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Inherited mitochondrial genomic instability and chemical exposures

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Abstract

There are approximately 1,500 proteins that are needed for mitochondrial structure and function, most of which are encoded in the nuclear genome (Calvo et al. 2006). Each mitochondrion has its own genome (mtDNA), which in humans encodes 13 polypeptides, 22 tRNAs and 2 rRNAs required for oxidative phosphorylation. The mitochondrial genome of humans and most vertebrates is approximately 16.5 kbp, double-stranded, circular, with few non-coding bases. Thus, maintaining mtDNA stability, that is, the ability of the cell to maintain adequate levels of mtDNA template for oxidative phosphorylation is essential and can be impacted by the level of mtDNA mutation currently within the cell or mitochondrion, but also from errors made during normal mtDNA replication, defects in mitochondrial quality control mechanisms, and exacerbated by exposures to exogenous and/or endogenous genotoxic agents. In this review, we expand on the origins and consequences of mtDNA instability, the current state of research regarding the mechanisms by which mtDNA instability can be overcome by cellular and chemical interventions, and the future of research and treatments for mtDNA instability.

Keywords

Mitochondria; Mitochondrial DNA; Toxicity; Gene-environment interactions; Therapeutics; Disease

2. Origins and consequences of mtDNA instability

Inherited mtDNA diseases are complex, poorly understood, and currently do not have good therapeutic options. Most mtDNA diseases predominantly affect the nervous system, which in large part is due to the high energy requirements of neurons, and the polar shape of neurons requiring mitochondria to travel larger distances than many other cell types (Chen and Chan 2009; Lax et al. 2017; Schwarz 2013). MtDNA diseases include childhood-onset Leigh syndrome and Alpers syndrome, as well as later onset ataxia-neuropathy spectrum

disorders, and progressive external ophthalmoplegia (Copeland 2014; Naviaux 2004; Stewart and Chinnery 2015; Wallace 1999). In addition to these rarer disorders, mtDNA instability has also been associated with Parkinson's Disease, cancer and ageing (Zapico and Ubelaker 2013). Ye et al. recently showed that 90% of healthy humans are heteroplasmic, that is, they carry more than one mtDNA variant in their cells and/or tissues. Of the same population sampled, 20% carried pathogenic mtDNA mutations associated with disease (Ye et al. 2014). Yet, these people did not show signs of ill-health or mitochondrial disease at the time of sampling. Why then are mitochondrial diseases not more prevalent? The answer likely lies in the level of heteroplasmy within a given cell, organ or tissue: developing increasing levels of mutated mtDNA can lead to a decline in the ability of the cell or organ to maintain energy levels needed to sustain overall health. Most of the people contributing samples for this study likely had levels of heteroplasmy that did not meet the threshold level for mitochondrial dysfunction. However, it is also important to understand the origins of an individual's mtDNA instability and how these factors could contribute to future decline in health and onset of disease.

2.1. Maternal inheritance of mtDNA mutations

Mitochondria and mtDNA are inherited maternally, although one case of inheritance of mtDNA from both the mother and the father has been documented (Schwartz and Vissing 2002). Dr. Douglas Wallace and coworkers revealed germline selection against inheritance of severe mtDNA mutations. His group showed that mitochondria that were respiration-proficient were preferentially replicated; consequently, the level of heteroplasmy in mature oocytes had fewer copies of mtDNA containing a severe mtDNA mutation than predicted from the level of mtDNA heteroplasmy observed in immature oocytes (Fan et al. 2008). However, mature oocytes containing mtDNA with milder, less deleterious mutations were maintained to similar proportions as found in the immature oocytes. Selective elimination of deleterious mtDNA mutations, or preferential replication of wild-type mtDNA could be a major contribution to the lower than expected levels of mtDNA instability in the general population as shown by Ye et al. (Ye et al. 2014)). During oogenesis, there is a substantial reduction in mtDNA numbers, known as the germline mtDNA bottleneck. From this small population of mitochondrial genomes (approximately 30–35 in humans (Rebolledo-Jaramillo et al. 2014)) all mtDNA for the offspring will originate. Thus, mechanisms for decreasing heteroplasmy at this bottleneck are important for reducing the transmission of mtDNA mutations and the incidence of mitochondrial disease. With aging, there is a decline in mitochondrial homeostasis (Bohovych et al. 2015), and this may greatly contribute to the increased number of heteroplasmies transmitted to offspring, as shown by a positive correlation with maternal age (Rebolledo-Jaramillo et al. 2014).

2.2. MtDNA instability arising from normal (or faulty) mtDNA replication

MtDNA mutation not only arises from maternal inheritance of mtDNA; a major source of mtDNA mutation and depletion originates from normal mtDNA replication (Zheng et al. 2006). Furthermore, mtDNA instability can be exacerbated by faulty mtDNA replication-associated proteins. In fact, several mitochondrial disease loci encode proteins that are directly and indirectly required for mtDNA replication (Copeland 2014). The three main proteins most directly involved in mtDNA replication with associated human disease

mutations are POLG (the catalytic subunit of the mtDNA polymerase, DNA polymerase gamma), POLG2 (the accessory subunit of DNA polymerase gamma), and TWNK (alternatively known as PEO1, the mtDNA helicase). There is only one replicative DNA polymerase in mitochondria, DNA polymerase gamma, which is essential for replication, proofreading, and repair of mtDNA. The DNA polymerase gamma catalytic subunit (encoded by *POLG*) has highly faithful DNA polymerase and 3′–5′ exonuclease activities (which removes misincorporated nucleotides) that are separated by a large linker region (Graziewicz et al. 2006; Young and Copeland 2016). POLG also contains a 5′dRP lyase activity needed for base excision repair, although the residue containing this activity is still unknown (Longley et al. 1998). The smaller accessory subunit (encoded by *POLG2*) is a dimer that confers tight DNA binding and processivity to the holoenzyme (Lim et al. 1999).

The first POLG disease mutations were reported in 2001 by Van Goethem and co-workers, including what remains the most common autosomal dominant mutation in POLG (Y955C mutation (Van Goethem et al. 2001)), which is associated with progressive external ophthalmoplegia, as well as parkinsonism, premature ovarian failure and premature menopause (Luoma et al. 2004; Pagnamenta et al. 2006). Approximately 300 human disease mutations have now been identified in *POLG*, and are scattered throughout the gene without any concentration within a particular domain or region (Young and Copeland 2016). We have since learned much about inherited mtDNA diseases from epidemiology studies, in vitro biochemistry and enzymology, as well as cellular and in vivo studies. Greater than 2% of the population carries a pathogenic *POLG* mutation (Cohen et al. 2010), which may contribute to the surprisingly high proportion of the human population with heteroplasmy (Ye et al. 2014). However, the level of heteroplasmy might not reach the threshold to cause dysfunction in most people, as most *POLG* mutations are autosomal recessive (Cohen et al. 2010). Much of what is known of the biochemistry and cell biology of proteins with mutations causing mtDNA diseases have been excellently summarized in recent reviews by the Copeland group (DeBalsi et al. 2017; Stumpf and Copeland 2014; Young and Copeland 2016). At least eight POLG2 mutations have been associated with human mitochondrial disease, and these have been also been carefully characterized by in vitro enzymology. Mutations in POLG2 that cause disease have been shown to be defective in their ability to bind the catalytic subunit, bind to double stranded DNA, and/or self-dimerize (Craig et al. 2012; Longley et al. 2006; Young et al. 2011). Cell and biochemical studies by Young et al. showed that mutant POLG2 heterodimers can either poison the enzyme activity of the mtDNA polymerase or fail to bind nucleoids, ultimately leading to failed mtDNA replication, decreased bioenergetics and contributes to mitochondrial disease seen in patients (Young et al. 2015). The TWNK mtDNA helicase, also known as Twinkle, is essential for mtDNA replication as it is needed to unwind the double helix ahead of the replication fork, as well as synthesis of nascent D-loop strands (Milenkovic et al. 2013). The mtDNA helicase is also a key regulator of mtDNA copy number (Tyynismaa et al. 2004). Recombinant TWNK proteins containing mutations associated with human disease were extensively characterized in vitro. Compared to WT TWNK protein, mutant proteins had significant differences in protein stability, DNA binding, nucleotide hydrolysis, and helicase activity (Holmlund et al. 2009; Korhonen et al. 2008; Longley et al. 2010; Matsushima et al. 2008). Cell studies, such as those by Spelbrink and co-workers, showed that TWNK

mutations also cause replication stalling and defects in mitochondrial transcription, and that TWNK is one of the proteins needed to tether mtDNA to the inner mitochondrial membrane (Goffart et al. 2009; Rajala et al. 2014).

The crystal structures of mammalian POLG and POLG2 have been solved, as well as the 3D structure of TWNK by electron microscopy and small angle X-ray scattering (Carrodeguas et al. 2001; Fan et al. 2006; Fernandez-Millan et al. 2015; Lee et al. 2009). Furthermore, Kaguni and colleagues have developed an algorithm based on mapping pathogenic POLG mutations to predict pathogenicity (Farnum et al. 2014; Nurminen et al. 2017). Replisome studies, for example, by the Falkenberg group, have also greatly added to our knowledge as to how defects in these proteins cause disease (Farge et al. 2007). However, despite this and much careful in vitro characterization of mutant proteins involved in disease, it has been difficult to determine the full pathogenic nature of some mutations, particularly for POLG. For example, some of the more common POLG disease mutations are associated with a wide spectrum of diseases with onset of symptoms occurring shortly after birth (such as mtDNA depletion syndromes) to diseases that occur late in adulthood (such as ataxia neuropathy disorders and progressive external ophthalmoplegia). This phenotypic variation occurs even when patients are homozygous for the same mutation (Cohen et al. 2010). Differences in symptoms can also occur between homozygous members of the same family, such as in (Schulte et al. 2009), and this is most evident for the three most common disease mutations for POLG, that is, the A467T, W748S and G848S mutations (Tzoulis et al. 2006). Biochemically, we and the Kaguni group showed that the A467T protein had severely reduced DNA polymerase activity (4%) compared to WT POLG protein (Chan et al. 2005a; Luoma et al. 2005). We also showed that the POLG A467T protein had defects in binding to the POLG2 accessory subunit (Chan et al. 2005a), which was later corroborated by crystallography experiments performed by the Yin lab (Lee et al. 2009). Likewise, the W748S POLG protein had severely reduced DNA polymerase activity (2.3%) compared to WT, which is tempered by the E1143G polymorphism, which is discussed later in this article (Chan et al. 2006). Gene dose of POLG mutation is also important. We analyzed fibroblasts from patients carrying the POLG A467T mutation *in trans* with either a POLG stop codon or intronic frame shift mutation, and showed that transcripts containing the stop codon or frame shift mutation undergo nonsense-mediated decay. These events ensured that virtually all POLG protein in the cell derived from the A467T allele, leading to haplotype insufficiency and severe Alpers syndrome in early childhood (Chan et al. 2009; Chan et al. 2005b). However, patients homozygous for A476T or W748S can develop a wide range of mitochondrial diseases, from early onset Alpers syndrome to mid-age onset ataxia neuropathy syndrome to adult onset progressive external ophthalmoplegia (Bereau et al. 2016; Blok et al. 2009; Gonzalez-Vioque et al. 2006; Henao et al. 2016; Lax et al. 2012; McHugh et al. 2010; Rajakulendran et al. 2016; Tang et al. 2011). We also found that single nucleotide polymorphisms can play a role in modulating disease. For example, the common single nucleotide polymorphism, E1143G, is generally found in cis with the W748S POLG mutation; we found using biochemical and structural methods that the E1143G SNP partially rescues the W748S defect (Chan et al. 2006). We also showed that other POLG mutations such as the common G848S mutation effectively inactivate the polymerase, in vitro the POLG G848S protein has only 0.01% activity compared to WT POLG

(Kasiviswanathan et al. 2009). Despite the deleterious effects on DNA polymerase activity, humans can survive past birth with two copies of POLG G848S: one study reported a child homozygous for the POLG G848S variant, who died of sudden infant death syndrome ((Tang et al. 2011) and personal communication from corresponding author, Dr. L.-J. Wong). Other DNA polymerases have recently been detected in vertebrate mitochondria, for example, PrimPol (Garcia-Gomez et al. 2013), and could potentially play a role in allowing animals to survive past birth. However, to date only POLG has the ability to perform highly accurate and processive DNA synthesis. Considering that POLG is the only replicative mtDNA polymerase in vertebrates, the compensatory mechanisms present in these patients are unknown and puzzling. Animal studies are thus essential for understanding these mechanisms, which could be targeted for therapies for mitochondrial disease, as well as understanding the involvement of outside factors, such as chemical exposures, on development of mitochondrial disease.

In mouse studies, knockout of POLG, POLG2, or TWNK leads to embryonic lethality around embryonic day 8.5 (Hance et al. 2005; Humble et al. 2013; Milenkovic et al. 2013; Tyynismaa et al. 2004). However, as indicated above, in humans with severe *POLG* mutations including homozygous mutations that have been shown to essentially inactivate the polymerase function, such as the G848S mutation, children can live for several years (Tang et al. 2011). However, knockout of *POLG* in other model systems, such as C. elegans and zebrafish, is not embryonic lethal, although these organisms do have shortened lifespans (Bratic et al. 2009; Rahn et al. 2015). For C. elegans, knockout of *polg-1* animals were sterile due to severe mtDNA depletion particularly in the gonad (Bratic et al. 2009). For zebrafish, a vertebrate model organism, *polg* knockout leads not only to severely decreased lifespan (rapid decline occurs just prior to reaching the juvenile stage at 1 month), but interestingly also leads to decreased regenerative capacity (Rahn et al. 2015). One of the most interesting abilities of the zebrafish is its ability to regenerate many of its tissues, including the heart, nervous tissue, liver, and fins (Gemberling et al. 2013). Serendipitously, while clipping tails from *polg* knockout zebrafish for genotyping, we noticed that the tail fin did not regenerate normally (Rahn et al. 2015). In mouse embryonic stem cells, Facucho-Oliveira et al. showed that steady state expression of POLG is essential for maintaining pluripotency, and that POLG was also important for cell fate (Facucho-Oliveira et al. 2007; Folmes et al. 2016). Further evidence linking mitochondrial function to fate and function of stem cells has been reviewed in this Special Issue by Dr. Weinhouse (PLEASE ADD WEINHOUSE REFERENCE HERE). Altogether, this suggests that POLG protein is needed for stem cell maintenance, differentiation, and tissue regeneration. Further investigation is needed to fully dissect the role of POLG in these processes.

Other mouse, yeast, fly, worm models of mutant POLG, POLG2 and TWNK have been developed and have been excellently reviewed recently (Nunnari and Suomalainen 2012; Sanchez-Martinez et al. 2012; Stumpf and Copeland 2014; Tyynismaa and Suomalainen 2009; Young and Copeland 2016). Of note, the exonuclease-deficient POLG mutant mouse developed by two groups showed an aging phenotype (Kujoth et al. 2005; Trifunovic et al. 2004). The heart-targeted POLG Y955C transgenic mouse showed mtDNA depletion, cardiomyopathy, as well as a reduced lifespan (Lewis et al. 2007). In addition, compared to other mouse models, a known disease mutation in POLG (Y955C) was now shown to be

linked with increased oxidative stress. Furthermore, cardiac nuclear DNA methylation changes were seen in this mouse (Koczor et al. 2013). These mice have been utilized in several gene-environment studies that will be discussed later in this review.

Jackson Laboratories defines conplastic strains as those that are developed by “backcrossing the nuclear genome from one inbred strain into the cytoplasm of another.” This allows comparisons between mice that have the same nuclear genome with different mtDNA backgrounds. Conplastic strains have been useful in determining that differences in mtDNA haplotypes greatly influence metabolism, health and aging (Latorre-Pellicer et al. 2016). Xenomitochondrial mice, where mtDNA-less embryonic stem cells are transplanted with mitochondria (McKenzie et al. 2004) have also shown that matching between mtDNA and nuclear DNA is important for fitness, and highlights the importance of heteroplasmy in disease progression (Sharpley et al. 2012). Ballinger and co-workers have also developed Mitochondrial – Nuclear eXchange (MNX) mice, where the nuclear and mitochondrial genomes have been exchanged from two different mouse strains that have wide differences in their susceptibility to diet-induced atherosclerosis. With this mouse, his group has found that the mtDNA background modulated bioenergetics, as well as susceptibility to cardiac overload (Fetterman et al. 2013) and non-alcoholic fatty liver disease (Betancourt et al. 2014).

2.3. MtDNA instability caused by endogenous and exogenous mutagens

The mitochondrial genome has a 10 times greater rate of mutation than nuclear DNA (Brown et al. 1979), due to the mitochondrial genome being susceptible to damage, with a more persistent and greater rate of damage than nuclear DNA (Yakes and Van Houten 1997). The location of the mitochondrial genome plays a role in this susceptibility, as mtDNA is replicated in nucleoids tethered to the inner mitochondrial membrane, near the site of oxidative phosphorylation, which not only produces ATP, but also reactive oxygen species (ROS) that can damage proteins lipids and nucleic acids. The mtDNA polymerase can be damaged by ROS (Graziewicz et al. 2002), and ROS production can lead to the production of oxidized nucleosides and nucleotides, such as 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG). 8-oxo-dG can be incorporated into mtDNA by normal DNA polymerase gamma to some extent (Graziewicz et al. 2007). When stably paired with adenosine, this can result in G to T transversion mutations if not removed. Many chemicals and toxicants can also induce mtDNA instability in cell and animal models by a number of oxidative and non-oxidative mechanisms (excellently reviewed by Meyer et al. (Meyer et al. 2013)). Because of this, clinicians should also consider potential chemical exposures during patient care; this has been discussed by Drs. Cunningham and Falk in this issue (PLEASE ADD CUNNINGHAM AND FALK REFERENCE HERE). Dr. Jayasundara, in this issue, also discusses the evolutionary and ecological perspectives on mtDNA mutagenesis (PLEASE ADD JAYASUNDARA REFERENCE HERE). Finally, base excision repair, the main repair system for removing and repairing oxidative lesions (Thapar and Demple 2017), is the major DNA repair mechanism within mitochondria (Cline 2012). Controversy surrounds whether other DNA repair mechanisms are accessible by mitochondria (Copeland and Longley 2014). Additionally, a new study from the Bielas group revealed that there are inherent mechanisms that inhibit chemically-induced mtDNA damage from conversion into mtDNA

mutation (Valente et al. 2016). The latest in this area has been excellently reviewed by Roubicek and Souza-Pinto in this issue (PLEASE ADD ROUBICEK AND SOUZA-PINTO REFERENCE HERE).

2.4. Gene-environment interactions in mtDNA instability

Here we consider two specific chemical exposures that are known to affect mtDNA stability and to which those with underlying POLG-related mtDNA instability are particularly susceptible.

Valproic acid—Human mitochondrial diseases are complex and poorly understood, and can be misdiagnosed, or diagnosed properly at a very late stage. One such disease is Alpers syndrome, a childhood mtDNA depletion disorder associated with mutations in POLG (Nguyen et al. 2006; Uusimaa et al. 2013; Zhang et al. 2011). The characteristic triad of symptoms in Alpers syndrome are epilepsy, liver disease and progressive developmental regression (Saneto et al. 2013). Valproic acid is a common drug used to treat epilepsy and bipolar disorder. Many side effects have been reported (Nanau and Neuman 2013). Unfortunately, prescription of valproic acid may include patients with as yet undiagnosed Alpers syndrome. In these patients, valproic acid can cause fatal hepatotoxicity and should not be prescribed when Alpers syndrome is suspected or when POLG mutation has been identified (Hynynen et al. 2014; Stewart et al. 2010). Liver transplantation in patients who have had vaproic acid-induced hepatotoxicity is controversial due to the systemic nature of Alpers syndrome, particularly the progression of neurological symptoms that can occur after liver transplantation (Hynynen et al. 2014). However, the data suggest that liver transplantation should not be completely ruled out for Alpers syndrome patients, if needed (Hynynen et al. 2014).

The reasons for toxicity are still unclear. Valproic acid is a histone deacetylase inhibitor and in great part due to this function, it is not recommended when pregnant due to its teratogenic effects (Alsdorf and Wyszynski 2005; Ornoy 2009). The high therapeutic dose requirement of valproic acid cautions its use in children. Valproic acid can target mitochondrial functions such as β-oxidation (Caldeira da Silva et al. 2008). However, Stewart et al. found that fatty acid oxidation was not the cause of toxicity (Stewart et al. 2010). Valproic acid causes defects in mitochondrial respiration as shown by in vitro HepG2 cell studies (Komulainen et al. 2015) and can stimulate mitochondrial biogenesis in POLG-deficient fibroblasts through expression of several regulators of mitochondrial biogenesis and POLG (but not through any modulation of DNA methylation) (Sitarz et al. 2014). Increased mtDNA copy number as well as increased metabolic rate was observed. However, the authors suggest that Alpers syndrome patient liver cells do not have the capacity to take advantage of this increased metabolic rate, in these cells the metabolic reserve capacity is exhausted. In liver cells, it is also thought that regenerative capacity is compromised with valproic acid, leading to liver failure. This was indeed revealed by studies from Stewart et al., who showed that valproic acid adversely affects liver regeneration in POLG-deficient fibroblasts (Stewart et al. 2010). As stated earlier, we also found that Polg deficiency in vivo in zebrafish gives rise to defects in tissue regeneration (Rahn et al. 2015), further indicating a key role for POLG protein in tissue regeneration.

Nucleoside reverse transcriptase inhibitors—Almost 40 million people are infected with HIV worldwide (UNAIDS 2016), with 17 million currently on anti-retroviral therapy (ART) (UNAIDS 2016). ART that includes nucleoside reverse transcriptase inhibitors (NRTIs; such as azidothymidine/AZT and lamivudine/3TC) have proven beneficial in extending the lives of HIV-infected patients. NRTIs are analogs of natural nucleosides that do not have the 3′OH required to make the phosphodiester bonds between nucleotides in the growing DNA chain. These analogs can be phosphorylated and incorporated into DNA, leading to chain termination and replication stalling. However, NRTIs not only readily inhibit viral reverse transcriptase, for example, the HIV reverse transcriptase, but they can also inhibit some cellular DNA polymerases (Copeland et al. 1992). NRTIs can inhibit DNA polymerases alpha and beta, but the most sensitive to NRTIs is DNA polymerase gamma, the replicative mtDNA polymerase (Kohler and Lewis 2007; Szymanski et al. 2015). Inhibition of mtDNA replication leads to mtDNA depletion (Chan and Copeland 2009), and in some cases can cause mtDNA lesions (Chan et al. 2007) and oxidative stress (Kline et al. 2009).

ART is currently the only effective treatment for preventing the vertical transmission of HIV from infected mothers to their infants (Luzuriaga and Mofenson 2016; Mandelbrot et al. 2015). Unfortunately, side effects from ART that include NRTIs are common, and include liver toxicity, lipodystrophy, lactic acidosis, bone marrow suppression, and neurotoxicity (Margolis et al. 2014). In 1999, a French group was the first to report eight cases of severe cognitive and neurological dysfunction among HIV-negative children after *in utero* ART (Blanche et al. 1999). These neurologic symptoms, including hypotonia, seizures, encephalopathy, and neuropathy, were similar to both congenital mitochondrial dysfunction in children and ART-induced mitochondrial toxicity in HIV-infected children and adults. Uninfected children exposed to HIV have subsequently been found to be lighter in weight at birth and had slightly accelerated growth in the first 2 years with less subcutaneous fat when compared with US references for these measures (Neri et al. 2013). An increased risk of cancer has also been implicated for in utero didanosine (ddI, analog of adenosine) exposure (Hleyhel et al. 2016).

People with certain POLG mutations and polymorphisms are susceptible to NRTIs. An HIV-infected patient with the POLG R964C mutation had hyperlactatemia following a one year course of d4T (stavudine, a thymidine analog) and 3TC anti-viral treatment (Yamanaka et al. 2007). Furthermore, people with the POLG E1143G single nucleotide polymorphism have been shown to be 4-fold more susceptible to d4T-induced lipodystrophy (Chiappini et al. 2009). The E1143G single nucleotide polymorphism is found in 3.7% of individuals of European descent, but is not found in Asian or African populations. Biochemically, we found that the POLG protein containing the E1143G polymorphism was 1.4-fold more active than WT POLG protein, and this increased polymerase activity could be due to higher thermal stability for E1143G pol gamma (Chan et al. 2006), which could correlate with the increased susceptibility to NRTI toxicity with this polymorphism. Studies in the yeast, Saccharomyces cerevisiae, showed that polymorphisms in Polg differentially affect mtDNA stability and NRTI-induced mitochondrial toxicity (Baruffini et al. 2015). Mouse studies have also attempted to address the role of mtDNA instability and NRTI susceptibility. Without treatment, transgenic mice with cardiac-targeted POLG Y955C mutation showed

severe mtDNA depletion and cardiac dysfunction (Lewis et al. 2007). Treating these mice with a combination ART consisting of AZT (0.22 mg/day), 3TC (0.11 mg/day) and indinavir/IDV (0.9 mg/day) exacerbated cardiac defects (Kohler et al. 2009; Kohler et al. 2008).

2.5. Gender considerations in mtDNA instability

We also showed in a study of WT mice perinatally treatment with either AZT, 3TC or AZT-3TC in combination that female mice, but not male mice, were susceptible to AZT-3TC treatment, which caused persistent mtDNA lesions long after the treatment period (at 10 weeks of age, which was 6 weeks after perinatal treatment was stopped) (Chan et al. 2007). The underlying gender-specific effects of certain NRTIs are currently not known, however inherited mtDNA instabilities, such as through POLG mutation, have been associated with gender-specific differences in phenotype. The current thinking regarding mtDNA and gender is that males may have greater detrimental consequences due to uniparental inheritance of mtDNA through females (Beekman et al. 2014). Some studies bolster this argument. For example, POLG exonuclease-deficient male mice (POLG D257A mice) showed cardiovascular defects that were not present in female mutants (Golob et al. 2015). POLG mutations (such as the number of CAG repeats at nucleotides 126 to 157 of exon 2) have also been associated with increased incidence of male infertility (Jensen et al. 2004; Rovio et al. 2001) and testicular cancer (Blomberg Jensen et al. 2008; Nowak et al. 2005). Conversely, POLG mutations such as R943H (Blok et al. 2009) and Y955C (Luoma et al. 2004) can lead to female-specific defects such as premature ovarian failure and premature menopause (Luoma et al. 2004; Pagnamenta et al. 2006). Thus, chemicals that are known to be more toxic to one gender versus the other may be important modifying factors in mitochondrial disease. For example, common endocrine disrupting chemicals such as bisphenol A can increase mitochondrial dysfunction and oxidative stress (Kaur et al. 2014). Interestingly, mtDNA was found to determine androgen dependence of prostate cancer cell lines: accumulation of mtDNA with large deletion or depletion appeared to be linked to androgen independence and the development of prostate cancers (Higuchi et al. 2006). Thus, gender is an important consideration for understanding inherited mtDNA instability and susceptibility to chemical exposures.

3. Promoting mtDNA stability

There currently are no effective therapies for mtDNA diseases (Kerr 2013). Antioxidant/nutritional supplement medicinal cocktails – attempting to protect or stimulate mitochondrial energy production – are sporadically effective at allaying symptoms but do not treat the disease (Tarnopolsky 2008). A successful disease-modifying treatment that slows or arrests tissue/organ dysfunction through increased mtDNA stability, could do so by one of several possible mechanisms, such as maintaining mitochondrial health, by bolstering oxidative phosphorylation and preventing/protecting against electron transport defects, or activating cellular mechanisms for promoting mitochondrial quality control. The mitochondrial quality control mechanisms used to keep mitochondria healthy are also important for maintaining mtDNA stability. In this issue, Meyer et al. have elegantly outlined these mitochondrial quality control mechanisms, such as mitochondrial fission and

fusion, mitochondrial biogenesis (for generating new, healthy mitochondria under stress) and mitophagy (for breaking down dysfunctional organelles) influence mtDNA quality control and stability (Kim and Lemasters 2010; Rasbach and Schnellmann 2007; Seo et al. 2010; Wills et al. 2012)(PLEASE ADD MEYER REFERENCE HERE). The mitochondrial unfolded protein response is also important for regulating mtDNA stability. The Haynes group showed in elegant C. elegans studies that activation of ATFS-1, the transcription factor that regulates the mitochondrial unfolded protein response, not only promotes mitochondrial biogenesis but under prolonged activation also enhances the maintenance and propagation of mtDNA containing deleterious mutations (Lin et al. 2016). The same group recently showed that in worms lacking ATFS-1, mammalian ATF5 could regulate mitochondrial unfolded protein response. Furthermore, ATF5 was needed by mammalian cells for mitochondrial homeostasis (Fiorese et al. 2016). Altogether this suggests that the mitochondrial unfolded protein response is highly conserved.

Lifestyle and environment

Lifestyle and environment can play a major role in controlling mtDNA mutagenesis and disease. Bratic et al. showed that mtDNA copy number in *polg-1* knockout worms could be greatly increased when exposed to environmental stimuli such as temperature (Bratic et al. 2009). Cold temperatures (15°C versus the normal 20°C) lead to a significant increase in mtDNA copy number in both WT and *polg-1* knockout worms. However, despite this increase in mtDNA copy number, the level of mtDNA was still significantly less than WT levels grown at 20°C. Thus, while cold exposure could force worms to compensate for mtDNA depletion, it could not increase mtDNA to WT levels. At higher than normal temperatures (25°C), *polg-1* knockout worms also showed compensation for this additional metabolic stress. In a mouse study by Tarnopolsky and co-workers, POLG exonuclease-deficient mice that normally have premature aging had longer lives, less pathology, less mtDNA depletion and deletions, as well as overall improved mitochondrial health when subjected to five months of endurance exercise training (Safdar et al. 2011). Exercise can promote mitochondrial quality control processes such as mitochondrial biogenesis, mitochondrial fission/fusion and mitophagy (Yan et al. 2012), and these quality control process help to maintain or improve mtDNA stability. Exercise as a therapeutic approach for mtDNA diseases has been studied in limited clinical trials (Kerr 2013); after 28 weeks of exercise training, patients had significantly improved exercise tolerance and oxygen utilization. Thus, this regimen may be safe and beneficial for mtDNA disease patients. However, there were no changes in heteroplasmy or mtDNA copy number, suggesting that longer term studies are needed (Taivassalo et al. 2006).

New chemical compounds for mtDNA diseases

One interesting drug candidate in Phase 2 clinical trials for Leigh Syndrome and pediatric mitochondrial disease is the EPI-743 drug candidate from Edison Pharmaceuticals (Enns et al. 2012). EPI-743 is thought to regulate cellular energetics by targeting the NADPH quinone oxidoreductase 1 (NQO1). Other drugs, such as bezafibrate, has been shown to be beneficial in some mouse models of mitochondrial disease (Viscomi et al. 2015). Bezafibrate activates peroxisomal proliferator activator receptors (PPARs, and thus PPAR gamma coactivator 1α (PGC1a), a key regulator of mitochondrial biogenesis). However, for the

POLG exonuclease deficient mouse, eight months of bezafibrate treatment was beneficial by improving aging phenotypes in some tissues, but not others. Interestingly, the authors suggest that bezafibrate improved phenotype in skin and spleen because of improvements in maintaining the stem cell population in these replicating tissues (Dillon et al. 2012). Other drugs that have been used in humans may be repurposed for mtDNA diseases. For examples, clofilium tosylate, an anti-arrhythmic agent, had efficacy by increasing mtDNA content in POLG deficient yeast, worms and human fibroblasts (Pitayu et al. 2016). Two other anti-arrhythmic agents were tested, dofetilide and ibutilide, which in the same study prevented some of the ethidium bromide-induced mtDNA depletion found in heterozygous *polg-1* mutant worms (Pitayu et al. 2016). This suggests that this class of compound could be therapeutic for mtDNA disease. In another study, induced pluripotent stem cells were developed from human Alpers syndrome patient cells with POLG mutations (Li et al. 2015). With these cells, it was found that the mitochondrial permeability transition pore inhibitor, cyclosporine A, as well as carnitine (fatty acid transport) and N-acetylcysteine (antioxidant), could prevent valproic acid-induced toxicity. We have also been testing new and old chemical compounds on Polg mutant zebrafish, which takes advantage of an in vivo vertebrate animal model that can be treated like cells in a dish due to its very small size (Garcia et al. 2016; Rahn et al. 2014; Rahn et al. 2015).

Gene therapy

In the 1990s, infertility clinics performed ooplasm donation (and thus mitochondrial donation) to patient eggs, in order to enhance fertility (Cohen et al. 1998). This practice likely lead to unwitting heteroplasmy. However, the downstream consequences in the resulting offspring are not currently known. The practice was subsequently investigated in 2001, when the FDA stated that “ooplasm transfer protocols should be done under Investigational New Drug (IND) exemptions and that an IND submission to the agency would be required to treat additional patients” (FDA 2002). Further concerns for this technique were highlighted, it was suggested that studies into mtDNA heteroplasmy and epigenetic modifications should be completed (Sills et al. 2004; St John 2002). Today, as stated above in “Maternal inheritance of mtDNA mutations,” a modified version of this ooplasm donation method has been approved in the United Kingdom. This type of gene therapy, whereby the maternal mitochondria and mtDNA are removed as much as possible from the oocyte or embryo, and the mtDNA of the resultant offspring are derived from donor mitochondria, could greatly decrease the incidence of inherited mitochondrial diseases (Amato et al. 2014). Carryover of mtDNA from the mother has been shown to be low, about 0.5% (Tachibana et al. 2013). Other therapies to decrease inheritance of mtDNA mutation have been studied in animals and patient cells, and could potentially be used instead of the donation method. For example, Moraes and co-workers showed that heteroplasmy could be altered by using a mitochondrial-targeted restriction endonuclease specific for mtDNA mutation (Srivastava and Moraes 2001). Other mitochondrial-targeted gene editing methods, such as using transcription activator-like effector nucleases (TALENs), CRISPR/Cas9 or zinc finger nucleases could also be used to specifically remove mtDNA with mutations in patient cells (Bacman et al. 2014; Bacman et al. 2013; Jo et al. 2015; Reddy et al. 2015). In addition, pluripotent stem cells generated from patients with mtDNA disease, in addition to somatic cell nuclear transfer was successfully implemented to replace mutant mtDNA with

WT mtDNA from a donor oocyte. The resultant cells had similar transcriptomic and energetic profiles to those in embryo-derived pluripotent stem cells with WT mtDNA, which suggests that nuclear-to-mitochondrial interactions were normal and that this could be a good alternative strategy for mitochondrial gene therapy (Ma et al. 2015).

Recent studies also showed that mitochondria can move between mammalian cells to improve or restore mitochondria and mitochondrial function. In vivo, it was shown that tumor generation in mice by cells lacking mtDNA is linked to acquisition of the host mtDNA (Tan et al. 2015). Recent work by the same group have now demonstrated that horizontal mtDNA transfer from donor cells to mtDNA depleted cells enabled increased mitochondrial respiration, which was essential for these cells to form tumors (Berridge et al. 2015; Dong et al. 2017). These findings highlight new and exciting cellular mechanisms, such as nanotube transmission of mtDNA from one cell to another (Rogers and Bhattacharya 2013; Rustom 2016; Rustom et al. 2004), that could be targeted to develop new and specific treatments of mitochondrial diseases.

4. Conclusions and Future Directions

There is still much that needs to be learned and discovered about mtDNA stability and how to maintain this in vivo. A major question that still needs work is the disconnect between POLG mutations and disease presentation. How do patients with the same homozygous mutation present with such disparate disease phenotypes, and why might one patient present with disease shortly after birth, while another might present with disease only in late adulthood? Concurrent genetic and environmental factors will be important for understanding disease progression; likewise, the mechanisms by which damaged mtDNA are removed or maintained needs further study. An excellent review by Mishra and Chan discusses the role of inheritance during organismal development, where stochastically uneven inheritance of mtDNA and mtDNA mutations could contribute to patients with the same mutation showing differential effects in different tissues (Mishra and Chan 2014). Interestingly, Koczor and co-workers with the heart-targeted POLG Y955C transgenic mouse recently showed that POLG mutation can lead to changes in gene expression and nuclear DNA methylation (Koczor et al. 2016). This suggests that mtDNA stability can indeed play a role in epigenetic modifications and may be important in the context of the wide range of phenotypes observed epidemiologically. Additionally, the newly developed models for mtDNA diseases can be used to screen for new mitochondrial targeted inhibitors and activators, which can be used not only for therapeutic purposes, but also for building an understanding of new mechanisms for promoting mtDNA stability.

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