent/Cell culture	Humans
Parkinson's disease	• Complex I inhibitors induce parkinsonian symptoms in rodents [142,143]	• Reduced complex I activity [144] • mtDNA deletions in the striatum [145] and substantia niagra [146] significantly higher in Parkinson's disease than aging alone. • mtDNA polymerase PolG mutations [148] • G11778A mtDNA mutation, which impairs complex I, has been reported to cause Parkinson's disease [147].
Alzheimer's disease	• Mitochondrial dysfunction in APP mice [151] • Mutation of mtDNA proof reading accelerates amyloid pathology [218]. • mtDNA deletions produce mitochondrial dysfunction but do not alter Aβ plaque formation [219].	• Complex IV dysfunction [149,150] • Increased mtDNA mutations [152] • Increased mtDNA in cytoplasm [153] • Increased mtDNA oxidative damage [154,220] • No difference in mtDNA damage between healthy elderly patients and elderly patients with AD [155] • H5 haplogroup enriched in AD patients [157]
Sarcopenia	• Mitochondrial abnormalities and mtDNA deletions correlate with age-related muscle loss [158]. • Clonal expansion of mtDNA deletions occur in muscles with age [158].	• Fibers deficient in complex IV activity increase with age and possess mtDNA deletions [159]. • mtDNA deletions accumulate in muscle with age, eventually accounting for ~90% of total mtDNA [161].
Heart failure	• Cardiac specific deletion of DNase II causes mortality, myocarditis and cardio-myopathy under heart pressure overload [168]. • Depletion of mtDNA nucleoid proteins causes mtDNA escape to the cytosol, triggering pro-inflammatory response [169].	• The mtDNA A3243G mutation leads to cardiomyopathy [163]. • OPA1, involved in mitochondrial fusion dynamics, is diminished in heart disease patients and OPA1 mutations are linked with a decline in mtDNA and age-related cardiac dysfunction [164]. • Increased circulating extracellular mtDNA in patients with coronary heart disease [165,166].
Diabetes	• High glucose increases mtDNA damage [171]. • Transfer of mtDNA from a mouse tumor into mouse embryonic cells predisposed mice to diabetes, lymphoma and metastasis [178].	• Elevated mtDNA damage [170] • Lower mtDNA copy number [177] • Increased circulating extracellular mtDNA in patients with diabetes [167] • Mutation at mtDNA position 3243 is linked with diabetes and deafness [172,173]. • Mutation at mtDNA position 3316 increases diabetes risk [174]. • Large mtDNA deletions associated with familial diabetes [175,176].
Cancer		• mtDNA mutations reported in esophageal, endometrial [181,182], colorectal [183], prostate [221], breast [222], ovarian [223], gastric [224], hepatocellular [225], pancreatic [226] and lung cancers [227] as well as several others (reviewed in [228])

8.2. Alzheimer's disease

A broad range of studies have reported mitochondrial dysfunction to occur in Alzheimer's disease, including reduced activity of cytochrome *c* oxidase (respiratory complex IV) [149,150]. In addition, mitochondrial dysfunction has been widely reported both in human and animal studies as an early event in Alzheimer's progression [151]. However, whether mitochondrial dysfunction is a key driver of Alzheimer's pathology or just a symptom of the disease remains unclear, as does the relative role of mtDNA damage. While some studies have shown that mtDNA damage increases with age and to a greater extent in Alzheimer's disease [152–154], others have not shown an increase beyond that seen with aging [155]. A study from Khan and colleagues has reported a causative relationship between mtDNA damage in Alzheimer's disease and mitochondrial function. They created cybrids cells using the platelet mitochondria of 5 sporadic Alzheimer's disease subjects and 5 age-matched neurologically normal subjects. They reported the cells created using mitochondria derived from Alzheimer's disease patients had mitochondria dysfunction along with increased production of β-amyloid [156]. Independent of the role of mtDNA damage in Alzheimer's disease, it has been reported that the mtDNA haplogroup H5 is represented at a threefold higher frequency in patients with Alzheimer's disease [157] (Fig. 4) and may therefore be a risk factor for development of the disease.

8.3. Sarcopenia

Sarcopenia is the progressive decline in muscle mass and function over the course of aging. There is a reasonable body of evidence implicating mitochondria dysfunction as a causative factor of this muscle loss. Wanagat and colleagues reported a 33% decline in muscle mass with age in rats with an associated 30% decline in muscle fiber number. The group reported that this occurred in parallel with a significant elevation in mitochondrial abnormalities and an increase in mtDNA deletions. Significantly, the group reported that muscle fibers harboring mtDNA deletions displayed a higher frequency of muscle atrophy, fiber splitting and oxidative damage than fibers without mtDNA deletions [158]. Though far from conclusive, these findings support a causative link between mtDNA deletions and sarcopenia. Further investigation into mitochondrial dysfunction in skeletal muscle aging led to observations of an age-related increase in ragged red fibers (RRF), which stain red under incubation with Gomori Trichrome and represent fibers deficient in the cytochrome *c* oxidase activity. Such fibers appear at around age 40 and become more prevalent with age. At later ages researchers report that almost all RRF fibers possess a large mitochondrial DNA deletion and this deletion is correlated with deficiency in cytochrome *c* oxidase activity. This suggests the occurrence of mtDNA deletions are an important factor in sarcopenia, although they are likely driven by other changes to muscle fiber [159]. Other researchers likewise noted an enrichment of mtDNA changes in RRF but noted that the pattern of mtDNA changes was distinct in most individuals [160]. Studies of these changes in rats support an argument that the accumulation of mtDNA deletions represent a clonal expansion of mitochondria carrying large sequence deletions through the mechanism behind this expansion remains unclear [158]. It has been reported that mtDNA deletions accumulate in muscle with age to highly detrimental levels, eventually accounting for over 90% of total mtDNA [161].

8.4. Heart failure

In contrast to sarcopenia, there has been very little data characterizing whether prevalence of mtDNA damage or mutations in cardiac

tissue increases with age and plays a role in age-related increases in heart disease prevalence. Conversely, there is a reasonable level of evidence supporting an important role for mtDNA in heart function as many mtDNA mutations result in changes to cardiac function (reviewed in [162]). As an example, the mtDNA A3243G mutation in the Leu^{URR} tRNA gene (Fig. 4) impairs translation of respiratory complex subunits, resulting in disrupted glucose metabolism and ultimately cardiomyopathy [163]. The nuclear-encoded gene optic atrophy 1 (OPA1), involved in mitochondrial fusion dynamics, has been found to be diminished in heart disease patients and mutation of this gene has been reported to cause both a decline in mtDNA and age-related cardiac dysfunction although it is unclear whether these are causally linked [164]. While it is unclear if mtDNA damage or mutation has a role in driving age-related cardiac dysfunction, there is a reasonable body of evidence showing that alterations in mtDNA levels are an important part of cardiac disease progression. Epidemiological studies have reported a significant decline in mtDNA levels in leukocytes and peripheral blood cells with coronary heart disease and have argued that there is a dose response relationship for risk of coronary heart disease; they also report mtDNA content to be impacted by smoking [165,166]. It has been reported that accumulation of circulating extracellular mtDNA, likely caused as a product of cell death and necrosis, is elevated in patients with diabetes and is further elevated in diabetic patients with coronary heart disease. Levels of free mtDNA are argued by this group to be a predictor for developing coronary heart failure in patients with diabetes [167]. Oka and colleagues argue that the elevation of circulating free mtDNA is not simply a predictor of coronary heart disease but a contributing factor. The group argued that circulating mtDNA which escapes degradation by the authophagial system would trigger an inflammatory response in heart tissue producing myocarditis and dilated cardiomyopathy. In support of this they showed that cardiac specific deletion of DNase II (a critical enzyme in removal of mtDNA in apoptotic cells) caused mortality, myocarditis and cardiomyopathy under heart pressure overload but no changes in cardiac function under normal conditions. They went on to show that these effects were ablated by inhibition of the TLR9 inflammatory pathway, suggesting that the toxic effect of free mtDNA was driven by recruitment of pro-inflammatory pathways [168]. The increase in free mtDNA may be partly driven by changes in the mtDNA nucleoid; there is a study reporting that depletion of certain mtDNA nucleoid proteins such as TFAM causes mtDNA escape to the cytosol triggering pro-inflammatory response through type I interferon responses [169]. This suggests that in situations of acute mitochondrial damage, mtDNA may serve as a signaling molecule to potentiate immune defense mechanisms.

8.5. Diabetes

Variations in mtDNA are reported in patients with diabetes, including mutations, deletions and changes in mtDNA abundance, all argued to be driven by oxidative damage [170]. This is supported by studies showing that exposure to high glucose results in an accumulation of mtDNA damage which is blocked by overexpression of the antioxidant scavenger MnSOD [171]. However, it is unclear from such studies whether elevated mtDNA damage is just a symptom of the disease or has pathological consequences. It is plausible that mutations in mtDNA-encoded genes would produce further dysfunction in glucose metabolism and exacerbate diabetes progression, but this is difficult to directly test.

In support of a causative role for mtDNA in diabetes, there are a number of familial studies which show mtDNA mutations to predispose individuals to development of the disease. The most well described of these is a substitution at mtDNA position 3243, in the gene for Leu^{URR} tRNA (Fig. 4). This mutation results in impaired respiratory complex translation, diabetes and deafness [172,173]. Similar deficits have also been noted with mutation of position 3316, in the gene encoded the complex I subunit ND1 [174] (Fig. 4). Increased diabetes risk has also been shown in a number of mtDNA deletion mutations, including a familial form of diabetes which co-segregates with a 10.4 kb mtDNA deletion [175] and a 7.6 kb mtDNA deletion producing Wolfram syndrome with accompanying respiratory enzyme dysfunction and diabetes [176]. In support of a causative role for mtDNA changes in diabetes, a longitudinal study reported that pre-diabetic individuals who went on to develop diabetes had lower mtDNA quantities than controls who did not, suggesting that a decline in mtDNA quantity may contribute to diabetes development [177].

A study by Hashizume and colleagues is one of the few demonstrations of a direct link between mtDNA damage and diabetes: The group transferred mtDNA from a mouse tumor cell with a mutation thought to induce metastasis through excessive ROS production. This mtDNA was introduced into mouse embryonic cells, creating a strain of animals predisposed to diabetes as well as lymphoma and metastasis [178]. More research is needed to determine whether mitochondrial deficits consequent of mtDNA make a significant contribution to primarily age-associated diabetes development.

8.6. Cancer

Otto Warburg was the first to propose that cancer cells make a metabolic switch driven in part by mitochondrial dysfunction [179]. This is thought to enable cancer cells to undergo rapid growth [180]. As a consequence of this, many studies have searched for evidence of mitochondrial genome instability in cancer. Esophageal and endometrial carcinomas have indeed been associated with the appearance of mtDNA point mutations, especially in the D-Loop region [181,182]. Strikingly, in a study of human colorectal cancer, it was reported that tumor tissue had a lower random mutation frequency than non-neoplasic tissue [183]. The authors argued that mtDNA genome stability in such tumors may have risen due to lower ROS production and therefore less oxidative damage. However, more studies are needed to address whether this represents an oddity of colorectal cancer or a feature of multiple malignant tumor types.

9. Mitochondrial DNA epigenetics

The role of epigenetics in mitochondrial DNA and aging is a controversial topic. Several studies have identified DNA methyltransferases which possess mitochondrial localization tags and have been found in mitochondria, most notably Dnmt1 and Dnmt3a [184–186]. Some studies have reported alteration in expression of these enzymes over the course of age. A study looking at 4- and 24-month-old rats showed region specific changes in expression of these enzymes in different regions of the rat brain [187]. In humans, hypermethylation of the D-loop and ND6 have been suggested to contribute to pathology in diabetes [188,189] and non-alcoholic fatty liver disease [190], respectively. Using retinal endothelial cells, one group found that high glucose treatment increased Dnmt1 mitochondrial localization and binding to the D-Loop, leading to cytosine methylation and eventual apoptosis. Significantly, both cytosine methylation and apoptosis were attenuated by Dnmt1 inhibition [189]. In humans, mtDNA methylation has been found to vary by tissue [191] while any changes with aging appear to be highly variable [192]. However, there is controversy regarding what role DNA methyltransferases play in mitochondria in vivo [193,194] and whether measures of mtDNA methylation are accurate or primarily experimental artifacts [195,196].

10. Mitochondrial DNA maternal inheritance: aged mothers

In mammals, mtDNA inheritance is solely maternal [197]. It was previously thought to be a simple consequence of the oocyte providing the cytoplasm to the newly forming embryo but is increasingly evident to be the result of a selective process that eliminates paternal

mitochondria. Endonuclease G was recently described in the breakdown of paternal mitochondria during fertilization in *C. elegans* [198]. It has long been known that fertility decreases with age, and therefore awareness of maternal age and its influence on the quality of the mtDNA being inherited has risen. Inheritance of mutated mtDNA can also lead to the so-called mitochondrial diseases.

In 2000, Brenner and colleagues found that a higher percentage of single human oocytes aged mothers (36 to 42 years old) contained the mtDNA T414G transversion point mutation present in the control region (D-loop) as compared to younger mothers (26 to 35) using regular Sanger sequencing [199]. Thanks to ultra-deep sequencing, Makova and colleagues were able to conduct a population study in mother-child pairs and scored different mtDNA copy variants (heteroplasmicity) with an allele frequency higher than 1%. They found a positive association between the amount of heteroplasmicity in a child and maternal age at fertilization. This is normally due to the appearance of point mutations, suggesting that older mothers accumulate more mutations in their germ-line, likely attributable to oocyte aging [200]. They additionally found that the older mothers accumulated more mutations in their somatic tissues relative to their children than the younger mothers. In addition, they found the number of point heteroplasmicities to triple over 30 years of life.

Goran Larson and colleagues used mice heterozygous for the mtDNA mutator allele ($Polg^{mut/+}$) to maternally transmit mtDNA mutations [201]. Through doing this, they observed several mild aging phenotypes even though they had a wild-type nDNA. They also observed reduced fertility at earlier ages.

The above findings suggest a double impact of maternal age effects: A decline in fertility with age and the transfer of higher levels of mtDNA mutations. Fertility and embryo quality have also been studied in the context of assisted reproductive methods. The cells that surround oocytes at ovulation, human cumulus granulosa cells (CGC) were studied for their influence in oocyte quality. mtDNA copy number on these cells was indeed found to be positively correlated to oocyte quality [202]. However, it has also been reported that the quantity of CGC mtDNA is negatively influenced by BMI and smoking, potentially suggesting that the individual's health is important to maintain healthier mitochondria. Moreover, a new bovine model to study the effect of both age and ovarian stimulation found that ovarian aging, but not ovarian hormonal stimulation, increased the number of oocytes with mtDNA deletions [203].

11. Conclusions

Changes in mtDNA over the course of aging are well supported in the literature. Such changes include an accumulation of oxidized base pairs, base mismatches, strand breaks and deletions. These changes have been linked with mitochondrial dysfunction. However, most studies linking mtDNA damage to aging are correlative and those studies which have attempted to manipulate accumulation of mtDNA damage have a number of confounding issues which make interpretation difficult. For this reason, the role of mtDNA damage as a driving force for age-related functional decline remains controversial.

There is also a reasonable body of evidence for specific mutations and mtDNA haplogroups as predictors of both lifespan and risk of various age-associated disease including cancer, diabetes, heart failure, sarcopenia and Parkinson's disease. However, for many of these diseases, most reports on a role for mtDNA haplogroups and other sequence variations in aging and lifespan determination are again correlative.

The ability to make targeted sequence alterations in mtDNA – as is routinely done in nDNA with techniques such as CRISPR-Cas9 [204,205] – would be a significant advancement in the field and allow direct testing for a causative role of mtDNA variations and mutations in determining lifespan. Several studies have reported success with various techniques in eliminating specific mtDNA from heteroplasmic cells [206–208]. However, difficulty in targeting RNA to mitochondria and uncertainty whether mtDNA can undergo homologous recombination in many species present significant obstacles to making specific point mutations in the mitochondrial genome [209].

In summation, the role of mtDNA in aging and age-associated disease remains a fascinating topic with a large body of correlative data suggesting a key role. However, the lack of methods and techniques to directly manipulate the role of mtDNA in this area continues to present a substantial challenge.

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Transparency document

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