Mitochondrial Diseases, Treatments, and FDA Orphan Legislation

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ABSTRACT

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Mitochondria originated during a key endosymbiotic event, when an enveloped bacterium or invasive parasite adjusted to its intracellular surroundings and formed the first eukaryotic cell. The symbiote evolved into a specialist organelle supplying the vast majority of a cell's ATP energy supply as well as regulation of cellular differentiation, cell death, the cell cycle, and cell growth. Due to its double layered membranes and separate DNA control systems, it has proved an elusive target for medicines, and is responsible for a host of diseases and may be a key contributor to the aging process.

Mitochondrial disorders, though a diverse family ranging from cardiac diseases to a subset of Parkinson's syndrome, individually affect relatively small populations and may therefore fall under the purview of the Orphan Drug legislation enforced by the Food and Drug Administration. While no viable delivery mechanism for macromolecules larger than proteins currently exists for the inner mitochondrial matrix, several academic research papers suggest that the unique morphological challenges of mitochondria may be overcome. A survey of these approaches is presented within the context of mitochondrial structure, and a path towards economical drug development via the Orphan Drug system is proposed.

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To N.L.A.H., both.

INTRODUCTION TO MITOCHONDRIA

Mitochondria, along with chloroplasts, share the unique property of being the only subcellular organelles with DNA independent of the nucleus. Two theories were proposed account for this non-nuclear DNA; that it had migrated from the nucleus itself, or was the result of an event where one anaerobic proto-eukaryotic cell engulfed another aerobic bacterium, known as primary endosymbiosis and championed by Lynn Margulis[1] despite much skepticism. A conclusive meta-study in 1992[2] found the endosymbiotic theory compelling, and it is accepted today as one of the seminal moments in the formation of complex life. Evidence of the mitochondrion's bacterial past abound, such as its reliance on binary fission for replication, double membrane outside structure, circular DNA structure, lack of histone proteins, and non-eukaryotic ribosomes along with non-eukaryotic amino acid codon triplet translation[3].

Recent sequencing work finds that *Rickettsia prowazekii* is the most genetically similar known relative of mitochondria[4], implying the possibility that endosymbiosis was not a chance event of a prey bacterium surviving attempted consumption by a predator proto-eukaryote, but that of an invasive, perhaps flagellated parasite that adopted a mutualistic relationship after successfully entering the proto-eukaryotic cell.

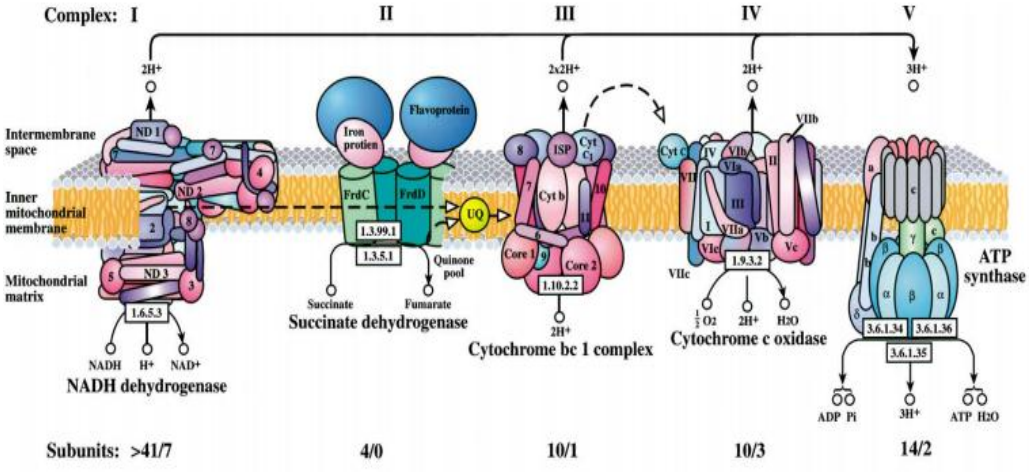
Regardless of the nature of the endosymbiotic event, the bacterial origins of mitochondria are seminal in its evolution, function, morphology, and prospects as a therapeutic target. 1.5 billion years of coevolution has stripped the organelle of virtually all of its original genes, transferring them to the nucleus or removing them entirely[5]. Varied mitochondrial functions separate from its canonical role as the primary aerobic

energy producer of the cell have developed, as a result of protein import into the mitochondria and its incorporation into cell cycle regulation.

Mitochondrial Functions

As the organelle responsible for both the citric acid cycle and oxidative phosphorylation (OXPHOS), mitochondria produce the vast majority of ATP energy available to a healthy cell[6]. Mitochondrial genes concern themselves with the OXPHOS process, the means by which ATP is processed due to the oxygen atom's singular ability to accept H+ ions. Put simply, the organelle's primary duty is to provide a series of 'water mills', whereby ions flow down an electrical gradient. The mills provide the motive power with which ATP synthase can attach a third phosphorus atom to ADP, allowing for the formation of an ATP energy carrier used ubiquitously throughout the cell. Interruption of this process, such as through asphyxiation, results in quick cell death – even more rapidly with those cells such as neurons with high energy requirements.

Figure 1: Electron Transport Chain[7]



As may be seen in the above diagram (Figure 1), the proteins Complex I (NADH), Complex III (Cytochrome b-c complex), and Complex IV (Cytochrome C Oxidase complex) exist to pump H+ ions across the inner mitochondrial membrane barrier, with Complex IV reducing oxygen to water. This forms a voltage gradient, whereby H+ ions seek to return to the matrix. This is facilitated via Complex V (ATP Synthase), a complex radial protein analogous to the watermill. Driving a rotating subunit akin to a radial piston, the proton flow provides the force allowing the enzyme to combine ADP and phosphate to form ATP[8].

Mitochondria are capable of a surprising amount of autonomous and cell-directed morphogenesis. Mere contact between two mitochondria can set a process of fusion in motion, incidentally providing evidence that macromolecular import (in this case, mitochondrial mtDNA plasmids) into these structures is indeed possible[9]. Cristae are able to invaginate and deform, and there is some evidence that the quantity of Complexes I through V are not static[10]. As with bacteria, mitochondrial replication takes place via fissioning; however the signals are nuclear in origin. Upregulation by mitochondrial

fission factor Mff and its associated Drp1 protein, and downregulation by Mitofusin 1, regulate mitochondrial cell count. With all these processes at its disposal, a cell can modulate energy output based on both its differentiated role and changing energy demands.

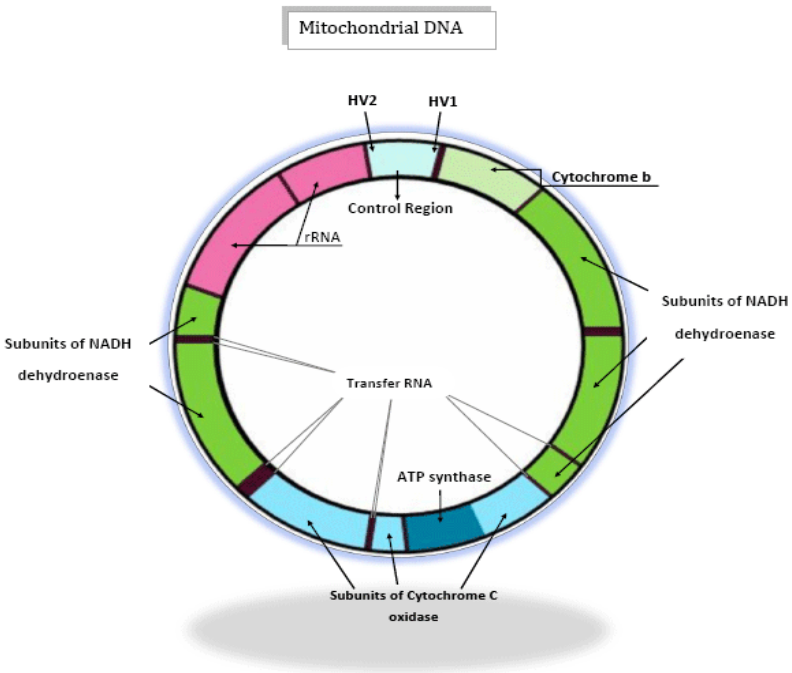
Evolution has curiously co-opted Cytochrome c, present within the inter-membrane space and the structural unit associated with Complex IV, into a cell death signaling device, whereby pro-apoptotic factors result in translocation of increased levels of Ca2+ from the endoplasmic reticulum into the cell and mitochondria. This triggers Cytochrome c release into the cell cytosol, causing caspase release. A positive feedback ensues that causes calcium to soar to cytotoxic levels. The recruited caspases function as complexes that actively destroy cellular structures and complete apoptosis[11]. Common causes of the initial Cytochrome c release include cellular infection and DNA damage; the pathway as a result is critical in cancer defense. It has been speculated that one of the causes of the Warburg effect, whereby cancerous cells shut down mitochondrial respiration and rely on glycolysis alone despite oxygen availability, is the reduction of voltage differentials in the mitochondria and inhibition of Cytochrome c release[12]. Any macromolecular delivery system will have to contend with the inter membrane space as a result. Rupture of the outer membrane could trigger the above process rendering an inadequately designed vector cytotoxic.

Mitochondria are also implicated in pyrimidine and heme synthesis; disrupted organellar function reduces the rate-limiting enzymes that power these processes. Cholesterol and neurotransmitter metabolism as well as liver ammonia detoxification are further known roles[13].

Mitochondrial DNA

Consisting of a circular double-stranded DNA (mtDNA) 16,569 base pairs long, and covered by a histone-like mitochondrial transcription factor A protein (TFAM), an average of 5 copies[14] of mtDNA reside within the inner matrix of a mitochondrial organelle. The DNA encodes for very few functional proteins, along with a control region, and tRNA and ribosomal machinery to support transcription and translation, respectively. All mtDNA is maternally inherited, as sperm mitochondria are actively destroyed during insemination to prevent chimerism.

Figure 2: Mitochondrial DNA[15]



Mammalian mitochondrial DNA, regardless of species complexity, appears to retain 13 core protein-coding genes[16] (Figure 2), while being relieved of all other protein synthesis by the nucleus. This strongly implies the functional importance of having

synthesis of these 13 proteins occur within the mitochondrial inner matrix itself and not within the nucleus. It may be speculated that either their structural functions cannot be replicated through mitochondrial import, or up and down-regulation of their ubiquity in the mitochondrial structure cannot be modulated with adequate speed from the nucleus, or both.

These genes may be grouped in 5 distinct components of aerobic energy production: complexes I, III and IV, and the ATP synthase enzyme in complex V[17]. Complex II indirectly contributes to energy production via catalyzing succinate to fumarate.

Critically, mtDNA suffers from a considerably higher mutation and particularly deletion rate than nuclear nDNA. This may be due to several factors. DNA polymerase gamma (POLG), encoded in the nucleus, may be less able to faithfully replicate guanine-dominated mtDNA, resulting in base-pair mismatches[18]. Much of the extensive protein machinery within the nucleus for mutation detection and repair appears unavailable within the mitochondrial inner matrix. This weakness is compounded by local free radical production during the OXPHOS process.

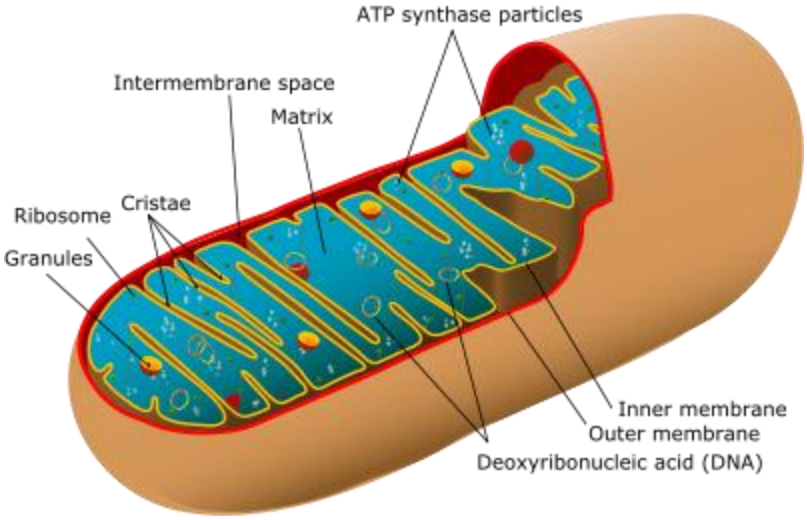
Table 1: Mitochondrial Genes

Component	Category	Gene
Complex I	NADH Dehydrogenase	MT-ND[1,2,3,4,4L,5,6]
Complex III	Coenzyme q Cytochrome b-c	MT-CYB
Complex IV	Cytochrome c Oxidase	MT-CO[1,2,3]
Complex V	ATP Synthase	MT-ATP[6,8]

Structure and Membrane Composition

Along with chloroplasts, mitochondria feature a double membrane enclosing an inter-membrane space. The inner membrane forms a series of folds, called cristae, greatly increasing the surface area of the inner matrix within it. As described later, this enlarged surface area serves enhances the voltage gradient that powers energy production. Cells with greater energy demands, such as muscle tissue, increase the surface area of this reaction via a greater number of cristae as well as a higher mitochondrial count. A variable number of mtDNA molecules are contained within the matrix, along with ribosomal protein assemblies, ATP synthase, and granules that appear to modulate inter-membrane enzymes. The entire structure is about 3-4 µm in length and 1µm in width[19].

Figure 3: Mitochondria Cross-Section[20]

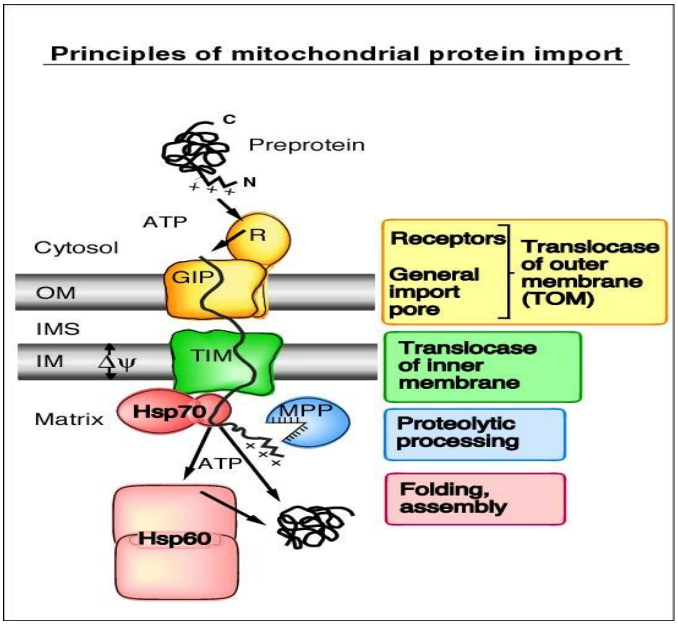


The inter-membrane space (Figure 3), acidic due to proton import from the matrix, retains structural and signaling proteins from the cytosol as well as mtDNA

proteins and Hsp70 protein stabilizers. It also serves as a store of the small heme protein Cytochrome c discussed later[21].

The outer membrane has a similar protein-to-phospholipid ratio to that of the eurkaryotic plasma membrane (50:50 in weight), while containing integral porins that allow sub-5,000 dalton molecules to pass through. Larger molecules require mitochondrial membrane transport proteins to shuttle across the membrane, referred to as the TIM/TOM complex[22]. Signaling sequences recognized by the Translocase of the Outer Membrane complex (TOM) cause active transport of the molecule into the intermembrane space and further to the Translocase of the Inner Membrane complexes (TIM) known as TIM23 and TIM22, located nearby, facilitating entry of the protein via ATP into the inner matrix where it can unfold. Hsp70 stabilizes the molecule, preventing Hsp60-mediated unfolding until arrival at the inner matrix, while processing peptidases cleave the signaling pre-sequences. The TIM/TOM system (Figure 4) allows for circa 1,500 mitochondria-related proteins encoded by the nucleus to localize; mutations in this complex are usually fatal.

Figure 4: Mitochondrial Protein Import[23]



Unlike that of the outer membrane, the mitochondrial inner membrane's protein-to-lipid concentrations are 80:20; similar to bacteria and further evidence for the endosymbiotic theory. Along with TIM structures, Complexes I through V belonging to the electron transport chain dot its surface. Multimeric mitofilin proteins present throughout the membrane stabilize the cristae. High protein levels reflect this structural backbone as well as the various permeable membrane-spanning proteins needed for respiration and proton exchange with the inter-membrane space[24].

As a result of this double-layered membrane and its size and signaling requirements for molecular import, only proteins with correct amino acid signaling sequences assembled in the cytosol are available to the inner matrix. This presents one of the key challenges to mitochondrial druggability – how to effectively import macromolecules without signaling sequences, or how to design therapeutic molecule compounds with the correct targeting sequences.

MITOCHONDRIAL DISEASES

Nuclear and Mitochondrial Origins

Recognition of the role of mitochondria in a plethora of diseases has been a fairly recent phenomenon. As may be seen in the table below and consistent with the role of the organelle, mitochondrial diseases are preferentially implicated in tissues with high energy demand: the brain, heart, liver, skeletal muscles, respiratory and endocrine, and kidney systems. A slew of symptoms may range from motor control loss, general muscle weakness and pain, exercise intolerance, gastro-intestinal indications, failure to thrive and poor growth, heart and liver disease, diabetes, seizures, to a weakened immune system[25]. Ragged red fibers, where clumps of diseased mitochondria accumulate in the subsarcolemmal region of muscle fiber[26], are identifiable under the microscope for a large number of the diseases. While 13 proteins are encoded by mtDNA, nearly 3,000 genes and 1,500 proteins from the nucleus have been discovered[27]. The field is in its early stages and often the root genetic locations of mitochondrial dysfunction are not fully known. More than 36 diseases have been identified; for the sake of brevity, the most understood syndromes are listed. Those with maternally-inherited characteristics strongly implicate mtDNA. Population sizes are based either on published figures or on extrapolations of published sample population rates. Table 2a identifies larger diseases with mitochondrial pathologies, while Table 2b lists afflictions directly attributable to mitochondrial malfunction.

Table 2a: Diseases with Mitochondrial Pathologies

Disease	Notes	Estimated U.S. Population
Alzheimer's Disease	Amyloid-β inhibition of Complex IV, among others[28]	5,000,000
Parkinson's Disease	Decreased repair & maintenance of mitochondria[29]	500,000 – 1,000,000
Athlerosclerotic Heart Disease and Mitochondria-related Cardiomyopathy	Varied causes. Observed loss of mitochondrial mass and mtDNA damage[30]	~ 500,000 deaths annually
CKD: Chronic Kidney Disease	Varied causes. Apparent up-regulation of nuclear mitochondrial genes and increased ROS[31]	1,000,000

Table 2b: Diseases due to Mitochondrial Malfunction

Disease	Notes	Estimated U.S. Population
MELAS: Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke	Mutant mtDNA. May be widely under-diagnosed, with mild to moderate hearing loss as the most minor symptom[32]	< 10,000[33 34]
MIDD: Maternally Inherited Diabetes and Deafness	May be mis-diagnosed as regular Type 1 or Type 2 diabetes. mtDNA tRNA mutation[35]	~130,000 - 700,000 0.5% to 2.8% of Type 2 diabetic population
LHON: Leber's Hereditary Optic Neuropathy	mtDNA mutations of Complex I	7,000 - 10,000 Frequency estimated at 1:30,000/1:50,000 in Europe[36]
Leigh's Disease	mtDNA or nDNA mutations.	~ 7,500 1 in 40,000 births. Higher in Quebec[37]
MERRF: Myoclonic Epilepsy with Ragged Red Fibers	mtDNA mutations affecting tRNA lyseine[38]	~ 750 ?
KSS: Kearns-Sayre Syndrome	Eyelid & eye muscle control malfunctions. mtDNA deletions[39]	< 100
Pearson Syndrome	Usually fatal in infancy. mtDNA deletions[40]	< 100
CPEO: Chronic Progressive External Ophalmoplegia	Related to Kearns-Sayre Syndrome. Ragged red fibers. mtDNA deletions[41]	< 100

Beyond the above figures, it is estimated that 1 in 4,000 children in the United States will develop mitochondrial disease by age 10, giving an affected U.S. youth population of circa 12,500[42]. Some children are classified within the above disease framework and others are labeled generally as a 'failure to thrive'. Mitochondrial myopathy, defined as

genetic mutations that directly affect the electron transport chain, may affect 1 in 8,000 in the general population[43], giving an affected U.S. population of 37,500.

Notably, most diseases whose root causes lie in mtDNA mutations alone such as LHON and Leigh's disease feature small population sizes. It may be speculated that strong selection pressure has kept rates low as maternal carriers would be at a multigenerational procreative disadvantage. Diseases with significant population counts, namely MIDD and MELAS, are survivable and may not have encountered such pressure.

As with much of medicinal research, causality in the progression of a disease may be very unclear. Does decreased mitochondrial function initiate malaise, or is it a byproduct? If a byproduct, does it then exacerbate the condition? If so, is the organelle worth targeting by a therapeutic, or ought instead the focus to be on mitochondrial genes within the nucleus?

Alzheimer's and Parkinson's disease bear further investigation and are the focus of much mitochondrial research. Mice engineered to overexpress amyloid-β clusters, a hallmark of Alzheimer's, then had neural mitochondria excised at various life stages. Well before old age, these synaptic mitochondria had accumulated five times the amount of clusters as non-neural mitochondria and had compromised energy production as a result[44]. Complex IV's functioning appears to be particularly disrupted by amyloid-β[45], and inhibition of correctly functioning mitochondrial enzymes appears to raise reactive oxygen species (ROS) that wreak further havoc on the mitochondria and beyond. Systemically, a significant decrease of energy metabolism in the frontal and temporal lobes of the brain is common. This leads to senescence and death of the neurons and breakdown of neuronal communications[46].

More specifically to mtDNA and to potential treatment of Alzheimer's and other diseases, ROS production leads to oxidation of mtDNA itself[47]. Control regions of mtDNA have shown elevated mutation rates in afflicted patients. A potential treatment of later-stage Alzheimer's could involve the provision of intact mtDNA into damaged mitochondria, while it may be speculated that amyloid-β-countering molecules could address earlier stages of the disease. Both require an as-yet-undeveloped mitochondrial delivery system.

An alternative theory postulates that a root cause of Alzheimer's is the disruption of mitochondrial motility. Normally functioning mitochondria in tissues with high energy demand may actively travel via the microtubule infrastructure to regions with high Ca2+ concentrations, such as axonal or dendritic regions of neurons. Defects in protein regulation of mitochondria-endoplasmic reticulum interactions in regions termed mitochondria-associated membranes (MAM)[48] may render mitochondria unable to escape their association with the ER and are therefore unable to travel to sites with high energy demands and/or other mitochondria-mediated requirements[49].

Parkinson's Disease appears to demonstrate nuclear-encoded mitochondrial gene malfunction. A root cause of some Parkinson's may relate to 10 sets of genes including Parkin and PINK-1, controlled by master regulator PCG-1α[50]. As a result, enzymes critical to OXPHOS are suppressed and neurons abandon mitochondrial respiratory pathways with a concomitant energy supply loss, leading to widespread death of dopamine-producing cells within the brain.

In a strikingly similar manner to Alzheimers, but involving Complex I and III instead of Complex IV, inhibition of those systems leads to dramatically increased ROS

production[51]. A downward spiral occurs: increased malfunction, leading to increased ROS production, resulting in damage to the surviving OXPHOS systems, attendant malfunction, and so on. Oxidative damage is not restricted to the mitochondria and can damage other cellular organelles, proteins, lipids, and nuclear nDNA. A potential treatment could redress this enzymatic balance by artificially increasing the amount of Parkin-type enzymes; this may be accomplished by remodulating PCG-1α or introducing those enzymes externally via medication. A recent study found widespread DNA repair assembly deficiencies in Parkinson's mitochondria as well as OXPHOS damage of mtDNA[52].

MELAS and MIDD sufferers, the other large mitochondrially-derived disease populations, may suffer from misdiagnosis as symptoms are often identical to similar, more common diseases[53], and severity may be dependent on the relative populations of corrupted and functional mtDNA within the target tissues. Improved online genealogic tools and more widespread genetic counseling may firm up the estimated numbers presented in this paper as well as open up these populations to tailored therapy.

Cardiomyopathy and heart disease, the primary cause of death in the United States, is due to an exhaustive range of genetic, environmental, and lifestyle factors. Recently mitochondrial abnormalities have been implicated as the sole cause or a contributing factor towards incidences of the disease[54]. Decreased metabolic rates and increased ROS production are implicated. Online tools such as MITOMASTER, combined with mtDNA sequencing and phylogenetic data could improve diagnosis[55]. Chronic Kidney Disease appears to have a similar relationship to mitochondria.

The relationship between mitochondria and cancer is extensive and beyond the scope of this paper. Manipulation of mitochondria disabled by the Warburg effect may provide a directed apoptosis-inducing weapon. High incidences of mtDNA mutations in cancerous cells have been observed[56]. A class of molecules termed 'mitocans'[57] are specifically lethal to mitochondria and induce cell death, and could be utilized as an anti-cancer strategy.

As is implied by the estimated prevalence rates in Tables 2a and 2b, maternally-inherited mtDNA mutations that are homoplasmic – where all mtDNA molecules share the same defect – are extremely rare in surviving populations. Far more likely is the condition of heteroplasmy, where a certain percentage of mtDNA remain wild-type while the rest entertain one or more defects[58]. This wt/- mtDNA ratio may be resilient; as many as 70-80% of mtDNA may be corrupted[59] before a threshold is reached and mitochondrial dysfunction ensues. A logical therapeutic approach would be to redress this heteroplasmic ratio by either the provision of wild-type mtDNA or the selective destruction of damaged mtDNA.

Alternatively, upregulation of mitochondrial fission activity could disperse the population of wild-type mtDNA within cellular mitochondria and alter the ratio as well, and possibly segregate salvageable mitochondria from those destined for mitophagy. After this triage, fission of functioning mitochondria could repopulate the cell. Disruption of this process may be causative for Parkinson's Disease[60].

Mitochondria and Aging

A common paradigm in the above discussed diseases is the starving of the cell due to insufficient energy production; whether the cause or a deleterious effect of a syndrome, an ATP deficit may directly result in cellular senescence and death. This is aggravated in cells with high energy requirements and it is no coincidence that mitochondrial disorders affect the central nervous or cardiovascular systems. A natural question arises: if cardiomyopathy is the leading cause of death in aged populations, and Alzheimer's & Parkinson's the leading neurological syndromes of the old, why do these diseases appear primarily in the elderly?

The mitochondrial theory of aging attempts to unify the diverse strands of evidence, by postulating that the inadequate repair machinery available to mtDNA, combined with its proximity to the OXPOHOS site of ROS production, leads to progressive, inevitable mtDNA mutation and deletion. Given that fully 93% of mtDNA is functional coding without introns, and even displays overlap of genes, virtually any point mutation will be deleterious. Either there will be a defect in Complex I, II, or IV formation through disruption of those structural genes, or a defect in the ribosomal machinery necessary to translate them, or a defect in the tRNA necessary to translate the proteins, or a defect in the control mechanism.

Redundancy only exists in the mtDNA copy number: if sufficient mtDNA molecules within mitochondria are intact for a particular gene, they may be expressed in wild-type in adequate quantity to maintain mitochondrial function. Virtually any recoding of the molecule stands a high chance of being phenotypic; compounding the effect is the

proximity of mtDNA to the source of ROS in the inner membrane and a concomitant sixteen-fold increase in the mtDNA mutation rate[61].

Disruption in the Complexes raises ROS production, engaging the aforementioned vicious cycle of increased protein and DNA damage, followed by further increase in ROS levels. The results are damaged or defective mitochondria and a cellular environment with hostile ROS interacting deleteriously with the nucleus and cytoplasm. ROS by itself is a key cellular death signal[62], often released by the cell intentionally to cement its own demise after DNA damage.

If the above is insufficient to kill the cell, the gradual reduction of ATP production may induce apoptosis in cells requiring higher energy levels. Further, once an energetic threshold has been reached, cells may enter senescence while up-regulating cellular maintenance genes[63] and down-regulating functions related to the cell's organismal role. This senescent 'house-keeping' stage, if repeated by a group of cells, may lead to tissue failure and eventually organ failure. Muscles are weakened and tone decreases. All these are classic hallmarks of aging.

In a direct test of the mitochondrial theory of aging and ROS production, mice heterozygous for the SOD2 antioxidant gene, therefore unable to produce SOD2 at levels seen with wild-type mice, demonstrated increased oxidative damage but did *not* significantly decrease their lifespans. New theories, identifying ROS production as a key cellular signal for OXPHOS regulation[64] and a homeostatic control, cast doubt on the current dietary fad of ingesting enormous amounts of antioxidants to stave off aging[65]. All this may do is further tax the cell by forcing it to export the compounds out of the cell as waste products. Further animal studies have replicated these disappointing results, and

meta-studies of human antioxidant consumption have found zero, to slightly negative health results among those with adequate base diets[66]. Therefore ROS may not be the first cause of mitochondrial degeneration.

Morphologically, mitochondria in the young and aged are distinct. Whereas a large number of small mitochondria are present in young cells, a small number of large mitochondria are visible in old cells of both rats and humans, while the total volume of mitochondria (~20% of cell volume) remains constant[67]. The larger, old mitochondria respire considerably less in aged liver tissue[68].

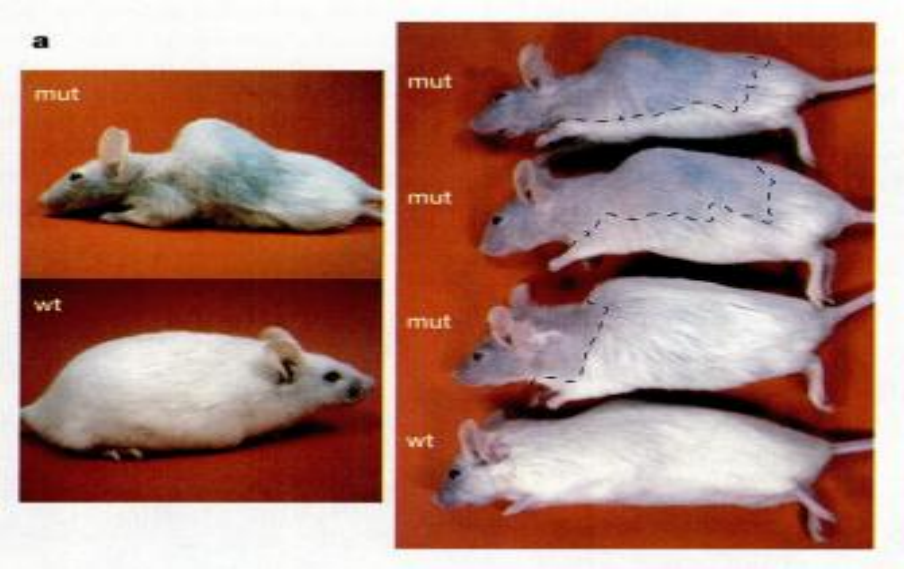
Aging, as far as it is mitochondrially related, may therefore not require cell-catastrophic levels of ROS and a vicious cycle, but the slow, inevitable creep of deletions of mtDNA and mutations of nuclear-encoded mitochondrial nDNA. Past an energetic threshold that may be determined by the degree of heteroplasmy, the cell enters senescence or dies. The effect on the circa 1.5% of the cells classified as stem cells is worthy of speculation; if large portions of these cells senesce then tissue regeneration, central nervous cell replenishment, muscle tone (inclusive of muscles contributing to tight, non-wrinkled skin), and wound healing would all be affected. These too are hallmarks of aging.

If the above is true, then mammals with longevity characteristics ought to have evolved with lower mutation rates of mtDNA, and not necessarily reduced metabolic rates. A cross-species analysis found reduced mtDNA base repeats in longer-lived creatures; base repeats are considerably more prone to mutation[69]. Indeed, reduced mtDNA mutation rates were found in longer-lived animals[70]. In favour of steady-decline models are studies supporting the notion of widespread mtDNA deletion over time, and

more worryingly, rates of mutation increase in energy-intensive tissues. One paper found a 2,000-fold increase in cerebral mutation rates over the cerebellum, with as much as 3% of total mtDNA bases mutated in elderly samples[71]. Other studies have found tissue-specific mutations that appear only in certain aging periods[72]. The process appears in other species as well, including monkeys[73], rats[74], mice[75] and nematodes[76]. Nevertheless, it could be argued that mtDNA mutations and deletion might be an effect, not a cause of aging.

An elegant study using homozygous knock-in mice addressed this central issue. Modification of the nuclear Polg polymerase, the only protein able to copy mtDNA and vital for tissue formation and mitochondrial operation, rendered it unable to proofread effectively[77]. The mice consequently suffered a far higher mutation rate of mtDNA. These modified mice developed classic signs of mouse aging by only 25 weeks, including spine kurtosis, weight and hair loss, osteoporosis, anaemia, alopecia, heart enlargement and sarcopenia (Figure 5). A median lifespan of 46 weeks was observed with no mouse living past 61 weeks. Greater than 90% of control mice were still living at the 61 week mark[78].

Figure 5: Mutant versus Wild-Type Mice of Same Age[79]



The mitochondrial theory of aging does not lay claim to the entire aging effect and complementary theories include build-up of intracellular and intercellular wastes, cross-linking of compounds, ROS damage of cellular machinery, nuclear DNA mutation and general mis-repair accumulation[80]. Nevertheless, a therapy that could repair mtDNA, restore sufficient homoplasmy, and potentially reboot cells might result in measurable life extension and delay of age-related decay. Such an invention would have immense societal and economic consequences.

DRUG DEVELOPMENT PROSPECTS

Micromolecule Delivery Strategies

As mentioned previously, access to the mitochondrial matrix is challenging due to the inner and outer mitochondrial membranes. Any potential drug regardless of vector will have to negotiate this barrier after successfully entering the cytosol through the cell's

outer membrane. Two methods have been researched: entry via the TIM/TOM membrane gates, and outright fusion with specially formulated liposomes.

Using the gates requires the correct 'key', a pre-sequence tag that is recognized by the gate proteins. Many exist, and are usually 10-80 amino acid residues in length and fold into an amphipathic α-helix with a hydrophilic and hydrophobic face[81]. Localization to the mitochondria is facilitated by the molecular chaperone Hsp90. Passing through the TOM gate, the protein's precursor is then cleaved by a peptidase at the TIM gate before accession to the inner matrix. Proteins destined for other compartments, such as the outer or inner membrane and intermembrane space, contain poorly understood internal sequencing signals.

Drug designers pursuing this route are able to choose from viral or non-viral vectors for entry into the cytoplasm as long as the cargo with its pre-sequence are delivered intact. Designed for protein transport, the system could in theory be used for snippets of DNA, RNA, siRNA/miRNA, or nucleases.

Protein import, which the system was designed to handle, may be the cargo of choice. A therapy might include provision of replacement or supplementary endogenous proteins that for reasons of mutation or mis-regulation are misshapen or in insufficient quantity, or supply of a custom small molecule(s) with a desired effect. The advantage of such small sizes is the possibility of oral dosing - key to adoption in the U.S. market[82].

Commercially several drugs are under development utilizing the TIM/TOM approach. Edison Pharmaceuticals is entering pivotal Phase 2 trials for an orally absorbed small molecule called EPI-743. It works as a CoQ analogue by targeting NADPH quinone oxidoreductase (NQO1) in Complex I, for treatment of 60 patients with Leber's

Hereditary Optic Neuropathy(LHON). The FDA granted orphan drug status to Edison for EPI-743. Little toxicity was noted and earlier trials demonstrated very promising results[83]. A second molecule is in Phase 2a, intending to treat Friedrich's ataxia. There do not appear to be other clinical trials specifically targeting mitochondria internally. The company does not give details out on the formulation of EPI-743, but given its definition as a small molecule and formulation, it is most-likely capable[84] of passively crossing the mitochondrial membranes.

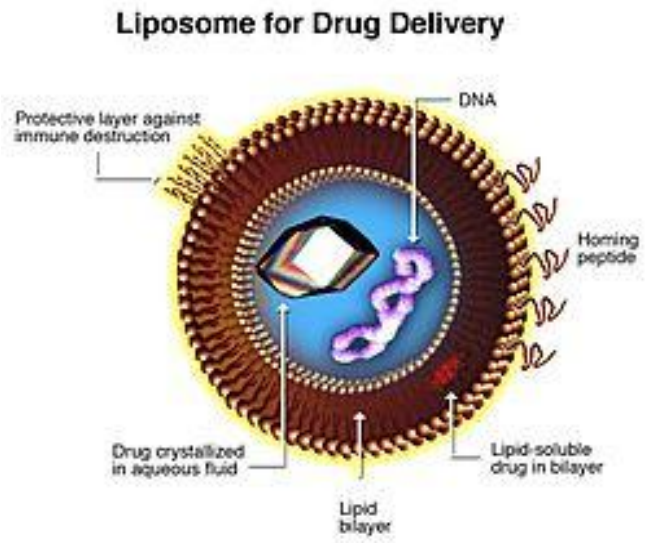
Recent academic research appears to have successfully inserted macromolecules by packaging an mtDNA gene called ND4 inside of a plasmid and then repackaging it inside of an adeno-associated virus(AAV) capsid containing the AAV protein fused to a mitochondrial targeting sequence[85]. Injection of the construct into vitreous mouse eyes resulted in successful recombination of the gene and its expression of the protein in mouse eyes along with attendant ATP synthesis. The experiment was repeated with mutant, defective ND4, and the mice developed LHON-like symptoms. The authors speculate that given the AAV vector's payload capacity of up to 5,000 base pairs, large deletions of mtDNA could be repaired.

However, use of such a vector implies local or temporary application due to immune response. The vitreous humour contains no white blood cells and is as a result a fertile target of gene therapy. Deployment of viral vectors elsewhere in the body will provoke an immune response and this has prevented use in a global treatment. Perhaps a non-viral vector studded with MTS could achieve a similar effect. Alternative small-molecule delivery strategies include lipophilic cations, where the delocalized positive charge preferentially accumulates in the negatively charged mitochondria of cancer cells.

Macromolecule Delivery Strategies

For truly large payloads, such as full, intact 16.5kb mtDNA molecule, a non-viral vector will be needed. Historically this has meant liposomes, dual-layer hydrophilic/hydrophobic lipids that resemble tiny cells with an inner core containing the drug of choice. These vectors feature lower immunogenicity but sacrifice transfection rate versus the viral approach. The inner core may be filled with a variety of compounds and sizing is very flexible. Intact mtDNA could be shuttled, as well as RNA, or for down-regulation, siRNA, miRNA or nucleases targeting deleterious mutations. Alternatively a protein or cocktail of proteins could be considered, or a variety of small molecules. Structural elements of the OXPHOS Complexes could be introduced.

Figure 6: Liposomes[86]



Composition of the outward-facing lipid layer (Figure 6) is key to the construction of liposomes. It must avoid immune detection long enough to reach target tissues, and then competently meld with a cell's plasma membrane to deliver its contents, and do so without being engulfed by a lysosome other otherwise destroyed once inside the cytosol. These difficult challenges have resulted in low transfection rates for liposomes and gene therapy efforts are primarily viral in nature. If a global, multi-tissue delivery system is the goal, transfection rates will have to approach reasonable levels. Recent approaches have included 'ghosting', where the outermost layer consists of remnant red blood cell membranes and considerably longer half-lives of the assembled liposome[87].

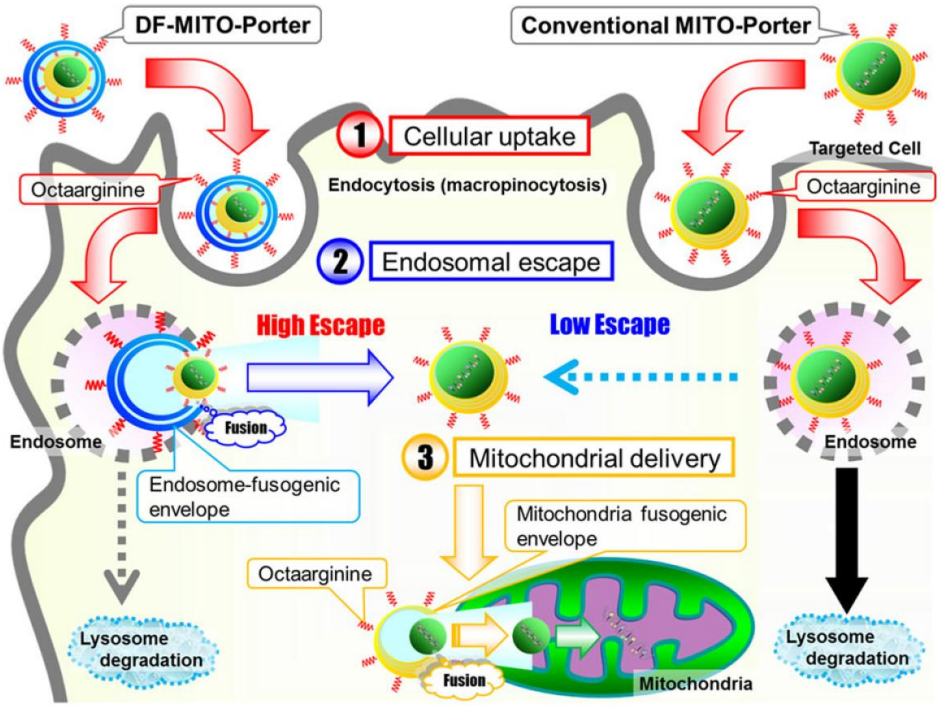
The first attempt to assemble a mitochondrially-targeted liposome began in 2003. A bola-liposome with a cationic outer layer was assembled, termed a DQAsome for its use of dequalinium chloride. The macromolecule fulfilled many of the criteria of a delivery system: it bound and condensed plasmid DNA, protected it from DNAse digestion in the cytoplasm, and successfully transported it to mitochondrial organelles. In vitro, cardiolipin-rich liposomal mitochondria simulants caused the DQAsomes to release their DNA cargo[88]. However no evidence was found of actual DNA migration into the inner matrix space of mitochondria and it is not obvious how a single-layer design could survive both endocytosis and bypass two mitochondrial membranes. Research has been discontinued[89].

In 2008 a group of researchers from the University of Hokkaido embarked on a new liposomic approach involving novel multilayered liposomes entitled MITO-Porter[90]. Uniquely, entry into the cell is mediated by pinocytosis and not the more common phagocytosis. The pinocytotic pathway is generally used for smaller-sized molecular

import of fluids and nutrients, as opposed to the phagocytotic pathway. Pinocytosis is nonspecific in its import requirements. Usually the pinocytotic pathway results in engulfing by lysosome for degradation and digestion of the nutrients while phagocytosis is a key element in immune system defense and clathrin-mediated garbage collection. It may be speculated that reliance upon phagocytosis has been a key stumbling block in liposomic delivery system transfection rates due to vesicle degradation of the contents.

Upon engulfment by the lysosome, the significance of the multi-layered nature of MITO-Porter is revealed. The outermost layer is dissolved upon entry to the cytosol through the cellular membrane, whereupon a second layer merges with the lysosome, facilitating escape of the macromolecule from the digestive vesicle. A third layer directs cellular machinery to transport the remaining liposome to mitochondrial locations, whereupon it melds with the mitochondrial outer membrane. A fourth and final layer permits melding with the inner membrane and delivery of the payload into the mitochondrial inner matrix[91].

Figure 7: MITO-Porter Delivery System[92]



The left side of Figure 7 demonstrates the pathway that the latest version of the MITO-Porter platform, DF-MITO-Porter, utilizes to gain entry into the matrix.

The Japanese research team has demonstrated steady progress in vitro of the technology. In 2008 a simpler single-layer octaarginine-coated liposome demonstrated cell entry and fusion with mitochondria, and expression of the green fluorescent protein (GFP) payload in mitochondrial membranes[93]. By 2010, dye whose fluorescence would only be activated in conjunction with DNA was successfully delivered into the inner matrix[94].

A year later the current multilayered platform, DF-MITO-Porter was employed to deliver DNAse 1 into the mitochondrial inner matrix. Statistically significant reduction of mitochondrial respiration was observed, implying that the delivered DNAse had

dissolved mtDNA molecules and impaired or halted mitochondrial function as a result. GFP expression was confirmed by confocal laser scanning microscopy. Use of DNAse 1, though an elegant idea, raises the objection that respiratory decrease might have occurred due to nuclear DNA destruction by DNAse delivered to the nucleus incorrectly. In an e-mail exchange, a member of the group indicated they would answer the toxicity question in their next study[95].

The team's latest paper, published in the summer of 2012, sought to more comprehensively determine delivery success rates, validate the pathway presumed to be the mode of action, and deliver 24-base pair oligonucleotides complexed with fluorescent Cy5. The inner layers of the liposome contained a mitochondria-fusogenic composition, while the outer envelopes were optimized for plasma membrane entry and lysosomic escape. Notably, no toxicity was observed with the HeLa cells used[96].

Using the fluorescently-tagged oligonucleotides, a more quantifiable understanding of liposomal fate and transfection efficiency was achieved. 83% of cells treated contained liposomes, up from 25% using the earlier formulation. Of these liposome-positive cells, only circa 20% of the oligo DNA actually arrived inside the mitochondria. The paper draws the conclusion that the rate-limiting step is in the cytosol: the liposomes enter successfully but only rarely associate with mitochondria[97].

In the immediate future, the team will attempt to rectify DF-MITO-Porter's cytosolic inefficiencies by experimenting with new formulations of the inner layers of the liposome, perhaps incorporating mitochondria targeting signals (MTS) found in nature. Resolution of this issue is critical to the viability of the delivery system but it does not appear insurmountable. There is also a potential issue with the long-term effects of a

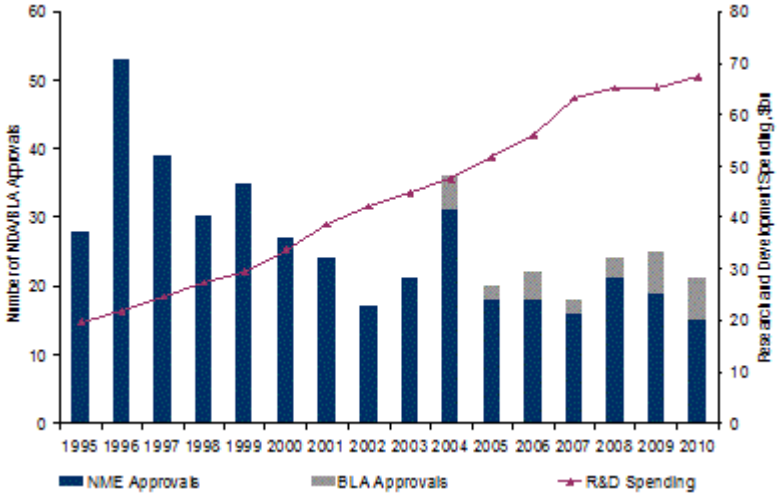
successful payload delivery on an individual mitochondrion. Does such an event impair its voltage gradients? Further study is needed.

A further question concerns the use of the pinocytotic pathway. Are there size limitations inherent in its use, and will that limit the payload that may be accommodated? The question will be posed at a face-to-face meeting at the World Mitochondria Congress in Berlin in November, 2012.

Orphan Drugs Act and Mitochondrial Drug Development

There are two primary avenues for drug development in the United States and abroad: large-scale, insourced drug candidates targeting diseases with large populations, and start-up companies associated with usually university-sourced drug candidates. The former model is pursued by large pharmaceutical companies, and the economics are daunting. Trade association PhRMA reports its members investing $49.5 billion in research and development in 2011[98], and recent estimates of the total cost of a single new, approved drug inclusive of all other expenses such as failed drug programs, of $1.2 billion for a novel medicine[99].

Figure 8: R&D versus Approvals[100]



Ballooning costs may be due to several causes, such as reduced drug pipelines, diminishing returns of high-throughput screening, and perhaps most directly, increasing Food and Drug Administration (FDA) requirements for patient numbers and efficacy data during clinical trials[101]. Increased R&D allocation has not resulted in increased FDA approval of new drugs. Figure 8 demonstrates this alarming trend of declining novel medicines amid an avalanche of R&D spending. This dynamic, as well as the number of blockbuster drugs that are approaching the end of their patent lives and loss of attendant profitability have sparked a wave of acquisitions by large pharmaceutical companies of listed and startup biotechnology companies in an attempt to rejuvenate their product pipelines. It is premature to judge whether this form of insourcing of potential new medicines, instead of internal development, will be the saviour of the industry.

An alternative approach to drug development lies within the rubric of the Orphan Drug Act passed in 1983. Given the inordinate costs of orthodox drug development, there is little case to be made for research of medicines for small patient populations. These so-called orphan diseases are neglected given the risk-reward calculations biotech companies must make. In order for a medicine to be declared an Orphan Drug, it must be specifically designed to treat diseases with fewer than 200,000 sufferers within the United States[102].

Once an Orphan Drug designation is achieved, numerous benefits accrue to the medical sponsor. The Prescription Drug User Fees Act (PDUFA) fees, required at the beginning of a drug approval cycle called the New Drug Application (NDA), are waived. For 2013, PDUFA fees stood at $2 million per drug[103]. Tax credits are available with a 15-year carry-forward provision and a 3-year carry-back provision applied to a profitable

year. Further a 7-year market exclusivity is granted at the date of approval, not the date of the patent, and any competitor wishing to introduce a drug in the same field is forced to prove therapeutic superiority, not just equivalency. A policy of unified filing of ophan drug documents for both the FDA and the European Medicines Agency (EMA) has been announced[104]. Finally the FDA is required to set specific endpoints in clinical trials, that if reached, require it to approve the drug. Clinical trials may also be of a smaller scope and therefore considerably less costly[105].

Before 1983 only 38 drugs were approved that might be considered orphan, and after the legislation passed through to 2010, 353 orphan drugs were approved with 2,116 compounds given orphan designation[106]. The global orphan drug market reached $84.9 billion in 2009, with an estimated growth rate of 6% yearly[107]. Intriguingly a number of orphan drug approvals led to blockbuster, widely-utilized medicines: Abilify, Provigil, Vioxx, Botox and Cialis among others. Orphan designates have historically enjoyed considerably higher success rates and faster approval; orphans average fewer than 5 years from Phase 2 to market, as opposed to 6 to 8 years for conventional drugs, an 82% success rate from Phase 2 forward rather than 35% for traditionals, are usually first to market, and often address chronic diseases with lifetime dosage requirements[108].

Therefore an alternative model for drug development, suitable for small start-up companies wishing to exploit university-based research, may be to focus on a compound with orphan druggability. Mitochondrial diseases by their nature are an ideal target, with the six smallest mtDNA-related diseases accounting for circa 15,000 to 18,000 U.S. patients and MIDD possibly remaining just below the orphan drug limit (see Table 2). A drug targeting LHON will most-likely face direct competition subject to FDA approval

from the small molecule marketed by Edison Pharmaceuticals and perhaps Leigh's Disease ought to be a first focus for FDA approval.

With a circa 7,500 sized patient population, Leigh's is characterized by movement disorders, continuous crying, seizures, rigidity, tremors, vomiting, and in later cases, lactic acidosis, kidney failure, and heart problems leading to a circa one year life expectancy after diagnosis[109]. The disease may be caused by either mtDNA or nDNA mutations. It afflicts infants, juveniles, and adults and there is no cure. As with most mitochondrial diseases, various combinations of CoQ10 and L-carnitine, vitamin B complex, vitamin C and vitamin K1 have been used as palliative with little to zero effect[110].

Drug pricing and business strategy is beyond the scope of this paper but in order to provide rough estimates of the value of monopoly positions on orphan drugs, an industry-standard metric called the Quality-Adjusted Life Year may be used. The World Health Organization considers a three times per-person income per QALY life-year gained to be a limit for cost-effective intervention[111]. For the United States, the 2011 per capita GDP of $48,100 implies a $144,300 QALY value. If 50% of the 7,500 patients with Leigh's were afflicted due to mtDNA defects, and the medicine was able to deliver 2 years of QALY, and its nature as the only treatment available led to a 75% adoption rate, then the revenue value of this tiny disease stands at $750 million. Given the internal dynamics of the U.S. insurance system and the constitutionally validated Patient Protection and Affordable Care Act, a 2-QALY life-saving medicine's up-front cost of $288,600 is not an unreasonable price point. All these numbers are for the United States alone. Efficient development programs run parallel with the European and Japanese drug

approval regimes. While payouts may be decreased in these markets, if disease rates are constant, their total population sizes triple the patient pool[112].

The aforementioned numbers are not meant to be a rigorous market assessment but to elucidate the potential value derivable from even small orphan diseases. A biotechnology start-up could expedite its time to market through the orphan drug route, with a view to expanding the range of its official approvals after the drug has reached the market. In this way drug development costs may be reduced and attendant equity dilution of the founders reduced. Further studies, sponsored industry symposia domestically and abroad, and physician off-label prescriptions, as well as careful promotional policies that remain within FDA and Federal Trade Commission law, may provide the ground work for entry into major diseases such as MELAS with mitochondrial implications and perhaps one day, aging itself. The compelling dynamics of orphan drugs have not gone unnoticed in the current acquisitions spree by large pharmaceuticals; Pfizer's acquisition of Protalix for its Phase III Gaucher disease compound and Shire's 2005 purchase of Transkaryotic Therapies demonstrates the industry's appetite for niche drugs.

What might a multi-disease, multi-symptom mitochondrial drug be? The requirements most-likely rule out small-molecule designs due to their specificity and a start-up's lack of an edge versus large pharmaceuticals' extensive high-throughput facilities. Instead a novel delivery platform of macromolecules to the mitochondria seems ideal. After extensive survey of the available literature[113] [114] [115] [116] the only academic candidate at this time appears to be DF-MITO-Porter. Recognition of the value of the drug both as the intellectual property of a flexible-compound delivery platform and separately as an effective mtDNA therapeutic, potentially allows for co-branding, cross-

licencing, and other alternative financing arrangements without equity dilution. The above is dependent on a sufficiently impregnable patent ring with blocking capability for the pathways in question and a zealous legal enforcement of those patents.

Further modification of the delivery system, such as with the addition of bezafibrate agonists of PCG-1α[117], could up-regulate mitochondrial count at the same time that re-programming of the mitochondria with intact mtDNA or other drugs is undertaken. Rather than mtDNA delivery, a therapy accomplishing selective destruction of defective mtDNA, allowing for cellular resupply of adequate wild-type mtDNA molecules, could restore adequate heteroplasmy and therefore function. Finally, the delivery system might be re-tasked, with sufficient modification of surface compositions, to deliver compounds to other organelles or even the nucleus.

CONCLUSION

The significance of the ‘other genome’ in medicine is only now the subject of extensive research. As powerhouses of the cell, mitochondria are intimately related to optimal cellular function and the cell death cycle. Widespread disruption of mitochondria through damage to mtDNA or nDNA, inherited or as a result of inevitable somatic processes, can cause systemic failure of high-energy tissues and crippling, currently incurable diseases. The process of aging itself may be partially mediated by the slow decay of these fascinating organelles.

While currently unavailable, the development of a cure or treatment of a variety of these diseases is not impossible. Advancements in small-scale chemistry and nanotechnology as well as increased understanding of the basic science involved have opened up new, promising avenues for the treatment of mitochondrial disease. Use of the Orphan Drugs Act may expedite the process from lab bench to bed side, and the potential profit available to successful pioneers of the space may further widen the technology’s applicability and potentially improve the quality of life of millions. Its pursuit is not only worthwhile but essential.

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