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REVIEW ARTICLE OPEN ACCESS

Recent developments in mitochondrial medicine (Part 1)

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Abstract – Research into elucidating structure and function of mitochondria has been quite steady between the time of discovery during the end of the 19th century until towards the late 1980's. During the 1990s there was talk about a "comeback" of this organelle reflecting a widely revitalized interest into mitochondrial research which was based on two major discoveries made during that time. The first was the etiological association between human diseases and mitochondrial DNA mutations, while the second revealed the crucial function of mitochondria during apoptosis. The March 5th, 1999 issue of Science even featured a textbook image of a mitochondrion on its front cover and was entirely dedicated to this organelle. Whilst the term "comeback" might have been appropriate to describe the general excitement surrounding the new mitochondrial discoveries made during the 1990s, a term for describing the progress made in mitochondrial research during the last two decades is difficult to find. Between 2000 and 2020 the number of publications on mitochondria has skyrocketed. It is now widely accepted that there hardly exists any human disease for which either the etiology or pathogenesis does not seem to be associated with mitochondrial malfunction. In this review we will discuss and follow several lines of mitochondrial research from their early beginnings up to the present. We hope to be able to convince the reader of what we expressed about a decade ago, that the future of medicine will come through mitochondria.

Keywords: Biochemistry, Cells, DQAsome, Lipoplexe, Heteroplasmy, miRNA, Mitochondrial medicine, Mitochondrial diseases, Mitochondrial DNA, Mitochondrial dysfunction, Mitochondrial research, Mitochondrion, Mitochondrium, mitomiRs, mtDNA, Organelle, Physiology, PNA, Research, Science

Introduction

All human cells, except erythrocytes, harbor mitochondria in the form of a tubular network constantly undergoing fission and fusion. Known as the "*powerhouse of the cell*", they are at the juncture of energy-producing pathways, as the "*cell's arsenal*" they play a decisive role in programmed cell death (apoptosis). In addition, mitochondria are involved in a multitude of anabolic, catabolic, and signaling pathways.

According to the endosymbiotic theory mitochondria are descended from once free-living bacteria which were engulfed by prokaryotic cells. This today generally accepted evolutionary theory was proposed for the first time in 1966 by Lynn (Sagan) Margulis who had, as she remembers [1], to submit her corresponding manuscript to about 15 different journals before it was accepted by the Journal of Theoretical Biology [2].

Mitochondria contain their own DNA and as shown by Doug Wallace and his group in 1980 [3] are maternally inherited. A recently claimed biparental inheritance of mitochondria in humans [4] however has triggered some contentious discussions [5, 6]. Based on correlating mitochondrial DNA (mtDNA) variations between populations from around the world Wallace was able to reconstruct origin and migration patterns of women in ancient times, which gave rise to the speculation about a "Mitochondrial Eve" the most recent female from whom all humans might have descended.

Mitochondrial DNA encodes for 13 polypeptides essential for energy production; all other mitochondrial proteins encoded by nuclear DNA are imported from the cytoplasm. The amount of mitochondrial mass per cell organized as a sub-micro- to nano-structured tubular network extending three-dimensionally around the nucleus and throughout the cytoplasm depends on the energy demands of the cell. Organs with the highest energy consumption are the brain, skeletal muscle, the kidney cortex, liver, heart and the

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visual system. Though mitochondrial malfunction as the cause of human disease was described for the first time in 1962 [7], the genetic basis for mitochondrial diseases remained elusive until 1988 [8]. Subsequent research over the last three decades has revealed that there is hardly any human disease the etiology or pathogenesis of which is not associated with mitochondrial malfunction, a realization leading us [Marvin Edeas and Volkmar Weissig (ME & VW)] to conclude that "the future of medicine will come through mitochondria" [9].

In this review, written for "non-mitochondriologists", we outline about 170 years of mitochondrial research whilst emphasizing the major groundbreaking discoveries and developments made since the beginning of the new century. Because we (ME & VW) have been hosting in Berlin (Germany) the Annual World Conference on Targeting Mitochondria since 2010 (the 12th edition will take place in October 2021), we are fortunate to know on a personal level many of the major investigators who are pushing mitochondrial research forward. In this review, we highlight their contributions to the field of mitochondrial medicine.

From "thread-like granules" to "cristae junctions"

Studies using light microscopy on cells during the second half of the 19th century uncovered the existence of small subcellular granules [10] which in shape and size were similar to bacteria as observed by Richard Altmann (1852–1900) who named them "*bioblasts*" in 1890 [10, 11]. Based on the Greek, eight years later, Carl Benda (1857–1932) coined the term "mitochondria" in 1898 [12], with "*mitos*" meaning thread and "*chondrion*" meaning grain. Noteworthy, although obviously already recognized at the end of the 19th century, the thread-like morphology of mitochondria only became fully appreciated at the beginning of the 21st century when it was revealed that the maintenance of the tubular (thread-like) network characteristic of mitochondria is critical for human health [13–15]. The appearance of mitochondria as individual objects usually indicates a severely damaged mitochondrial network as present in unhealthy, dying cells or as an artifact of sample processing for microscopic images. Unfortunately, the depiction of mitochondria in text books as single bean-like shaped bodies seems to be still very common.

By 1930, most cytologists had recognized mitochondria as well-defined and ubiquitous cellular structures, but there was no agreement as to their function. In 1890, Richard Altmann proposed them to be the basic units of cellular activity, other proposals made suggested them to be the center for genetic information, protein synthesis, respiration and lipid synthesis [10].

The average single mitochondrion, disconnected from the tubular mitochondrial network by fixation and staining procedures of tissues samples, appears as a bean-shaped organelle with approximately the size of Escherichia coli, up to 2 μm long and 1 μm wide, i.e. it is large enough to be visible under the light microscope. Light microscopy however does not allow for a more detailed structural analysis of mitochondria.

Their major structural features, which are an outer membrane and a highly convoluted inner membrane, were made visible for the first time during the early 1950's when George Emil Palade (1912–2008, Nobel Prize Physiology 1974) investigated the organelle under the electron microscope [16, 17]. Based on Palade's work a model for the inner structure of mitochondria was developed which dominated the literature for up to 50 years (Fig. 1A). According to this model, mitochondria have two aqueous sub compartments, the intermembrane space (IMS) between the outer and the inner membrane and the so-called mitochondrial matrix. Here it should be noted that although the term "aqueous sub compartments" is still widely used, it is misleading. Due to the presence of between 1100 and 1400 [18] distinct proteins, the mitochondrial matrix "*constitutes a biochemical reaction environment with a highly complex structure*" [19]. The viscosity of the mitochondrial matrix solvent has been determined to be between 2.69 and 3.32 cP, which can, under a variety of physiological and pathological conditions increase up to 30-fold [19]. For comparison, the viscosity of water is about 1 cP.

In Palade's model no barrier exists between the space inside the cristae and the IMS. This model became obsolete towards the end of the 1990's when Perkins and Frey investigated mitochondrial morphology by electron microscope tomography [20, 21] and discovered junctions at the neck of cristae (Fig. 1B). Due to their narrow diameter of about 12–40 nm, these junctions were proposed to impede the flow of proteins and metabolites towards and away from cristae thereby creating an aqueous compartment distinct from the IMS and also separated from the mitochondrial matrix.

Studies conducted during the last decade, [22] have revealed major protein complexes which control the formation of cristae and their junctions, among them optic atrophy 1 protein (OPA1) and the mitochondrial contact site and cristae organization system (MICOS) (Fig. 1B).

Controlling cristae junctions is crucial for mitochondrial function. In the mid-1960s, Charles Hackenbrock reported that ultrastructural changes of mitochondrial cristae were associated with metabolic changes [23–25]. In 2002, Stanley Korsmeyer and co-workers showed that cristae remodeling is an essential step during a highly complex pathway leading to apoptosis (programmed cell death) [26]. Finally, a large body of work during the last 20 years (the reader is referred to an excellent recently published review [22]) revealed that malfunctioning cristae formation or cristae remodeling is associated with several human diseases. Just to name a few, mutations in the OPA1 gene have been linked to optic atrophy associated with deafness and dementia, as well as encephalomyopathy, and cardiomyopathy (*reviewed in* [22]). Further studies about mitochondrial cristae formation will have "*important implications for understanding human disease linked to various forms of mitochondrial dysfunction*" [22].

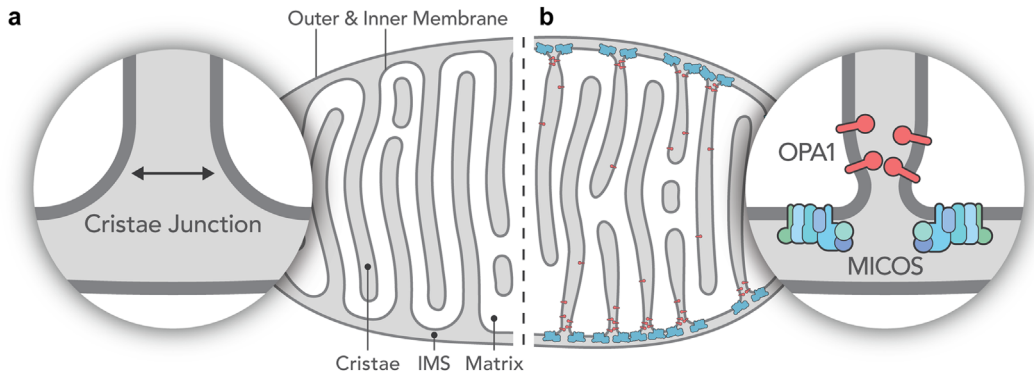

Figure 1. (A) The "old" and (B) the "new" model of the inner structure of mitochondria. Instead of the large openings connecting the intercristae space to the intermembrane space present in the old model (A), narrow tubular openings (crista junctions) connect these spaces in the new model (B) thereby creating a third aqueous intramitochondrial compartment. Panel (B) also shows the presence of two out of several protein complexes which control the formation and remodeling of the cristae junctions. This schematic illustration is based on figures in [22].

From "Atmungsferment" to "Respirasomes"

Exactly how substrates are being oxidized in aerobic organism was a hot topic discussion in the early 1900s. For example, Heinrich Otto Wieland (1877–1957, Nobel laureate in Chemistry in 1927) proposed that oxidation in living tissues involves the removal of the hydrogen atom (dehydrogenation) instead of the addition of oxygen [10]. Otto Warburg (1883–1970, Nobel Prize in Physiology or Medicine in 1931) advocated in 1914 for an iron compound, which he termed "*Atmungsferment*" ("*Breathing Enzyme*") as being the chief catalyst for activating and transferring oxygen in biological systems [27]. Subsequently, Warburg succeeded in identifying cytochrome oxidase as the enzyme that reacts with oxygen. In addition to finding the final cytochrome (cytochrome a3), Warburg also characterized nicotinamide adenine dinucleotide (NAD) as a hydrogen carrier in biological oxidation reactions. Interestingly, cytochromes were already described back in 1884 by Mac Munn as respiratory pigments that function in the transfer of oxygen [28]. Charles Alexander Mac Munn's (1852–1911) work however wasn't fully recognized and accepted until 40 years later when David Keilin (1887–1963) started in 1925 publishing a series of investigations about cytochrome systems of pigments in aerobic cells [29].

The next milestone towards revealing the secrets of biological oxidation and respiration was the description of the citric acid cycle or tricarboxyllic acid (TCA) or Krebs cycle by Sir Hans Adolf Krebs (1900–1981, Nobel Prize in Physiology or Medicine in 1953) in 1937 [30–34], who in turn built upon the earlier work by Albert Szent-Gyoergyi (1893–1986, Nobel Prize in Physiology or Medicine in 1937), Carl Martius (1906–1993) and Georg Franz Knoop (1875–1964) [7]. Hans Karl Heinrich Adolf Lohman (1898–1978) discovered ATP in 1929 [35] but its connection to and vital role in cell respiration wasn't established until 1937 [36]. Two years later Volodymyr Oleksandrovych Belitser (1906–1988) and Elena Tsibakova introduced the term "oxidative phosphorylation" [37] and about during the same time, between 1939 and 1941 Fritz Albert Lipmann (1899–1986, Nobel Prize in Physiology or Medicine in 1953) identified ATP as the main bearer of chemical energy in the cell, coining also the term "energy-rich phosphate bonds" [38–40].

Also during the 1940s, based on progress made in development of cell fractionation techniques, the activities of enzymes for fatty acid oxidation and the TCA cycle were found to be associated with mitochondrial fractions [10] and in 1953 Edward Charles Slater (1917–2016) proposed a scheme for the mechanism of oxidative phosphorylation involving chemical intermediates [41, 42]. These developments in mitochondrial research did not go unnoticed; the legendary term "*Powerhouse of the cell*" was coined in an article for Scientific American by Philip Siekevitz (1918–2009) in 1957 [43]. In 1961 it was suggested by Robert Joseph Paton Williams (1926–2015) that protons instead of Slater's energy-rich intermediates served to deliver energy to ATP synthase [44]. In the same year Peter Dennis Mitchell (1920–1992) postulated his chemiosmotic hypothesis [45–47] for which he was awarded the Nobel Prize in Chemistry in 1978. By and in 1962 the major players of the respiratory chain, i.e. enzymes with coenzymes, were purified, reconstituted and termed Complexes I–IV by Youssef Hatefi *et al.* [48–51] and by 1976 the fluid model of the inner mitochondrial membrane (IMM) organization was established [52, 53].

Since then it has become a widely believed dogma that all enzyme complexes, CI-CIV including the ATP synthase (Complex V) are independent diffusible proteins associated with the IMM and connected by small electron carriers shuttling between them. However, this paradigm of how the yeast and mammalian system of oxidative phosphorylation is organized fundamentally changed around 2000. Using a mild one-step protocol for the isolation of membrane protein complexes, namely blue-native polyacrylamide gel electrophoresis (BN-PAGE), Schragger et al were able to isolate supramolecular structures of the mitochondrial oxidative phosphorylation electron transfer chain (OXPHOS-ETC). They found that in mammalian mitochondria, almost all complex I is assembled into supercomplexes comprising

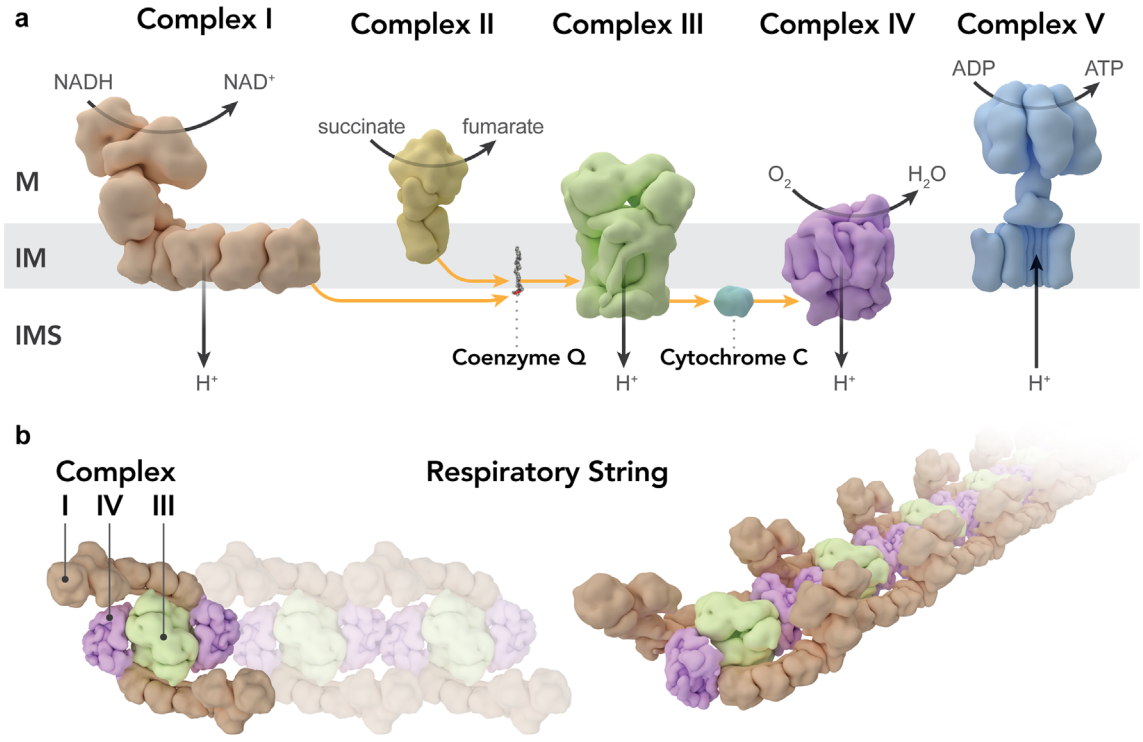

Figure 2. (A) "Classical" schematic representation of the mitochondrial oxidative phosphorylation (OXPHOS) system showing its individual components separated from each other, (M) matrix, (IMS) intermembrane space, (IM) inner membrane. (B) Schematic model of the organization of individual components of the respiratory chain (complexes I, III and IV) into a respiratory string. Both figure panels were created based on corresponding figures in [55].

complexes I and III and up to four copies of complex IV, which led the authors to propose a model for a network of respiratory chain complexes they named "*respirasome*" [54]. During the next 10 years (2000–2010) additional studies demonstrated that that complexes I and III in mammals and I, III, and IV in plants actually behave as single units kinetically, which led to the assumption of substrate channeling. Furthermore, recently it has been found that respirasomes seem to be organized into respiratory strings, as schematically shown in Figure 2 (*reviewed in* [55].

Finally, in 2016 the first detailed organization of a respirasome was published by Yang's group from Tsinghua University. The authors presented "*a 5.4 Å cryo-electron microscopy structure of the major 1.7 MDa SCI1III2IV1 respirasome purified from porcine heart*" [56]. Disruption of the formation of such supercomplexes or strings of respirasomes ultimately can cause OXPHOS malfunction resulting in pathologic changes [57]. Studying the detailed interactions within this huge molecular machinery will eventually provide important information for drug design and screening [58].

The recognition of mitochondrial dysfunctions as a cause of human diseases

Rolf Luft (1914–2007), a neurologist and endocrinologist at the Karolinska Institute in Sweden, discovered towards the end of the 1950s malfunctioning mitochondria as a cause of human diseases. In 1962, he published a paper entitled "*A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: a correlated clinical, biochemical, and morphological study*" [7]. The ailment described in his paper, caused by an extensive uncoupling of mitochondrial respiration in skeletal muscle tissue, soon became known as "*Luft disease*". Rolf Luft is nowadays widely acknowledged as the "*Father of Mitochondrial Medicine*" and the Rolf Luft Foundation invites nominations for the "Rolf Luft Award in Diabetes and Endocrinology Research" each year. Luft's paper from 1962 spurred intense investigations into the possible role of mitochondria in the etiology of human disease. About 25 years later Hans Rudolf Scholte, in a comprehensive review of the biochemical basis of mitochondrial disease, was able to classify over 120 diseases. Luft himself wrote in 1994 "*From Scholte's and subsequent reviews several basic principles in mitochondrial pathology emerged. First, some mitochondrial diseases affect only one tissue, most often skeletal muscle and brain but also liver, heart, kidneys or endocrine glands. Other organs may be involved secondarily. The disease may originate as a specific defect in mitochondrial function, but a variety of genetic and environmental factors may contribute to the phenotype*" [59].

However, the genetic basis for mitochondrial diseases remained elusive until 1988 (see below).

The "Decade of the Mitochondrion"

On March 5th in 1999 the editors of Science chose a textbook image of a mitochondrion as the front cover, which was unprecedented for this journal. It was a way for the Science editors to "officially" acknowledge the 1990s as the "decade of the mitochondrion". Two key discoveries were made during this decade: the first one actually continues where Luft and Scholte (see above) left off. Two papers, one in Science and the other one in Nature (both came out in 1988) revealed that mutations and deletions in mitochondrial DNA were associated with human diseases. Ian James Holt and co-workers described the link of deletion mutations of mitochondrial DNA (mtDNA) and mitochondrial myopathies [60], and Douglas Wallace and his team reported the association of Leber's Hereditary Optic Neuropathy (LHON) with mtDNA point mutations [8]. It should be noted that mitochondrial DNA was discovered back in 1963 by Nass & Nass, who described "*intramitochondrial fibers with DNA characteristics*" [61] and was sequenced in 1981 [62]. Consequently, during the 1990's most of Luft's and Scholte's "classical" mitochondrial disorders were found to be linked to mtDNA mutations.

The second key discovery involves the crucial role of mitochondria during programmed cell death (apoptosis), which however we will not further discuss in this review. The interested reader is referred to a review from 2007 by Susan Elmore [63]. To highlight the excitement this key discovery has triggered, we would like to cite what Brown, Nicholls ad Cooper, three key players in this field, wrote in 1999 in their preface to "Mitochondria and Cell Death" [64]:

> "Who would have believed it?! Mitochondria, the powerhouses of cellular life, are also motors of cell death. Few would have accepted this even 5 years ago".

Manipulation of the mitochondrial genome

Finding an effective and reliable way to manipulate the mitochondrial genome has been the "Holy Grail" of mitochondrial medicine ever since defective mitochondrial DNA has been linked to human diseases. Due to the multi-copy nature of mtDNA, several principal strategies can be followed, which are correcting mtDNA mutations / deletions, selectively degrading mutated mtDNA circles, selectively inhibiting the replication of mutated mtDNA and replenishing mitochondria with wild-type copies of mtDNA (Fig. 3). All of these approaches have been and are still being explored since the 1990s and though all of them were and still are promising, the "hunt for the Holy Grail" is ongoing.

Mitochondrial gene therapy

Worldwide, during the 1990's over 1500 nuclear-targeted gene therapy trials have been conducted exploring a variety of nuclear-targeted viral and non-viral transfection vectors. Relating to a potentially similar approach towards mitochondria-targeted gene therapy, Peter Seibel wrote in 1995

> "Successes in classical gene therapies have been achieved by placing a corrected copy of a defective nuclear gene in cells. A similar gene replacement approach for a mutant mitochondrial genome is invariably linked to the use of a yet unavailable mitochondrial transfection vector" [65].

Seibel's group launched the hunt for the "Holy Grail" by demonstrating that DNA coupled covalently to a short "mitochondrial leader peptide" can enter mitochondria via the protein import pathway, which at that time seemed to open a novel way for gene-, antisense-RNA- or antisense-DNA-delivery for mitochondrial molecular therapies." [65]

A mitochondria-targeted transfection vector, in 1995 according to Seibel et al. still unavailable [65], presented itself during the same time when one of us (VW) discovered by chance that dequalinium chloride, a mitochondriotropic cationic bolaamphiphile, was able to self-assemble into cationic liposome-like vesicles, named "*DQAsomes*" (DeQAlinium-based liposomes) [66, 67]. Liposomes are man-made vesicles between 50 and 1000 nm in diameter in which one or more phospholipid bilayer membranes encapsulate an aqueous inner space. Following the synthesis of cationic phospholipid derivatives in 1987 by Phillip Felgner's group [68], liposomes could be rendered cationic which in turn enabled them to form complexes with negatively charged DNA. The striking similarity of DQAsomes with cationic liposomes widely used during the 1990s for nuclear-targeted gene therapy seemed to qualify DQAsomes as the mitochondrial transfection vector of choice [69, 70]. DQAsomes have been shown to meet all pre-requisites for a mitochondrial transfection vector, they bind and condense pDNA forming "DQAplexes", which are taken up by cells followed by their early endosomal release (just like it is known from cationic liposome/DNA complexes aka "*Lipoplexes*") and upon contact with mitochondrial membranes DQAplexes become destabilized releasing a portion of the DNA from its cationic carrier [71–74].

Finally, it was demonstrated that oligonucleotides as well as plasmid DNA conjugated to a mitochondrial leader sequence co-localize with mitochondria when delivered into mammalian cells via DQAsomes [75]. Yet the barrier lying between co-localization with mitochondrial membranes and functional DNA expression inside mitochondria proved to be unsurmountable at that time. However, several years later two groups have reported DQAsome-based transgene expression in mammalian mitochondria. In 2011, Lyrawati et al. described the expression of Green Fluorescence Protein (GFP) GFP in the mitochondrial compartment using DQAsome-mediated delivery of an artificial mini-mitochondrial genome [76] and Bae reported in 2017, an enhanced mitochondrial transfection efficiency in HeLa cells and dermal fibroblasts when using a modified DQAsome formulation called DQA80 [77]. Though both reports seem to be a promising start, despite their limitations as discussed most recently [78], any routine mitochondrial

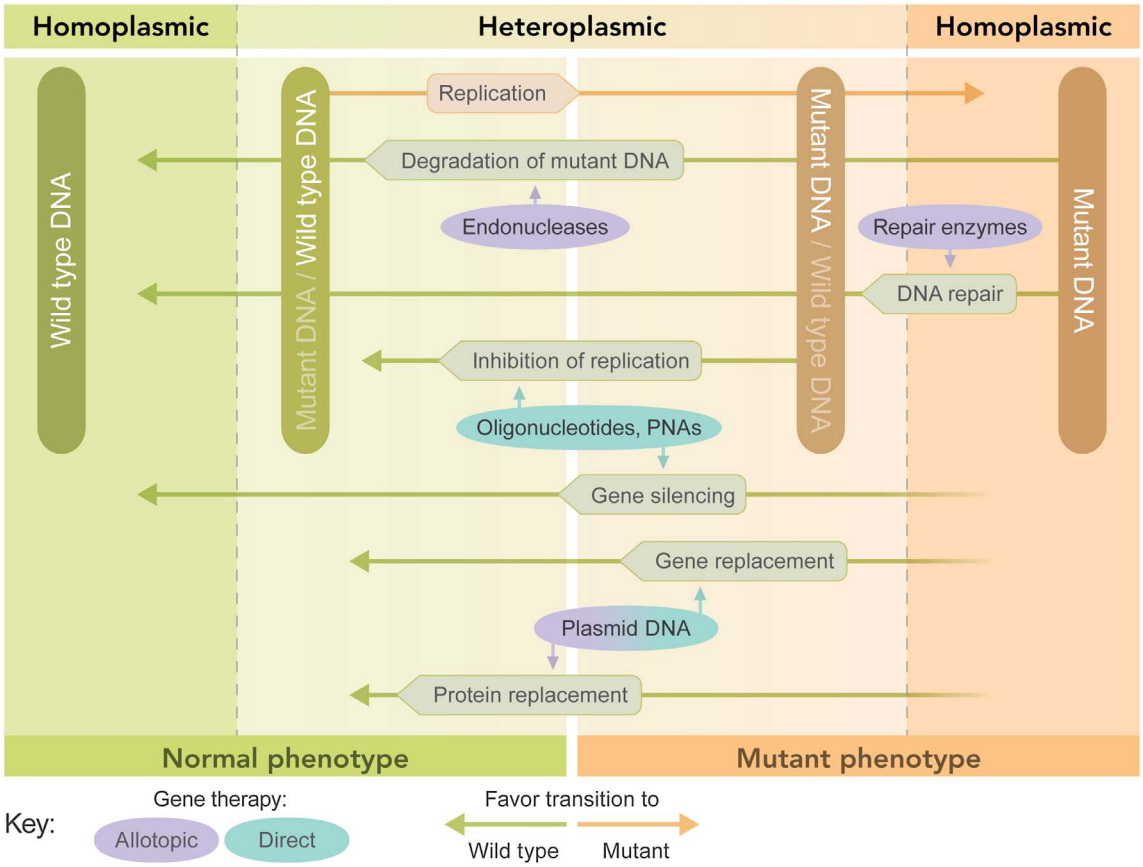

Figure 3. Graphical representation of the effect of various mitochondrial gene therapeutic approaches on mitochondrial heteroplasmy. A cell can go from homoplasmic wild type (dark Green) to homoplasmic mutant (dark ochre) and pass stages of heteroplasmy where either wild type (light green) or mutant (light ochre) mtDNA is dominant. The threshold determines the transition from normal phenotype to mutant phenotype. Ovals represent therapeutic intervention and rectangles represent natural processes. Arrows that do not contact the DNA boxes indicate that the process does not change the ratio or content of DNA. Figure recreated based on a corresponding figure in [145].

transgene expression in the laboratory and above all any mitochondrial gene therapy-based treatment of primary mitochondrial DNA diseases in the clinic have to be judged as being elusive. An alternative mitochondria-targeted nanocarrier, called Mito-Porter, has been designed by Hideyoshi Harashima's group. The Mito-Porter is a liposome-based carrier that delivers its macromolecular cargo to the mitochondrial interior via membrane fusion mediated by an octaarginine peptide (R8) on the liposome surface [79, 80]. More recently, in 2020, Harashima's group encapsulated wild-type mitochondrial pre-tRNA(Phe) into MITO-Porters and then transfected cells with mitochondria carrying a mutated version of tRNA (Phe). As result, the mutation rate of tRNA(Phe) was decreased, and this therapeutic effect was sustained even on the 8th day after transfection [81].

RNA-based approaches towards the manipulation of the mitochondrial genome

The majority of proteins functioning in mitochondria are imported from the cytoplasm and by the end of the 1990's the mechanism of mitochondrial protein import have been studied extensively [82–84]. In contrast to mitochondrial proteins imported from the cytoplasm, no imported tRNAs have been found inside mammalian mitochondria [85] though evidence had been presented for the transport of nuclear encoded tRNA into mitochondria in a variety of non-mammalian organism [86]. The mechanisms by which negatively charged RNAs can cross both hydrophobic mitochondrial membranes began to be unraveled in the late 1990's by Ivan Tarassov's group [87, 88]. During the last 20 years, a substantial amount of data has been accumulated demonstrating that in all eukaryotes non-coding RNAs are being transcribed from the nuclear genome followed by their transport into mitochondria, where they play a role in mitochondrial gene expression [89]. Today it has been well established that "*the mitochondrial RNome represents an intricate mixture of the intrinsic transcriptome and the extrinsic RNA importome*" [89] and efforts are aimed at exploring mitochondrially imported RNAs as a tool to further study mitochondrial DNA and pathologies associated with its malfunction. For example, exploiting the pathway of RNA import into mitochondria

a recent study investigated the possibility of establishing a CRISPR-Cas9 system targeting mtDNA. Ivan Tarassov's group tested *in vitro* and *in vivo* mitochondrially targeted Cas9 versions and a set of mitochondria-specific guide RNAs regarding their effect on mtDNA copy numbers. The authors describe this system as *"more complicated for use than previously found for nuclear DNA"* and chose as title for their paper "*Can Mitochondrial DNA be CRISPRized: Pro and Contra*" [90].

Shifting heteroplasmy

Since mutations or deletions normally do not affect all mtDNAs, wild-type and mutated mtDNA can coexist in a cell, a phenomenon called heteroplasmy. Depending on the cell type, in particular on the cell's energy demand, a minimal number of mtDNA circles have to be damaged to cause clinical symptoms, which is called the threshold effect. Shifting the level of heteroplasmy by selectively inhibiting the replication of the mutant mtDNA and thereby allowing propagation of only the wild-type molecule was proposed for the first time in 1997 by Robert Lightowlers group in Newcastle, UK [91]. To confirm the validity of such a novel strategy they synthesized peptide nucleic acids (PNAs) complementary to human mtDNA templates containing certain mutations known to cause human pathologies. Using an *in vitro* replication run-off assay under physiological conditions, the authors confirmed that their antigenomic PNAs specifically inhibited replication of mutant but not wild-type mtDNA templates [91]. Following Lightowlers' pioneering work, designing ways to manipulate heteroplasmy in cells became a hot area of research for the next 20 years, "*but until recently, a viable method has proven elusive*" [92]. Most recent efforts towards the selective degradation of mutated mtDNA involve zinc-finger nucleases (ZFNs) [93] and transcription activator-like effector nuclease (TALENS) [94, 95], both of which are enzymes able to selectively target single nucleotide mutations. Both enzymes have genetically been modified to express at their N termini a mitochondrial leader peptide to ensure their transport into the mitochondrial matrix and both enzymes have recently been successfully tested in mice carrying a heteroplasmic pathogenic variant in mt-tRNA^{Ala} [94, 95]. Both enzymes are introduced into cells via nuclear-targeted transfection using viral vectors, and the remaining problem is the crossing of the BBB by the vector, which is of importance considering that neurodegenerative diseases have been linked to mtDNA mutations / deletions within the CNS [92].

Bypassing manipulating of the mitochondrial genome via allotopic expression

In contrast to the above described direct mitochondrial gene therapy this approach involves the nuclear-cytosolic expression of a protein originally encoded by mtDNA now mutated and ensuring its transport into the mitochondria matrix by utilizing mitochondrial leader peptides recognized by the mitochondrial protein import machinery. Limited success of allotopically expressing mitochondrial genes was described for the first time in yeast in 1988 [96, 97], endorsed for biomedical use by Aubrey de Grey in 2000 [98] and successfully applied to human cells for the first time in 2002 as described in two papers. Manfredi et al. demonstrated the rescue of a deficiency in ATP synthesis by transfer of the mitochondrial ATP6 gene to the nucleus [99]. Guy et al. allotopically expressed the mitochondrial ND4 subunit gene of complex I, which was also the first point mutation in the mitochondrial genome linked to Leber's Hereditary Optic Neuropathy [100]. The latter approach advanced in 2018 to clinical Phase I, II and III trials (reviewed in [101]). The authors of these studies found an improvement in vision when they injected the viral vector carrying a modified ND4 gene into only one eye while using the other eye as a control for disease progression [102, 103].

5.5. Mitochondrial transplantation

A review paper published in August 2020 asked the question *"if (can)... transplant(ing) fully functional mitochondria directly into defective cells (to treat mitochondrial disorders). Could this be too good to be true?"* [104]. Two years earlier, a paper carried the headline "*Mitochondrial transplantation in humans: "magical" cure or cause for concern?"* [105]. Both of these papers mirror the skepticism with which many reports claiming the effective transplantation of mitochondria into cells, tissues and patients have been met. The paper from August 2020 states

> "... all these data (about effective mitochondrial transplantation) challenge our understanding of how organelles behave in cells, and we should consequently demand high levels of rigour to support these claims" [104].

It comes to mind that Laplace once said "*the weight of evidence for an extraordinary claim must be proportional to its strangeness*", which much later was rephrased by Carl Sagan (1934–1996), into "extraordinary claims require extraordinary evidence" [106].

It is not our aim here to investigate exactly how extraordinary all those claims are, we just want to briefly outline the history of mitochondrial transplantation. First of all, to anyone who believes in Lynn Petra Margulis' (1938–2011) (1967 Lynn Sagan) endosymbiotic theory of the origin of eukaryotic cells [2] it should not come as a total surprise that mammalian cells might have the ability to internalize exogenous mitochondria considering that their progenitor (prokaryotic) cells must have had that ability. However, experimental evidence was lacking until the beginning of the 1980s, when two papers provided data in support of a cells ability to internalize exogenous mitochondria [107, 108]. Both of these papers apparently fell into oblivion, because a 2006 PNAS paper [109] was asking the question whether cells with nonfunctional mitochondria could be repaired by transfer of functional mitochondria without referencing them. The authors answered their own question by demonstrating data which led them to

conclude that mitochondrial transfer between cells can rescue aerobic respiration [109] though the exact mechanism of mitochondrial transfer was left open. This reported mitochondrial transfer between cells however was immediately doubted in the same year by Csordas who wrote that "*these results do not establish whether it was only mtDNA or whole functional mitochondria that were transferred*" [110].

During the same period one of us (VW) conducted his own investigations in collaboration with Keshav Singh's laboratory and we were able to demonstrate that xenogeneic transfer of murine mitochondria into human cells lacking functional mitochondria can, for a limited time, functionally restore respiration in cells lacking mtDNA [111]. Two years later, in 2009, James McCully and his team at the Harvard Institute of Medicine tested the hypothesis whether transplantation of mitochondria isolated from remote tissue unaffected by ischemia into the ischemic zone before reperfusion could enhance cellular viability during reperfusion. They succeeded in showing that injection of respiration-competent mitochondria could significantly decrease infarct size in a surgical ischemia/reperfusion model [112]. Subsequently, McCully's group published a series of papers validating their approach [113–123] and several other groups, using different clinical models, followed [124–127]. In conclusion we believe that it is certainly justified to keep demanding "*high levels of rigor to support all data*" [104] but we are not so sure whether implying the involvement of "magic" for mitochondrial transplantation [105] is appropriate.

Gene replacement – The "3 Parent Baby"

Tremendous success has been achieved in recent years in trying to avoid pathogenic mtDNA transmission between generations via so-called mitochondrial replacement therapy (MRT), which involves several different reproductive technologies designed to replace the mitochondria in eggs from affected women. This revolutionary approach to essentially stop the maternally transmission of defect mtDNA to their newborns was pioneered at Newcastle University [128–130]. The children born by utilizing this approach will have nuclear DNA from both parents and healthy mtDNA from another woman as a third person, hence the term "3 Parent Baby". Currently, an ongoing clinical trial in the UK is designed to assess the outcome of such mitochondrial donation from a healthy woman to a woman carrying mtDNA mutations on the first 75 children born [92].

Mitochondrial MicroRNAs (mitomiRs)

MicroRNAs (miRNAs) are noncoding RNA molecules containing between 18 and 25 nucleotides which act generally as post-transcriptional regulators of gene expression via pairing with complementary mRNA thereby affecting multiple cellular processes like cell growth, proliferation, differentiation, embryonic development and apoptosis [131]. The first miRNA (not mitochondrial!) was discovered in the early 1990s in *C. elegans* [132] and miRNAs were recognized as a new specific group of biological regulators by the early 2000's [133–136]. As of 2015, approximately 600 human microRNAs have been identified [137]. The existence of microRNA in mitochondria, termed "mitomiR" was established between 2009 and 2012 [138–143]. This new field of mitochondrial microRNAs is currently flourishing. Our 11th World Congress on Targeting Mitochondria held virtually on October 29–30, 2020 dedicated an entire session to the role of non-coding RNAs in the nuclear-mitochondrial cross talk and their potential application for RNA medicine (https://www.targeting-mitochondria.com). The major focus on current mitomiR research lies on elucidating the mechanistic details of the transport of microRNAs from the nucleus into mitochondria, on exploring the possibility of mitochondrial genome generated microRNAs and on trying to understand how mitomiRs regulate mitochondrial gene expression, mitochondrial biogenesis and mitochondrial functions [131].

Conclusion

In 2001 one of us (VW) wrote in the preface for a Theme Issue about "Drug and DNA delivery to mitochondria" that "*the field of mitochondria is currently one of the fastest growing discipline in biomedicine*" [144]. This statement from 20 years ago was made perhaps a bit prematurely, considering the remarkable progress the field of mitochondrial medicine has made during the last two decades. In Part II of our review we will continue to outline and highlight most recent developments in mitochondrial research.

Abbreviations

BBB	Blood Brain Barrier
BN-PAGE	Blue-Native Polyacrylamide Gel Electrophoresis
C. elegans	*Caenorhabditis elegans*
Cas9	Caspase 9
CNS	Central Nervous System
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
GFP	Green Fluorescence Protein
IMM	Inner Mitochondrial Membrane
IMS	Intermembrane Space
MICOS	Mitochondrial contact site and Cristae Organization System
mtDNA	Mitochondrial DNA
NAD	Nicotinamide Adenine Dinucleotide
OPA 1	Optic Atrophy (gene, protein) 1
OXPHOS-ETC	Oxidative Phosphorylation Electron Transfer Chain
TCA	Tricarboxylic Acid
tRNA	transfer Ribonucleic Acid

Conflict of interest

Volkmar Weissig is Senior Board member at Life Sciences-Medicine of 4open, published by EDP Sciences.

Both authors did not take any action to influence the standard submission or peer-review processes and reports no conflict of interest. The authors alone are responsible for the content and writing of this manuscript. This manuscript contains original material that has been previously published and is appropriately cited. The authors have no conflicts of interest to declare.

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