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Review

Mitochondrial DNA mutations and human disease

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ABSTRACT

Mitochondrial disorders are a group of clinically heterogeneous diseases, commonly defined by a lack of cellular energy due to oxidative phosphorylation (OXPHOS) defects. Since the identification of the first human pathological mitochondrial DNA (mtDNA) mutations in 1988, significant efforts have been spent in cataloguing the vast array of causative genetic defects of these disorders. Currently, more than 250 pathogenic mtDNA mutations have been identified. An ever-increasing number of nuclear DNA mutations are also being reported as the majority of proteins involved in mitochondrial metabolism and maintenance are nuclear-encoded. Understanding the phenotypic diversity and elucidating the molecular mechanisms at the basis of these diseases has however proved challenging. Progress has been hampered by the peculiar features of mitochondrial genetics, an inability to manipulate the mitochondrial genome, and difficulties in obtaining suitable models of disease. In this review, we will first outline the unique features of mitochondrial genetics before detailing the diseases and their genetic causes, focusing specifically on primary mtDNA genetic defects. The functional consequences of mtDNA mutations that have been characterised to date will also be discussed, along with current and potential future diagnostic and therapeutic advances.

1. Introduction

Mitochondria are essential double-membraned subcellular organelles, present in all nucleated mammalian cells. Their primary function is to support aerobic respiration and produce, by oxidative phosphorylation (OXPHOS), the bulk of cellular ATP [1]. The OXPHOS machinery is made up of over 80 different polypeptides, organised into five transmembrane complexes. Electrons, resulting from oxidation of fat and carbohydrates, are shuttled to oxygen along the first four respiratory chain (RC) complexes (CI–CIV), producing water, and providing the energy necessary to pump protons across the inner membrane (IMM) from the matrix to the intermembrane space. The electrochemical gradient thus created enables the fifth RC complex, ATP synthase, to phosphorylate ADP forming ATP. In addition to OXPHOS metabolism, mitochondria control cytosolic calcium concentration [2] and regulate apoptotic cell death [3]. They are the major source of endogenous reactive oxygen species (ROS) [4] and they host other important biochemical pathways, including the tricarboxylic acid (TCA) cycle and parts of the urea cycle [5]. Furthermore, these organelles are central to iron–sulphur cluster biogenesis, the only entirely conserved function of eukaryote mitochondria [6].

Over the last 20 years, mitochondrial dysfunction has been increasingly recognised as an important contributor to an array of neuromuscular and neurodegenerative diseases. Because mitochondria are under the dual genetic control of both the mitochondrial and nuclear genomes, mutations within either DNA molecule may result in a RC deficiency. For this review, we will focus specifically on primary mitochondrial DNA (mtDNA) mutations in relation to human disease. Reviews on nuclear gene defects in mitochondrial disorders are available [7–9]. After outlining the peculiarities of mitochondrial genetics and describing the clinical syndromes caused by mtDNA mutations, we review the current understanding of the molecular mechanisms of mitochondrial pathogenesis. Finally, recent advances in the development of potential therapeutic approaches will also be discussed.

2. Basic mitochondrial genetics

Mitochondrial genetics differ considerably from Mendelian genetics. Uniparental inheritance, cellular polyploidy and a deviation from the standard genetic code are just some of the peculiarities of mitochondrial genetics. These unique features strongly dictate the functional consequences of pathogenic mtDNA mutations.

2.1. Mitochondrial genome

Considered evolutionary relics of ancient bacterial symbionts, mitochondria have gradually transferred the vast majority of their genetic material to the nucleus [10]. All that remains is a compact (16,569 bp), double-stranded (cytosine-rich light (L) and guanine-rich

Abbreviations: 2-DE, two-dimensional electrophoresis; DHU, dihydrouridine; H-strand, heavy strand; IMM, inner mitochondrial membrane; L-strand, light-strand; OXPHOS, oxidative phosphorylation; mtDNA, mitochondrial DNA; PD, Parkinson's disease; PGD, preimplantation genetic diagnosis; RC, respiratory complex; ROS, reactive oxygen species; TCA, tricarboxylic acid

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heavy (H) strands), circular genome, the complete sequence of which was fully elucidated in 1981 [11] and further revised in 1999 [12]. Several hundreds or thousands of copies of the mitochondrial genome are present in a single cell. When all mtDNA molecules within a cell are identical, a situation known as homoplasmy prevails. The presence of two or more mitochondrial genomes results in heteroplasmy.

Originally believed to be discrete entities with their own individual 'naked' and free-floating genetic identity, mitochondria are now recognised to be dynamic organelles that continuously move, fuse, and divide [13]. Multiple copies (~6–10) of mtDNA are organised in stable protein–DNA macrocomplexes, termed nucleoids, which are primarily IMM associated [14,15] and may be exchanged between mitochondria. Key proteins involved in mtDNA replication, maintenance, repair, and recombination, such as mitochondrial single-stranded binding protein (mt-SSB), mitochondrial polymerase γ (POLG), and mitochondrial transcription factor A (TFAM), are just some of the major constituent proteins associated with these structures [15–17].

Strictly inherited through the maternal lineage [18], human mtDNA contains only 37 genes, which encode 13 polypeptides, all core subunits of RC complexes I, III, IV, and V, and the RNA necessary for mtDNA translation, namely 2 rRNAs (12S and 16S) and 22 tRNAs. Nuclear genes encode the remaining ~70 OXPHOS components, and all other proteins required for mitochondrial metabolism and maintenance, which are imported to mitochondria via specialised import systems [19]. There are no introns within mtDNA genes and almost no intergenic noncoding nucleotides exist, with the exception of the 1.1 kb displacement loop (D-loop), which contains transcriptional promoters and at least one of the proposed replication origins (O_H) (Fig. 1).

2.2. MtDNA maintenance: replication, transcription, and translation

It is largely accepted that mtDNA is continuously turned over, independently of the nuclear genome, and its replication does not coincide with the cell cycle [20]. Evidence of mtDNA replication in postmitotic tissues (e.g., brain, skeletal muscle) supports this view [21]. However, increasing reports on the relationship of mitochondrial function to the cell cycle [22–24] suggest a connection between mtDNA replication and the cell cycle may well actually exist.

The exact mechanism of human mtDNA replication is still uncertain. Two distinct mechanisms have been proposed. In the traditional strand-asynchronous method, replication initiates at the heavy strand origin (O_H), causing displacement of the L-strand from the H-strand. The former remains single-stranded whilst replication of the H-strand proceeds almost two-thirds of the way around the mitochondrial genome, until exposure of the L-strand origin (O_L). L-strand synthesis can then begin in the opposite direction [25]. A second more conventional replication method has been suggested in which coupled leading-lagging strand DNA replication, also initiating at O_H, is coordinated [26,27]. In this model, lagging L-strand synthesis

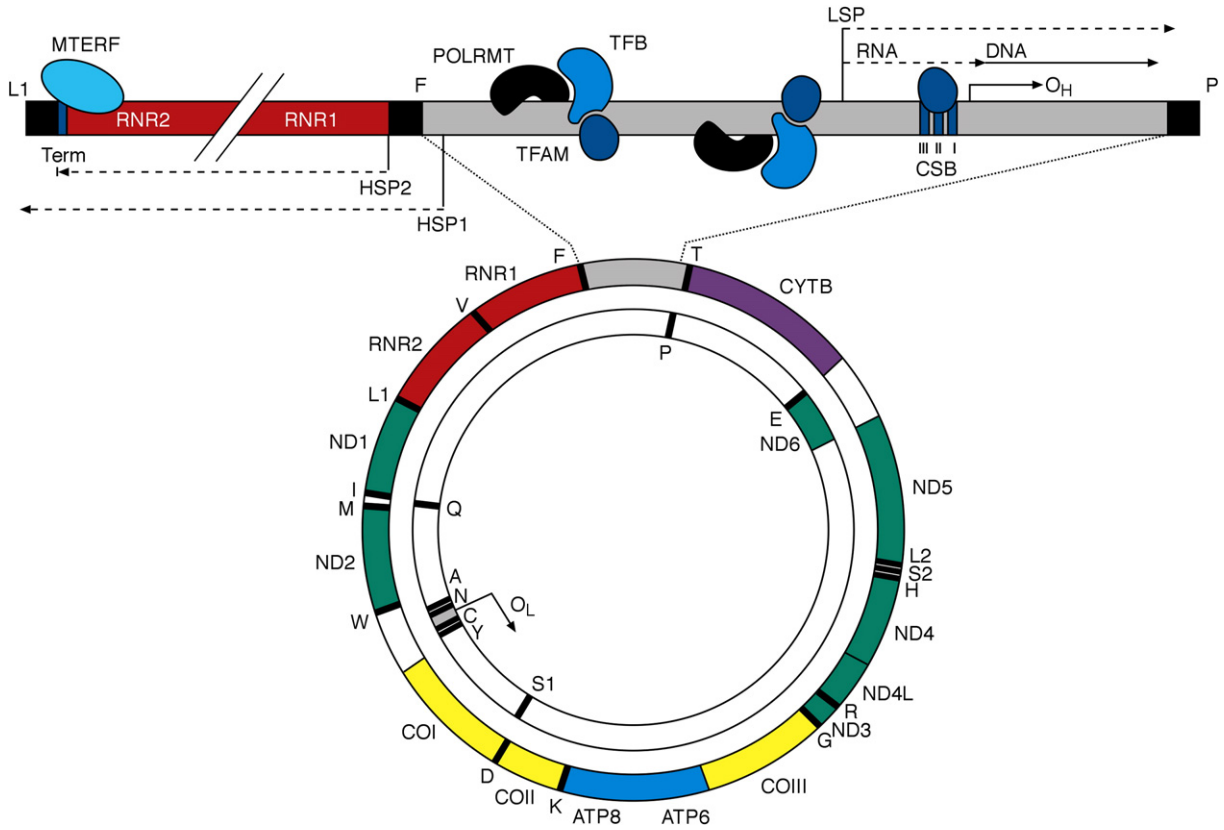

Fig. 1. Human mitochondrial genome. Represented is a schematic diagram of the 16.6 kb circular, double-stranded human mitochondrial genome with an enhanced view of the mammalian D-loop and transcription termination regions, shown in linear form. The circle represents the heavy (H) strand of the genome and the inner circle the light (L) strand. Human mtDNA encodes the 2 mt-rRNAs (red) RNR1 (12S rRNA) and RNR2 (16S rRNA), 22 mt-tRNAs (black bars), identified by their single letter abbreviation, and 13 essential RC polypeptides: ND1-ND4 and ND4L, subunits of complex I (green), CYTB, a subunit of complex III (purple), COI-COIII, catalytic subunits of complex IV (yellow), and ATP6 and ATP8, subunits of complex V (blue). Major noncoding regions of the genome (grey) include the 1.1 kb D-loop, and the origin of L-strand replication (O_L). The origin of H-strand replication is indicated within the D-loop (O_H). H-strand transcription is initiated either from HSP1, generating a short transcript that terminates at the RNR2/MTTL1 boundary (Term) under the guidance of the transcription termination factor MTERF, or from HSP2, generating polycistronic transcripts of the entire H-strand. LSP denotes the L-strand initiation point that produces polycistronic transcripts for this strand and also generates RNA precursors for H-strand replication initiation. Conserved sequence blocks (CSBs I-III) are conserved regions in human, mouse and rat that participate in the formation of RNA primers for replication. Transcription from all promoters requires the upstream binding of transcriptional activator TFAM, together with a single subunit RNA polymerase (POLRMT), which forms a heterodimeric complex with the transcription factor TFB2M (depicted as TFB). TFAM also binds to other regions of the D-loop; however, only binding to the CSB region is shown.

begins shortly after replication initiation and is the result of the generation of short Okazaki ribonucleotide fragments, subsequently converted to DNA [28]. The exact mechanism of mtDNA replication is still debated. However, it is likely that both models exist and their occurrence may be cell type-dependent, with the leading-lagging strand model more prevalent in cells which are in a steady-state and the strand-displacement mechanism more predominant in cells requiring rapid mtDNA synthesis (e.g., cells recovering from mitochondrial depletion) [26,27,29].

MtDNA replication is accomplished by nuclear-encoded factors. Core components of the mitochondrial replisome include POLG, consisting of a catalytic subunit with 5′–3′ exonuclease activity (PolgA) and a processivity subunit (PolgB), Twinkle, with 5′–3′ DNA helicase activity, and mt-SSB [30].

Mitochondrial transcription is initiated from one of two promoters on the H-strand (HSP1 and HSP2) and from a single promoter on the L-strand (LSP) (Fig. 1). Transcription from HSP2, located close to the 5′ end of the *MT-RNR1* gene, and from LSP, situated in the D-loop, generates almost genome-length polycistronic transcripts, which are processed to produce the individual mRNA and tRNA molecules [31,32]. HSP1 is located upstream of the *MT-TF* gene and produces only the two rRNAs, 12S and 16S, and the two mt-tRNAs, mt-tRNA^{Phe} and mt-tRNA^{Val}. The basal transcription machinery consists of a limited number of nuclear-encoded proteins: the dedicated mitochondrial RNA polymerase (POLRMT), the transcription activator TFAM, the transcription factor TFB2M (but probably not TFB1M as previously thought [33,34]), and the termination factor mTERF [35]. In-depth reviews on mtDNA transcription are available [36,37].

Our current understanding of the mechanistic details of human mitochondrial translation is far from complete. It is known to involve nuclear-encoded factors imported into the organelle. Besides ribosomal proteins, aminoacyl synthetases, and mt-tRNA modification proteins, these include two mitochondrial initiation factors (IF2 and IF3) [38,39], four mitochondrial elongation factors (EFG1, EFG2, EFTs, and EFTu) [40–42], and at least one termination release factor, mtRF1a [43]. Other recently identified nuclear-encoded factors are the translation regulator PTCD3 [44], the ribosome recycling factor mtRRF [45,46], and the methionine aminopeptidase MAP1D, which removes N-terminal methionines [47]. Mitochondrial translation is characterised by a number of distinct features including RNA-poor mitoribosomes [48], bicistronic transcripts [49], partial stop codons in some of the transcripts that are completed by polyadenylation [31,50], and deviation from the standard genetic code.

3. Clinical features of human mtDNA disease

The mitochondrial genome has a very high mutation rate, 10- to 17-fold higher than that observed in nuclear DNA. Although mtDNA repair systems do exist [51,52], they are not sufficient to counteract the oxidative damage sustained by the mitochondrial genome due to its proximity to the RC complexes in the IMM and the ROS they generate. Protective histones are also lacking.

Most mtDNA alterations are neutral polymorphisms, which have proved useful in tracking human migrations [53]. The first pathogenic mtDNA mutations were identified in 1988 [54,55]. Since then, over 250 pathogenic mtDNA mutations (point mutations and rearrangements) have been characterised [56], shown to cause a wide variety of diseases with a heterogeneity of phenotypes and a variable age of onset [57]. The exact prevalence of mtDNA disease is notoriously difficult to ascertain due to clinical heterogeneity of mitochondrial diseases and the plethora of known causative mutations. Estimates from the North East of England suggest that as many as 1 in 10,000 people have clinically manifesting mtDNA disease with a further 1 in 6000 at risk [58]. Recent birth prevalence studies have reported mutation frequencies of 0.14% for the m.3243A>G mutation [59] and ~0.2% for the m.1555A>G *MT-RNR1* mutation associated with aminoglycoside-induced sensorineural hearing loss [60,61], suggesting the perceived prevalence of mtDNA mutations remains an underestimate.

A complete description of all known pathogenic mtDNA mutations is beyond the scope of this review and a current compendium can be found on the MitoMAP database [56]. Salient features of mtDNA disease and their most common causative mutations (Fig. 2) will be described.

3.1. Specific features of mitochondrial disease

The onset of clinical symptoms, phenotypic variability, and variable penetrance of mitochondrial diseases are governed by a number of factors, including the threshold effect, mitotic segregation, clonal expansion, and a genetic bottleneck.

3.1.1. Threshold effect

Some deleterious mtDNA mutations are homoplasmic; however, the vast majority are found in some but not all genomes. In the presence of heteroplasmy, the ratio of wild-type to mutant mtDNA determines the onset of clinical symptoms. A minimum critical proportion of mutated mtDNAs is necessary before biochemical defects and tissue dysfunction become apparent. This threshold level varies for each mutation and differs amongst tissues, being lower in tissues highly dependent on OXPHOS metabolism than in tissues that can rely on anaerobic glycolysis. Typically, the threshold value is in the range of 60%–90% mutant to wild-type mtDNA. Although the threshold level can partly explain the disease phenotypes observed in patients, an exact correlation between clinical severity and the proportion of mtDNA mutation is lacking [62].

3.1.2. Mitotic segregation

During mitosis, mitochondria are randomly segregated, and in heteroplasmic cells, the proportion of mutant mtDNA in the daughter cells can thus shift. Should the mutant load exceed the pathogenic threshold for that tissue, clinical expression of the disease can occur. Conversely, mutant mtDNA may be lost, particularly in fast-dividing tissues. For example, an average annual 1% decrease in m.3243A>G mutation levels in blood has been described [63].

3.1.3. Clonal expansion

Clonal expansion refers to the preferential amplification of mtDNA mutations to a high level in post-mitotic tissues [64,65]. This expansion is thought to be a result of random genetic drift, dependent on relaxed replication of the mitochondrial genome [66].

3.1.4. MtDNA bottleneck

The observation of a rapid segregation in mammalian heteroplasmic mtDNA genotypes between generations, with a return to homoplasmy in some progeny, has suggested the existence of a mtDNA bottleneck during development [67–72]. The precise mechanism by which this genetic bottleneck occurs is currently being fiercely debated. A prevalent hypothesis is that the bottleneck occurs during embryonic development, driven by a marked reduction in mtDNA copy number in the germ line [73,74]. Paradoxically, it has also been suggested that the bottleneck occurs without reduction of germ line mtDNA copy number, but is a result of the preferential replication of a subgroup of mtDNAs during oogenesis [75]. Finally, a third alternate view on the genetic bottleneck has recently been proposed [76]. From observing the variations in mtDNA heteroplasmy and copy number that occur throughout oogenesis in single germ cells, it would seem the genetic bottleneck may actually take place during postnatal folliculogenesis and not during embryonic oogenesis, as a result of replication of a subpopulation of mitochondrial genomes. Further investigations are necessary to clarify the exact nature of the mtDNA bottleneck.

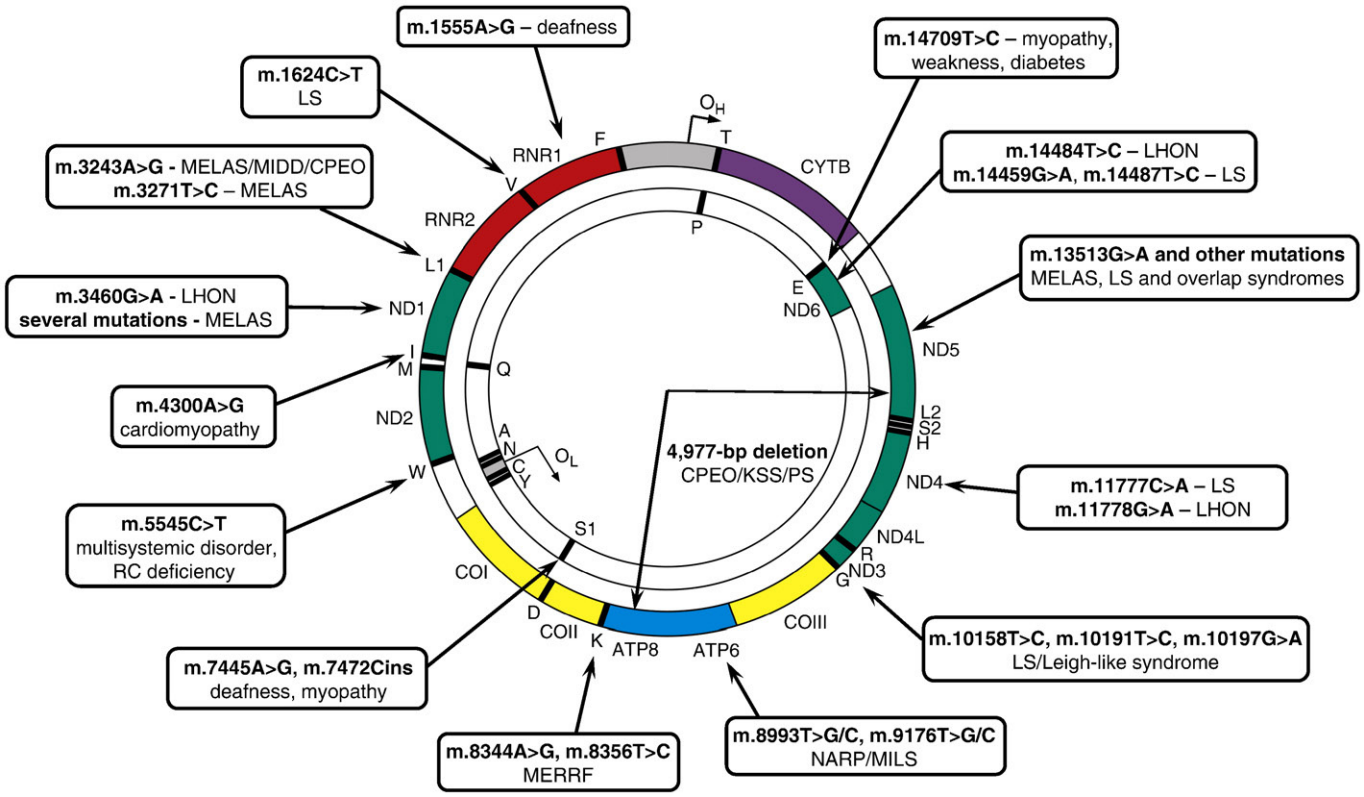

Fig. 2. Genotype:phenotype correlations in human mitochondrial disease. The circular, double-stranded human mitochondrial genome is depicted with sites of common mtDNA mutations highlighted. Additionally, two further mtDNA point mutations described in this review, m.1624C>T [77] and m.5545C>T [83], are shown. Associated clinical presentations are also highlighted. CPEO, chronic progressive external ophthalmoplegia; LHON, Leber hereditary optic neuropathy; LS, Leigh syndrome; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy and ragged red fibres; MILS, maternally inherited Leigh syndrome; NARP, neurogenic weakness, ataxia, and retinitis pigmentosa; PS, Pearson syndrome.

3.2. mtDNA mutations

3.2.1. Point mutations

MtDNA point mutations are usually maternally inherited. They may occur within protein, tRNA, or rRNA genes. However, more than half of disease-related point mutations reported are located within mt-tRNA genes. Phenotypically, point mutations in mitochondrial protein-coding genes specifically affect the function of the RC complex to which the corresponding protein belongs, whereas mt-tRNA mutations may impair overall mitochondrial translation by reducing the availability of functional mt-tRNAs.

Point mutations are mostly heteroplasmic, displaying considerable clinical heterogeneity, and are considered highly recessive. However, an increasing number of pathogenic homoplasmic mutations, often affecting just a single tissue and characterised by incomplete penetrance, are being recognised [77–82]. Given the high mutational rate of the mitochondrial genome and the presence of numerous family- or population-specific polymorphisms, the distinction between neutral mtDNA variant and disease-causing mutation can often be difficult. To complicate matters further, a dominant mt-tRNA mutation, m.5545C>T in the *MT-TW* gene, was recently reported with levels of mutation at <25% in clinically affected tissues [83]. Also, it has been frequently recognised that certain nucleotide changes that are not pathogenic per se may modulate the effects of deleterious mtDNA mutations [84,85].

3.2.2. MtDNA rearrangements

The majority of mtDNA rearrangement mutations are large-scale deletions, which vary in size from 1.3 to 8 kb and span several genes [86]. Single mtDNA deletions occur sporadically early in development, and the identical deletion is present in all cells within affected tissues [87]. The occurrence of multiple mtDNA deletions of varying lengths in affected tissues may be due to inherited mutations in nuclear genes, whose products are involved in mtDNA maintenance and replication (e.g., *POLG* and *PEO1* encoding Twinkle) and mitochondrial nucleotide metabolism (e.g., *SLC25A4*) [88–90]. An exponential accumulation of multiple mtDNA deletions has also been reported in aged postmitotic tissues and individuals with neurodegenerative diseases [91–93].

Despite different origins, most mtDNA deletions share common features. They mainly occur between the origins of replication, O_H and O_L, and are typically flanked by short direct repeats [94,95]. Their mechanism of generation is also thought to be identical, irrespective of the clinical phenotype [96], although the exact method by which deletions are formed is currently under debate. Whilst most researchers consider replication to be the most likely mechanism of deletion formation [97], Krishnan et al. [98] recently proposed that mtDNA deletions arise during the repair of damaged mtDNA.

The amount and tissue distribution of the deleted mtDNA are the most important factors in determining clinical symptoms, and not, as one might expect, the size and location of the deletions [99–101].

3.3. Clinical syndromes

The clinical syndromes associated with mtDNA mutations are extremely variable and patients can present at any stage in life. On the whole, the age of onset reflects the level of mutation and the severity of the biochemical defect, but other factors (presumably nuclear genetic or environmental) also effect the expression of disease. For the purposes of this review, we will concentrate solely on primary mtDNA diseases, the most common of which are depicted in Fig. 2, together with their associated mutations. It should be noted that

increasing evidence supports the involvement of accumulated mtDNA mutations in ageing, neurodegeneration, and tumourigenesis. However, an in-depth review of the role of mitochondrial mutations in these diseases is beyond the remit of this article. For excellent recent reviews on these topics, see references [102–104].

3.3.1. Mitochondrial diseases with onset in early infancy/childhood

3.3.1.1. Leigh syndrome. Leigh syndrome is a progressive neurodegenerative condition, which particularly affects the brainstem, diencephalon, and basal ganglia. There are characteristic neuropathological features, but newer neuroimaging techniques can now easily detect these lesions in life. Clinically, these infants and children have signs of brainstem and basal ganglia dysfunction and often deteriorate in a stepwise manner. Leigh syndrome is due to severe failure of oxidative metabolism and can be due to a variety of different genetic defects affecting either the mitochondrial (e.g., m.8993T>C/G, m.10158T>C, m.10191T>C) or nuclear genome (e.g., *SURF1* gene) [105].

3.3.1.2. Depletion syndromes. The clinical features associated with depletion syndromes depend upon the organ(s) which have mtDNA depletion. On the whole, these are severe disorders and present in childhood with severe muscle weakness, progressive encephalopathy, or liver failure. There are a number of different genetic defects identified in these patients and the clinical syndromes often reflect the genetic defect [106]. In view of the tissue-specific nature of the defect, these patients may respond to organ transplantation.

3.3.1.3. Kearns–Sayre syndrome (KSS). KSS is associated with the development of retinitis pigmentosa and progressive external ophthalmoplegia occurring before the age of twenty. Clinical examination usually detects a 'salt and pepper' retinopathy of the posterior fundus without the visual field defects, optic disk pallor, and attenuation of retinal vessels usually seen in retinitis pigmentosa. KSS is a multisystem disorder caused by single, large-scale deletions [107]. Patients often develop other neurological complications including cerebellar ataxia, cognitive impairment and deafness, as well as non-neurological features of cardiomyopathy, complete heart block, deafness, short stature, endocrinopathies, and dysphagia.

3.3.1.4. Pearson syndrome. This is a rare disorder of infancy characterised by sideroblastic anaemia with pancytopaenia and exocrine pancreatic failure. The clinical course in these children can be severe leading to early death. In those that survive the blood disorder improves but they later develop the clinical features of KSS. In these children, there is a very high level of large-scale single mtDNA deletion present in all tissues [108].

3.3.2. Mitochondrial diseases with onset in late childhood or adult life

3.3.2.1. Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). These patients often present with stroke-like episodes with seizures. They particularly affect the parieto-occipital region of the brain leading to visual field defects. Their lesions do not match a recognised vascular distribution, highlighting that they are not simple ischemic lesions. Seizures are frequent in these patients associated with the episode or as isolated phenomena. Other features include intermittent episodes of encephalopathy, vomiting, migraine like headaches, ataxia, and cognitive impairment. Over 80% of patients with MELAS have the m.3243A>G mutation in the *MT-TL1* gene [109,110]. Other mutations in this mt-tRNA gene (e.g., m.3271T>C) [111], other mt-tRNA genes (e.g., m.1642G>A, *MT-TV* gene) [112] and protein-encoding genes (e.g., m.9957T>C in the *MT-CO3* gene [113], several *MT-ND5* mutations (m.12770A>G, m.13045A>C, m.13513G>A and m.13514A>G) [114–117] and *MT-ND1* mutations [118]) may also cause MELAS.

3.3.2.2. Chronic progressive external ophthalmoplegia (CPEO). One of the most common presentations of mtDNA disease in adults is CPEO. CPEO is characterised by a progressive paralysis of the eye muscles leading to impaired eye movement and ptosis. Ptosis is frequently the presenting symptom and may be asymmetrical; however, patients usually progress to bilateral disease. CPEO is typically caused by sporadic large-scale single deletions or multiple mtDNA deletions [119,120], although mtDNA point mutations are detected in some patients (e.g., m.3243A>G [121], m.12316G>A [122]). In patients with CPEO, there may be other features depending on the underlying genetic defect but myopathy and fatigue are common in all patients.

3.3.2.3. Neuropathy, ataxia, and retinitis pigmentosa (NARP). This combination of symptoms has been described in several families and is usually due to the *MT-ATP*6 m.8993T>G mutation. Although peripheral neuropathy is the principle feature of this phenotype other neurological complications include developmental delay, seizures, and dementia. It has subsequently been recognised that patients with a mutant load greater than 95% m.8993T>G have an onset in childhood with maternally inherited Leigh syndrome (MILS) [123]. This reflects the importance of the degree of heteroplasmy influencing the phenotype, at least with this mtDNA mutation.

3.3.2.4. Leber's hereditary optic neuropathy (LHON). There are three primary LHON mtDNA mutations (m.11778G>A, m.3460G>A, and m.14484T>C), which in total are present in at least 95% of LHON cases [56,124]. LHON is predominantly an organ-specific disease, targeting the retinal ganglion cells of the optic nerve. Clinically, this presents with a subacute or acute, painless, central visual loss, which is typically unilateral with the other eye usually becoming affected within the next 2 months. This visual loss usually occurs between the ages of 20 and 40 and is more common in men. LHON is usually due to a homoplasmic mtDNA mutation and all maternal offspring will inherit the mutation; however, whilst 50% of males will be affected, only 10% of females will develop visual loss. The progression of the disease seems to depend upon the responsible mutation with 71% of patients with the m.14484T>C mutation showing some recovery, compared to 25% with m.11778G>A [125]. Whilst the majority of patients with m.11778A>G who have symptoms develop LHON, there have been a few reports of other neurodegenerative phenotypes including early-onset dystonia [80].

3.3.2.5. Myoclonic epilepsy and ragged red fibres (MERRF). MERRF is a progressive, neurodegenerative disease caused most commonly by a point mutation in the *MT-TK* gene, m.8344A>G [126–128]. Clinically, MERRF is a severe neurodegenerative disorder, which often presents in childhood or early adulthood following normal development. The characteristic myoclonus is often the presenting symptom. This progresses into a mixed picture of myopathy, often with pronounced proximal muscle wasting in a limb-girdle distribution, and central neurological features of focal and generalised epilepsy, cerebellar ataxia, optic atrophy, pyramidal signs, and hearing loss. Non-neurological manifestations include re-entrant atrioventricular tachycardias such as Wolff–Parkinson–White syndrome and multiple lipomas.

4. Diagnosis of mtDNA disease

Complicated by mtDNA heteroplasmy, the lack of clear genotype: phenotype correlations in many patients and the complex interactions between the nuclear and mitochondrial genome, the laboratory diagnosis of mtDNA disease often involves piecing together information from clinical, histochemical, and biochemical testing to inform molecular testing, allowing the development of rational diagnostic algorithms [129,130]. Family history and characteristic, histological changes in affected tissues are key signs that may prompt the

investigation of mitochondrial disease, but these are not always evident. Defining whether the primary molecular defect resides within the mitochondrial genome itself (single, large-scale mtDNA rearrangement or mtDNA point mutation) or in a nuclear gene—leading to secondary mtDNA abnormalities such as loss of mtDNA copy number or multiple mtDNA deletions—ultimately provides key information to families and clinicians concerning likely inheritance patterns, recurrence risks, and disease prognosis. Whilst by no means exhaustive, this short section provides a summary of the key histochemical and molecular approaches to making a mitochondrial genetic diagnosis.

4.1. Histochemical and biochemical testing

Many, but not all, patients with mtDNA disease show histological and histochemical changes in affected tissue, indicative of RC dysfunction. Classically, these are ragged red fibres—so called because of the appearance of fibres on Gomori trichrome staining showing subsarcolemmal accumulation of abnormal mitochondria—and cytochrome *c* oxidase (COX)-deficient fibres (Fig. 3). The COX enzyme contains subunits encoded for by both the mitochondrial and nuclear genome, and as such, a mosaic pattern of COX activity—where the COX-deficient fibres may also be "ragged red"—is highly suggestive of mtDNA involvement. There are notable exceptions to this rule; biopsies from patients with the MELAS phenotype and/or mutations in complex I and complex III mtDNA-encoded subunits may be normal or exhibit COX-positive ragged red fibres. A global decrease in the activity of COX is usually suggestive of a nuclear mutation in an ancillary protein required for COX assembly such as *SURF1* but can be associated with some homoplasmic mt-tRNA defects [77,78]. The sequential reaction of COX and SDH histochemical activities is helpful to identify low levels of COX-deficient fibres, although care must be made in the interpretation of these as the somatic accumulation of mtDNA mutation with age also leads to focal, COX-deficient fibres in muscle and other postmitotic tissues. Biochemical testing of clinically affected tissue will often involve the determination of individual RC enzyme complex activities in mitochondrial fractions or enriched homogenates prepared from fresh or frozen tissue samples [131]; some labs also investigate functional testing of intact organelles to include measurements of mitochondrial substrate oxidation, oxygen consumption, and ATP synthesis [132]. The identification of isolated or multiple RC abnormalities further narrows the options for identifying the molecular genetic defect.

4.2. Molecular genetic testing

Diagnostic algorithms and protocols are available for the molecular investigation of patients with suspected mtDNA disease [130,133,134]. Some mutations—mtDNA rearrangements and certain mtDNA point mutations—are only present in clinically affected tissues, necessitating the work-up on postmitotic (muscle) DNA samples. MtDNA rearrangements (deletions and duplications) have historically been assessed by Southern blotting [54], although long-range PCR is now often the method of choice. The presence of multiple mtDNA deletions would indicate the screening of appropriate nuclear mtDNA maintenance genes including *POLG*, *POLG2*, *SLC25A4*, *PEO1*, and *OPA1* [135]; the demonstration of a quantitative loss of mtDNA—usually by real-time PCR with reference to a nuclear housekeeping gene—is indicative of a recessive mtDNA depletion disorder; several known genes are now implicated, leading to either a myopathic or hepatocerebral phenotype [106].

Common mtDNA point mutations (m.3243A>G, m.8344A>G, m.8993T>G/C, and LHON mutations) are readily screened in blood using restriction fragment length polymorphism (RFLP) analysis, with the addition of a radio- or fluorescent label in the final PCR cycle to permit accurate quantification of mtDNA heteroplasmy; however, the m.3243A>G mutation is positively selected against in this tissue, prompting the investigation of other noninvasive tissue samples for diagnosis [136,137]. The assessment of m.3243A>G mutation load in urinary sediments not only provides a correlate with the level in muscle but also has proven to be an accurate indicator of clinical outcome for these patients [138].

Due to its small size, screening the entire mitochondrial genome can be undertaken relatively easily to identify rare or novel mtDNA mutations in patients with suspected mtDNA disease. Denaturing gradient gel electrophoresis (DGGE) [139,140] and denaturing high-performance liquid chromatography (dHPLC) [141,142] have been available for some years, whilst emerging technologies include resequencing microarrays such as the Affymetrix MitoChip 2.0 [143,144]. Many laboratories, however, continue to directly sequence the entire mitochondrial genome in affected tissues, using overlapping primer sets to ensure complete coverage of the 16.6 kb genome [145]. Given the highly polymorphic nature of the mitochondrial genome, careful assessment of newly identified mtDNA sequence variants must be undertaken to establish a clear link with human disease. A number of canonical criteria to support pathogenicity for a novel mtDNA variant, originally proposed by DiMauro and Schon [146] and applied by others [147,148], should be considered (Fig. 4) and a pathogenic role only assigned once sufficient evidence is available [149].

5. Functional characterisation of mtDNA mutations

Pathogenic mtDNA mutations invariably result in a general defect in mitochondrial respiration that leads to a reduced ability to produce cellular ATP. The clinical phenotypes of mitochondrial disorders are however extremely heterogeneous. Consequently, it is unlikely that a reduced respiration rate is solely responsible for this variability, but rather additional molecular mechanisms must also contribute to the

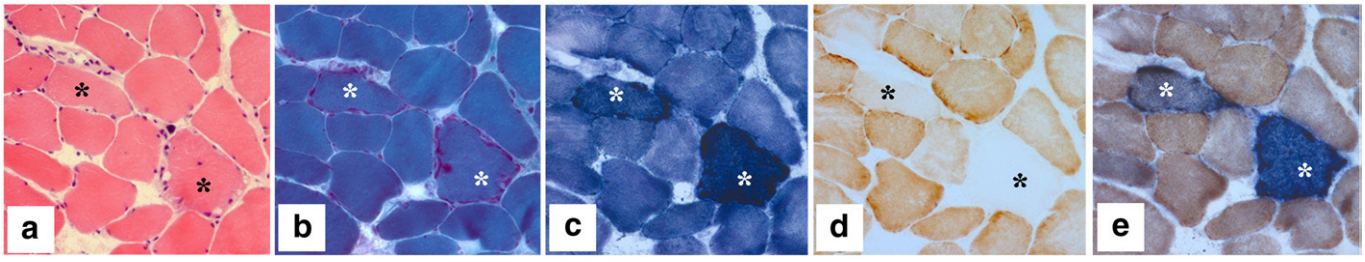

Fig. 3. Histopathological abnormalities associated with mtDNA disease. The histological and histochemical assessment of clinically affected tissue—often but not exclusively skeletal muscle—provides useful clues in the laboratory diagnosis of mtDNA disease. Two fibres (highlighted by an asterisk) are shown in this sequential series of transverse muscle sections from a patient with a single, large-scale mtDNA deletion highlighting the following stains or activities; a, H&E to show general muscle morphology; b, modified Gomori trichome stain highlighting classical ragged red muscle fibres; c, SDH, which reveals the subsarcolemmal accumulation of mitochondrial activity, is exclusively found in mitochondria; d, COX histochemistry showing COX-deficient fibres within a population of normal fibres, a typical "mosaic" distribution; e, sequential COX/SDH histochemistry highlighting individual COX-deficient fibres which retain SDH activity.

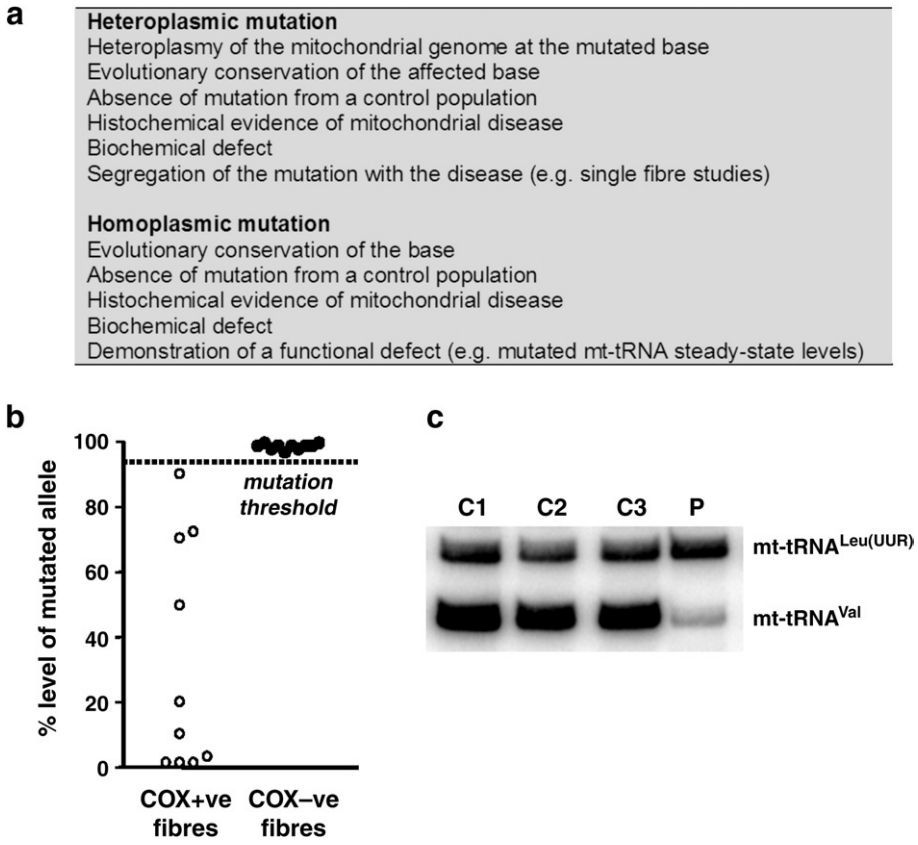

Fig. 4. Determination of mtDNA mutation pathogenicity. a, Accepted canonical criteria for assigning pathogenicity to novel mtDNA mutations. b, Example of the segregation of a specific mtDNA point mutation, m.15967G>A, with biochemical deficiency in individual cells as a tool for assigning pathogenicity. Graphical representation of single muscle fibre polymerase chain reaction data demonstrate a clear segregation of high mutation load in the COX-deficient muscle fibres [256]. c, Example of an mt-tRNA functional defect. High-resolution Northern blot of patient (P) and control (C) muscle total RNA demonstrates significantly reduced steady-state levels of mutated mt-tRNA^{Val} harbouring the m.1624C>T mutation.

development and clinical presentation of disease. In order to design and develop effective therapeutic approaches for mitochondrial disorders, it is important to uncover the biochemical and molecular mechanisms linking genotype to phenotype. Identification of the diverse functional effects of mtDNA mutations has proved challenging. Firstly, engineering mtDNA is problematic due to its polyploidy, inaccessibility within a double-membraned organelle, and apparent lack of, or minimal, recombination, which have prevented the successful transfection of autonomously replicating foreign DNA plasmids or modified mtDNA into mitochondria to date. Secondly, the development of suitable models of disease that accurately portray the pathogenesis of mitochondrial diseases has been quite slow. Only very recently has a naturally occurring canine model of mitochondrial disease been reported. A single heteroplasmic mt-tRNA^{Tyr} deletion (m.5304delT of dog mtDNA) has been found to be responsible for the neurological disorder sensory ataxic neuropathy in golden retrievers [150]. Whilst no human equivalent of this mutation has yet been identified, the model may be of use in the development of therapeutic approaches for human mitochondrial diseases.

Despite these difficulties, considerable progress has been made over the last few years in understanding the molecular mechanisms of mitochondrial disease. The experimental models that have enabled these developments will first be discussed, followed by the current understanding of mitochondrial pathogenesis.

5.1. Experimental models of mitochondrial disease

5.1.1. Transmitochondrial cybrids

In vitro models of mitochondrial disease are achieved through fusion of enucleated cytoplasts (derived from patient cell lines) with immortalised human cell lines, devoid of endogenous mtDNA (ρ^0 cells). Following pioneering work by Morais and colleagues between 1985 and 1988 establishing avian ρ^0 cells [151,152], the first transmitochondrial cytoplasmic hybrid cells (cybrids) using human ρ^0 cells were described in 1989 [153]. Cybrids have since proved an excellent tool for studying mtDNA mutations. By allowing selected mtDNA sequences to be expressed against fixed nuclear backgrounds, not only can cybrids be employed for establishing and/or confirming the genetic origin of a mitochondrial disorder, but they can also provide a controlled environment in which to study both the functional and physiological consequences of varying levels of heteroplasmy of specific mtDNA mutations as well as the precise molecular mechanisms by which these mutations impair cellular function. In addition, these cell models have challenged our established criteria for pathogenicity with the identification of the first mt-tRNA suppressor mutation, m.12300G>A in *MT-L2* [154], and the first functionally dominant mt-tRNA mutation, m.5545C>T in *MT-TW* [155]. For further details on the history, development and use of cybrids for the study of mtDNA mutations see Khan et al. [155] and Swerdlow [156].

Precautions are necessary when extrapolating from the cybrid system. Most cybrid models are generated using aneuploid tumour cells. The effects of aneuploidy on mitochondrial function are not clear; however, it is reasonable to assume that it can affect the stoichiometry of nuclear- and mitochondrial-encoded RC subunits, thus interfering with genotype–phenotype relationships. On a similar note, tumour cells are largely glycolysis-dependent, relying little on OXPHOS for generating the majority of their energy requirements. In contrast, mtDNA disorders typically occur in highly aerobic cells. It is important to consider what impact, if any, the primary energy

metabolic route of a host cell would have on the phenotypic expression of an mtDNA mutation [156].

It is also important to note that both the ρ^0 and cybridisation processes can impact strongly on the cellular transcriptome. Indeed, microarray studies have demonstrated that both processes can cause significant and stable up-regulation of many nuclear transcripts involved in OXHOS, even after repopulation of ρ^0 cells with wild-type mitochondria [157,158]. These observations might in part explain why in vivo phenotypes are not always replicated in an in vitro cybrid system. For example, cybrids harbouring the homoplasmic *MT-TV* m.1624C>T mutation, which caused a profound metabolic defect in skeletal and cardiac muscle, with low complex I and IV activities, and resulted in multiple neonatal deaths [159], were found to lack the marked respiratory defect despite retaining a striking reduction in mt-tRNA^{Val} steady-state levels [159].

5.1.2. Yeast models

Because the manipulation of the mammalian mitochondrial genome has proved frustratingly problematic, a number of researchers have turned to yeast as an alternative model system for the investigation of mtDNA mutations. *Saccharomyces cerevisiae* is currently the only known organism in which mitochondrial genetic transformation is possible. Most importantly, this can be achieved in a highly controlled fashion as DNA delivered to mitochondria via biolistic transformation can only incorporate into the mitochondrial genome by homologous recombination [160]. Thus, it is possible to introduce mutations at any desired position in yeast mtDNA genes. Recently, the biochemical consequences of a mutation in the protein-coding gene, *MT-ATP6* (m.8993T>C), have been investigated in yeast [161]. Moreover, given the sequence and structural similarities of some human and yeast mt-tRNAs, this in vivo system is particularly interesting for modelling mt-tRNA mutations equivalent to human pathogenic base substitutions [162–164]. Furthermore, in view of the ability of *S. cerevisiae* to survive in the complete absence of functional mtDNA, the study of very severe mitochondrial defects is possible.

To date, yeast models have been successfully employed in the identification of mt-tRNA mutation suppressors. Overexpression of both the mitochondrial translation elongation factor EFTu [162] and the mitochondrial leucyl-tRNA synthetase [163] has been reported to rescue mitochondrial dysfunction caused by the yeast equivalents of the m.3243A>G, m.3256C>T, and m.3291T>C MELAS mutations. Similar abilities for EFTu in human myoblasts harbouring the m.3243A>G mutation [165] and leucyl-tRNA synthetase in transmitochondrial WS277.546 human cell lines also containing the m.3243A>G mutation [166] have since been described.

Significant time and effort have been invested in validating the yeast model system for the investigation of human pathogenic mutations [162,164,167]. Nonetheless, several limitations do hamper the more widespread use of this organism as a model system of mitochondrial disorders. Not all human mt-tRNAs share strong homology with yeast mt-tRNAs. Also, *S. cerevisiae* are inherently homoplasmic, thus precluding threshold studies, although it is possible to modulate the severity of yeast respiratory phenotypes by studying mt-tRNA mutations in different nuclear contexts [163,164]. Finally, fermentative yeast such as *S. cerevisiae* lack complex I. However, the introduction over the past decade of an alternative model, the obligate aerobic yeast *Yarrowia lipolytica*, which possesses a vital proton-pumping NADH:ubiquinone oxidoreductase, has enabled important structural and functional analyses of mitochondrial complex I [168].

5.1.3. Mouse models

Whilst cybrids and yeast have helped to further our current understanding of mitochondrial genetics and the molecular mechanisms of disease, a mammalian in vivo model system is ultimately necessary for answering the many questions regarding mtDNA biology, mitochondrial disease pathogenesis and tissue specificity that still remain. For example, how can one mutation, such as m.3243A>G or m.8993T>C, result in multiple phenotypes (MELAS and MIDD, or NARP and MILS, respectively)? If all mtDNA mutations result in ATP deficiency, why are there so many different disease phenotypes? Why are some metabolically active tissues more affected than others?

Due to their rapid life cycle, ease of propagation, well-described genetics, and long-standing use in biological research, mice have become an important model system for understanding mitochondrial pathology. As it is not possible to directly manipulate their mitochondrial genome, researchers have been forced to adopt more ingenious and indirect ways of engineering mouse mtDNA.

The first heteroplasmic mice were generated by cytoplasmic fusion of two normal mouse strains carrying distinct haplotypes [74,169]. These mice harboured only polymorphic mtDNA variants. However, the repeated observation of directional selection for one mtDNA genotype in certain tissues [170], mediated by single tissue-specific factors [171], suggests these mouse models will be particularly important in our quest to understanding the basis of tissue specificity and mtDNA somatic segregation.

Additional transmitochondrial mouse models, showing signs of mitochondrial dysfunction and capable of transmitting mtDNA mutations to subsequent generations, have since been established, including the 'Mito-mice' model that harbours high levels of a single heteroplasmic mtDNA deletion and shows a multitude of symptoms including myopathy, cardiomyopathy, renal failure, anaemia, deafness, lactic acidosis, a mosaic pattern of COX deficiency, and a shortened life-span [172,173]. The 'Mito-mice' phenotype closely mimics that of early-onset Pearson syndrome, which is caused by a single heteroplasmic mtDNA deletion resulting in anaemia, myopathy, and pancreatic and renal insufficiency. Recently, transmitochondrial mouse models of mtDNA point mutations have been generated. Two heteroplasmic point mutations introduced in *MT-ND6* (m.13885insC) and *MT-CO1* (m.6589T>C) resulted in myopathy and cardiomyopathy [174], and the injection of m.3243A>G mutated mitochondria into C57BL/6J mouse zygotes led to respiratory dysfunction and enhanced ROS production in heteroplasmic tissues [175].

Alternative and more successful approaches to developing mouse models of mitochondrial disease involve manipulating the nuclear genes that regulate mtDNA maintenance and replication, such as *TFAM* [176–182], *POLG* ('Mutator' model) [183], and *PEO1* (Twinkle; 'Deletor' model) [184]. However, these models are beyond the scope of the current review; for further details see Vempati et al. [185] and Tyynismaa and Suomalainen [186].

5.2. Molecular mechanisms of mitochondrial disease

On account of the gradual development of these various experimental models, our understanding of the functional consequences of mtDNA mutations and the molecular mechanisms by which mitochondrial diseases develop is progressing. Biochemical effects of mtDNA mutations have been well described in all experimental systems and are invariably characterised by lower cellular respiration, compromised RC complex activity, and reduced ATP synthesis. Due to the role of mitochondria in the generation of ROS and the regulation of apoptotic cell death, the status of these pathways in patient cell lines, tissues, and mouse models of disease harbouring mtDNA mutations has been frequently investigated. Depending on the experimental model used, conflicting results have been observed. Increased ROS production has been described in neuronal NT2 cybrid cells containing m.3460G>A and m.11778G>A LHON mutations [187], in lymphocytes with m.8993T>C and m.8993T>G NARP mutations [188] and more recently in the chimeric m.3243A>G mice mentioned previously [175]. Conversely, normal

levels of ROS were measured in the *POLG1* mutator mice, which eventually accumulate sufficient mtDNA mutations to cause severe OXPHOS deficiency [189]. Furthermore, no increase in programmed cell death was identified in this mouse model, contrary to a double *TFAM* knockout mouse model in which substantial apoptosis occurred at embryonic day 9.5 [179,190].

5.2.1. Molecular impacts of mt-tRNA mutations

So far, investigations into the molecular mechanisms of mtDNA mutations have mainly concentrated on elucidating the molecular impacts of mt-tRNA mutations, in particular determining the structural and/or functional perturbations capable of interfering with mitochondrial protein synthesis. Transmitochondrial cybrids have proved instrumental in studying the various mt-tRNA mutations. In vitro transcribed wild-type and mutated mt-tRNAs have also been used.

Mt-tRNAs are characterised by a distinctive cloverleaf-shaped structure, maintained in part by Watson–Crick base pairing, and thus consist of four domains: an acceptor stem, a dihydrouridine (DHU) stem/loop, a TψC-stem/loop and an anticodon stem/loop. This secondary structure is further condensed into an L-shaped form through conserved tertiary interactions. The fundamental function of mt-tRNA molecules is to enable mitochondrial protein synthesis by the ribosome. Their ability to carry out this function is critically dependent on the appropriate processing of the primary polycistronic transcript into mature mt-tRNA as well as the adoption of the appropriate 3D structure.

Mutations have been found to affect all aspects of mt-tRNA biogenesis. Correct transcription of 16S mt-rRNA is impaired by the common mt-tRNA$^{Leu(UUR)}$ m.3243A>G mutation, which interferes with a transcription termination site located at the boundary of the 16S mt-rRNA and mt-tRNA$^{Leu(UUR)}$ genes, causing accumulation of an unprocessed RNA intermediate (RNA19) including 16S mt-rRNA, mt-tRNA$^{Leu(UUR)}$ and MTND1 [191–193]. Following transcription, endonucleolytic 5′- and 3′-end cleavage of precursor mt-tRNA molecules and subsequent 3′-CCA addition are necessary to produce mature mt-tRNAs. Mt-tRNA$^{Leu(UUR)}$ mutations m.3256C>T, m.3260A>T and m.3271T>C lead to impaired RNase P processing of 5′-leader sequences in vitro [194], whilst the mt-tRNA$^{Ser(UCN)}$ m.7445T>C transition reduces the efficiency of 3′-cleavage [195]. Mt-tRNA structural stability and the presence of recognition elements for interacting protein factors are achieved by post-transcriptional modification of mature transcripts (e.g., methylation, 5-taurimomethyluridine modification). Interestingly, m.3243A>G has been shown to impair methylation of mt-tRNA$^{Leu(UUR)}$ in cybrid osteosarcoma cells [196], but to prevent taurine modification of the anticodon wobble position (U34) in cybrid HeLa cells [197]. Similarly, post-transcriptional modifications of mt-tRNA^{Lys} m.8344A>G mutant mt-tRNAs are also dependent on nuclear background [196,198]. Mt-tRNA mutations invariably result in structural defects, which may range from mild to severe. The mt-tRNA$^{Ser(UCN)}$ m.7512T>C mutation causes slight localised kinking of the acceptor stem [199], whereas m.3243A>G, in the DHU stem, is responsible for a dramatic structural change, which disrupts both the L-shaped tertiary fold of mt-tRNA$^{Leu(UUR)}$ by preventing np A14/U8 interaction [200] and its quaternary structure by promoting the formation of a dimeric complex by intermolecular D-loop base pairing [201]. Finally, decreased mt-tRNA stability has been described with the m.8344A>G mutation both in vitro and in cybrid osteosarcoma cells [198,202].

The functional properties of mt-tRNAs, such as their capacity for aminoacylation and binding to various protein factors of the mitochondrial translation machinery, may also be affected by mt-tRNA mutations. Only mature and correctly folded mt-tRNAs can be esterified by their cognate aminoacyl synthetase. Given the ease with which mt-tRNA structure can be disrupted, numerous cases of decreased mt-tRNA aminoacylation have been reported. Recent examples include the mt-tRNA^{Phe} m.611G>A mutation, which decreases aminoacylation activity 100-fold in vitro [203], and the mt-tRNA^{Tyr} m.5874T>C and m.5877C>T transitions [204]. Following aminoacylation, mt-tRNAs are transported, by transient binding to translation factors (e.g., EFTu), to the ribosome, whereupon transfer of the amino acid to the growing polypeptide chain completes protein synthesis. By altering mt-tRNA^{Ile} T-stem structure, the mutation m.4269A>G impairs binding affinity for EFTu two-fold [205]. For more in-depth reviews of the effects of mutations on mt-tRNA structure and function see references [206–208].

5.2.2. Global effects of mtDNA mutations

Whilst the direct investigations of mtDNA mutations have revealed important molecular effects, it is becoming increasingly apparent that new global approaches are required to fully elucidate the molecular mechanisms of mtDNA mutations and to correlate these mechanisms with the phenotypic expression of mitochondrial diseases. Indeed, the constantly increasing numbers of causative mutations, the absence of unifying mechanistic features, the influence of the nuclear background on the mutation effects, and the increasing realisation that mitochondrial proteins possess a dual cellular localisation [209,210] make the use of the unbiased methodology of modern post-genomic approaches particularly appealing for application in the field of mitochondrial research. The global transcriptomic and comparative proteomic investigations performed to date to determine the wide-ranging mitochondrial and cellular effects of mtDNA mutations in the context of mitochondrial disorders are outlined below.

Microarray analyses have been performed using a variety of platforms, biological samples, and experimental designs, making direct comparisons between experiments difficult. Nonetheless, some common transcriptional responses to mitochondrial dysfunction are emerging. For example, genes involved in cellular metabolic pathways, such as the TCA cycle, energy production, and amino acid metabolism, are reportedly up-regulated not only in patients with the 4977 bp macrodeletion [211], but also in two lymphoblast cybrid cells containing the LHON mutations m.3460G>A (in the *MT-ND1* gene) and m.11778G>A (*MT-ND4* gene) [158], in a 143B osteosarcoma cybrid cell line with the m.8993T>G NARP mutation (*MT-ATP6* gene) [212], and in muscle [211], neuronal cells [213] and 143B cybrid cells [212,214] harbouring the m.3243A>G mutation.

Other shared transcriptional consequences of mitochondrial dysfunction include inhibition of both ubiquitin-mediated protein degradation and mitochondrial and nuclear ribosomal protein synthesis [214,215], as well as activation of the unfolded protein response [215,216]. An induction in the expression of several nuclear RC transcripts has also been observed in macrodeleted muscle [211] and in 143B cells harbouring the m.8993T>G NARP mutation [212]. This response has previously been reported in muscle with over 88% levels of the m.3243A>G mutation [217], but it is also thought to represent a sustained effect of the cybridisation process [158]. Nuclear OXPHOS gene up-regulation may thus be considered a generic compensatory effect for mitochondrial dysfunction.

Intriguingly, recent work published by Alemi et al. [215] does not agree with many of these communal transcriptional responses. The transcriptomes of muscle and six cybrid cell lines containing 4977 bp and 7.5 kb mtDNA deletions revealed a reduction in the expression of metabolic pathway genes and nuclear OXPHOS genes relative to controls. Furthermore, the mtDNA deletions were shown to exert a negative effect on cell proliferation and cell cycle. Similar findings were also later reported in two fibroblast cell lines harbouring the microdeletion m.9205delTA in *MT-ATP6* [218]. Why such discrepancies exist is not clear but they may represent intrinsic differences between cell types/tissue, varying levels of heteroplasmy or variations in experimental design. Moreover, the nature of the mtDNA mutations, in particular the m.9205delTA deletion, which eliminates

the *MT-ATP6* gene stop codon, placing *MT-CO3* immediately in frame, and causes a marked decrease in RNA14 (*MT-ATP8* and *-6*-encoding bicistronic transcript) steady-state levels [81], may account for these disparate findings.

Whilst transcriptomic analyses can provide invaluable information on the molecular mechanisms of disease, it is ultimately the effects on protein expression (known to not always reflect transcriptional fluctuations) and their post-translational modifications that dictate the phenotypic expressions of pathogenic mutations. The application of proteomics to further our understanding of numerous human diseases is well established. However, surprisingly few attempts to profile proteomic changes associated with primary mtDNA mutations have been reported. A large-scale quantitative proteomic study to investigate the effects of primary inherited mtDNA mutations on mitochondrial proteins was performed in 2002 by Rabilloud et al. [219]. Mitochondrial proteomes of wild-type and mutant cybrid cell lines, harbouring either the m.8344A>G MERRF or m.3243A>G MELAS mutations, were compared by silver-stained two-dimensional electrophoresis (2-DE). A number of up- and down-regulated proteins associated with both phenotypes were observed. In particular, the expression levels of two proteins, both nuclear-encoded subunits of CIV (Vb and VIa), were shown to be dramatically reduced in both mutant cell lines relative to their sibling wild-type. Subsequently, quantitative analysis of >800 total detectable spots revealed almost 5% with significant quantitative variations in m.8344A>G mutant cybrid cell lines versus control [220]. As would be expected, these included the 13 mitochondrially encoded OXPHOS subunits [221–223]. In addition, several nuclear-encoded mitochondrial proteins, including RC subunits, metabolic enzymes and a mitochondrial translation protein, were identified [220]. However, whilst these initial results appeared promising, the study unfortunately did not address the issue of aneuploidy inherent to cybrid cells.

To the best of our knowledge, this analysis remains the only proteomic study to date to investigate the wider effects of specific primary mtDNA defects. Recently, the global cellular proteomic response of CRL-8303 osteosarcoma cells with chemically reduced OXPHOS activity has been analysed by silver-stained 2-DE [224]. Inhibition of CI or CIV caused changes in the expression levels of apoptotic, cytoskeletal, and OXPHOS proteins, as well as proteins involved in TCA cycle, glycolysis, and oxidative stress responses. Mostly, current efforts are concentrating on elucidating the complete mammalian mitochondrial proteome, a critical step in furthering our understanding of the role of mitochondria in health and disease [209,225,226]. The most recent mammalian mitochondrial compendium (MitoCarta) consists of 1098 identified proteins [209].

These preliminary transcriptomic and proteomic investigations have proven to be useful approaches for mining new data and will hopefully open new perspectives for understanding mitochondrial disorders. Nonetheless, more global investigations are required to fully appreciate and comprehend not only the diverse molecular pathways that are dysregulated in the pathogenesis of mitochondrial disease and contribute to the expression of the disease but also any potential cellular compensatory effects to mitochondrial dysfunction. Ultimately, these approaches, if correctly applied, offer a promising strategy for the development of much-needed therapies.

6. Management of mtDNA disease

Despite considerable advances over the last decade in our understanding of mitochondrial genetics and function and the pathogenesis of mtDNA diseases, no effective treatment options are currently available for patients with mitochondrial dysfunction, except in rare cases where surgery or transplant may be indicated. Given the complexities of mitochondrial genetics and the phenotypic variability expressed in mtDNA disorders, the development of a generic ‘cure’ for mitochondrial disorders has been problematic. The administration of various pharmacological and biochemical agents, including vitamins, cofactors, metabolites, and electron acceptors, to correct or bypass the underlying RC defect has met with limited success (reviewed in [227]). Current focus is on developing mutation- and disease-specific therapies to minimise symptoms and prevent complications. Two main approaches are being actively pursued, namely exercise and gene therapy. Significant efforts are also being made to prevent mtDNA disease transmission.

6.1. Exercise therapy

Management of mtDNA disease through exercise training aims to improve physical capacity and quality of life in patients with high levels of heteroplasmic mtDNA mutations in muscle. Well tolerated and safe, endurance training for up to 14 weeks has been found to improve OXPHOS function, although no concomitant shift in mutated mtDNA levels in muscle has been observed and cessation in training does cause a loss of the physiological adaptations [228–230]. Long-term follow-up training studies are currently underway to determine if changes in mtDNA mutation load are possible. Recently, the benefits of daily aerobic exercise have been further demonstrated in conditional *COX10* knockout mice, which typically develop severe myopathy at ~2.5–3 months [231]. Muscle conditioning enhanced ATP levels, delayed disease onset, and increased life expectancy in these mice.

Resistance exercise protocols have shown similar promise. This approach is based on the stimulation of muscle regeneration in response to fibre injury. Undifferentiated myogenic cells, satellite cells, are known to proliferate in response to damage caused by concentric (shortening) or eccentric (lengthening) muscular contractions. In patients with high levels of sporadically mutated mtDNA in mature muscle fibres but with low or undetectable levels in satellite cells, activation of these stem cells and subsequent incorporation of wild-type satellite mtDNA into mature muscle may shift heteroplasmy levels sufficiently to recover muscular function (termed “gene shifting”) [232]. Twelve-week resistance training studies in patients with single, large-scale mtDNA deletions have been demonstrated to increase satellite cell number with concomitant improvements in muscular strength and oxidative capacity evidenced by a decrease in the proportion of COX-negative fibres [233]. Further work is required to determine the optimal exercise regime parameters.

6.2. Gene therapy

On account of the peculiarities of mitochondrial genetics new and inspired approaches to genetic-based strategies for mtDNA disease therapy have been necessary. Manipulation of heteroplasmy levels to shift the balance of mutant to wild-type genomes, critical in determining the expression of the biochemical defect, has been attempted by a number of different approaches. Antigenomic therapy, which involves using sequence-specific nucleic acid derivatives that selectively target and inhibit the replication of mutated mtDNA thus allowing the wild-type genome to propagate, showed promising initial results in vitro with peptide nucleic acids (PNAs) [234]. However, although PNAs colocalised to mitochondria in intact cells, they were not able to cross the IMM. Molecules with greater polarity have since been designed (cell membrane crossing oligomers, CMCOs), and CMCO:PNA hybrid molecules have been successfully targeted and imported into mitochondria in whole cells [235]. Further research is still necessary to establish their ability to inhibit mtDNA replication and their effectiveness in vivo. Alternative strategies for manipulating mtDNA heteroplasmy levels include mitochondria-targeted restriction endonucleases, capable of distinguishing between mutated and wild-type genomes. Successful in human cells harbouring pathogenic mutations, in hybrid cells with both rat and mouse mtDNA and in a mouse model heteroplasmic for the BALB and NZB mtDNA haplotypes, this approach is limited to mtDNA mutations that

introduce an additional restriction site although recent data demonstrate this restriction site need not be unique to the pathogenic genome [236]. Selective targeting of mutated mtDNA has also been successfully attempted with a targeted chimaeric zinc finger DNA methylase, which can bind and modify, in a sequence-specific manner, mutant genomes in cells harbouring the m.8993T>G NARP mutation [237]. Whilst very promising, the therapeutic use of this strategy is limited by the need for transfection and successful expression in defective tissues.

Complementation of mtDNA defects, by allotopic expression of a gene in a different cellular compartment to its target location, is another promising approach for treating mitochondrial diseases. Initial attempts to recover respiratory defects in cybrids harbouring *MT-ATP6* and *MT-ND1* mutations, by engineered nuclear expression of their wild-type proteins [238,239], have been improved recently by forcing mRNA localisation to the mitochondrial surface, which ensures maximal mitochondrial translocation of the highly hydrophobic polypeptides [240,241]. For mt-tRNA mutations, yeast $tRNA^{Lys}$ derivatives can be expressed and imported into mitochondria within cybrid cells and primary human fibroblasts harbouring the m.8344A>G MERRF mutation. Imported $tRNA^{Lys}$ molecules are correctly aminoacylated, able to participate in mitochondrial translation, and partially rescue mitochondrial functions [242]. Recently, Rubio et al. [243] have demonstrated the import of cytosolic $tRNA^{Gln(CUG)}$ and $tRNA^{Gln(UUG)}$ in rat and human mitochondria in vivo, by a mechanism distinct from that of protein import. This discovery has important implications for the development of future therapies for mt-tRNA mutations as this process was not thought to normally occur in human cells. Pathogenic mt-tRNA mutations may also be rescued through modification or overexpression of their cognate aminoacyl synthetase. Aminoacylation efficiency of mutant mt-$tRNA^{Phe}$, carrying the m.611G>A MERRF mutation, was significantly improved following modification of the mt-tRNA binding domain of the cognate phenylalanyl-tRNA synthetase [203]. Furthermore, two recent studies have demonstrated an increase in mutant mt-$tRNA^{Val}$ [159] and mt-$tRNA^{Leu(UUR)}$ [166] steady-state levels following overexpression of the valyl and leucyl synthetases, respectively. In mutant m.3243A>G cells, this overexpression was capable of recovering wild-type RC function. These findings suggest promising new therapeutic strategies to treat mt-tRNA diseases.

A variation of allotopic expression is the xenotropic expression of wild-type genes of other species, encoding proteins with similar function. Mitochondrial targeting of the *S.cerevisiae* single subunit NADH oxidase, Ndi1, has been shown to rescue complex I deficiency in human cells [244] and also protect against neurodegeneration in rats fed with MPTP, a hallucinogen known to cause a form of Parkinson's disease (PD) [245]. The use of yeast Ndi1 may thus represent a promising approach for rescuing any complex I deficiency in patients with PD. Similarly, xenotopic expression of the green alga *Chlamydomonas reinhardtii* ATPase 6 (CrATP6) in human cybrid cells harbouring the m.8993T>G mutation resulted in moderately improved ATP production whilst cellular viability was fully recovered [246]. These data suggest gene therapeutic approaches for the treatment of ATP synthase disorders such as NARP and MILS may be possible. Furthermore, an encouraging strategy for the potential rescue of mitochondrial COX defects may soon be possible following the successful transfection and IMM targeting in human cultured cells of the sea squirt *Ciona intestinalis* cyanide-insensitive alternative oxidase (AOX) [247]. Indeed, AOX was well tolerated and transfectants displayed remarkable levels of cyanide resistance to mitochondrial metabolite oxidation.

6.3. Prevention of mtDNA disease transmission

The outcome of specific pregnancies remains unpredictable due to the genetic bottleneck, which leads to considerable variation in the proportion of mutant mtDNA in different offspring [248]. This makes accurate genetic counselling for families affected by mtDNA mutations extremely challenging. Nonetheless, several approaches to help minimise the transmission of high mtDNA mutation loads are being considered. For example, prenatal genetic diagnosis from chorionic villus sampling or amniocentesis may be performed. Whilst there is concern whether the mutation load detected in the prenatal sample would accurately reflect the heteroplasmy levels in other foetal tissues, present evidence suggests that this should not be a problem [169,249]. Preimplantation genetic diagnosis (PGD) of mtDNA from the polar body of unfertilised oocytes or from one to two single cells removed from early embryos prior to implantation is an alternative approach. This technique may similarly be limited by tissue-specific differences in mutational load. However, studies in heteroplasmic mice have demonstrated almost identical levels of heteroplasmy between the ooplasm and polar body of the mature oocyte and also between individual blastomeres [250]. Despite its promise, PGD has only been successfully completed in one case of mtDNA disease [251].

Currently, mitochondrial gene replacement offers great potential for the prevention of human mtDNA disease transmission. Recently, spindle–chromosome complex transfer from a mature primate oocyte to an enucleated primate egg successfully produced healthy offspring, in which, importantly, spindle donor mtDNA was not detected [252]. An alternative approach involving pronuclear transfer, the transfer of the pronuclei from a fertilised zygote of a mother with mtDNA disease to an enucleated zygote from a healthy donor female, is presently being investigated in abnormally fertilised human embryos (Turnbull et al., unpublished data). Previous studies in heteroplasmic mice have suggested the feasibility of this approach [253]. For comprehensive reviews regarding the treatment of mitochondrial disease, see references [235,254,255].

7. Perspectives

Since identification of the first mtDNA mutations more than 20 years ago, significant progress has been made in cataloguing and understanding the mtDNA defects responsible for the development of mitochondrial diseases. The continually increasing number of reported pathogenic mutations within both the mitochondrial and nuclear genomes and the constantly evolving rules of pathogenicity make the diagnosis of these disorders ever more challenging. The implementation of more high-throughput screening approaches should hopefully improve the situation. Disappointingly, no effective therapies or cures are presently available for patients with mtDNA disease. Several exciting and promising experimental approaches for mtDNA disease treatment are currently being investigated and may hopefully make their way into the clinic in the near future. Furthermore, recent research efforts are focusing more and more on establishing the molecular mechanisms of disease and elucidating the complex relationships between genotypes and the diverse clinical phenotypes associated with these disorders. Elucidating the pathogenesis of mitochondrial disorders will undoubtedly uncover novel protein targets or molecular pathways that may be exploited for therapy development. In the meantime, approaches to prevent the transmission of mitochondrial disease, including PGD, pronuclear transfer between single cell embryos and spindle-chromosome transfer between oocytes, are being actively pursued.

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