Cell and animal models of mtDNA biology: progress and prospects

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MITOCHONDRIA OCCUPY A CENTRAL position in the biology of multicellular organisms. Most eukaryotic cells contain many mitochondria, which can move, fuse, and divide within a cell (9). Collectively, they can occupy as much as 25% of the volume of the cytoplasm. Structurally they consist of four compartments: an outer membrane, a protein-rich inner membrane, an intermembrane space between outer and inner membranes, and a matrix—the region inside the inner membrane. The implications of this structural uniqueness were recognized early on by the German pathologist Richard Altman. In his *Die Elementarorganismen* (2), Altman postulated that mitochondria possess not only metabolic autonomy but also genetic autonomy, presciently anticipating the recognition by Margulis (73) that the mitochondrion was an endosymbiotic organism possessing a separate genome.

The human mitochondrial genome, similar to that of other mammals, is intronless and circular and encodes 37 genes (131). Twenty-four genes encode the translational machinery of the mitochondrial DNA (mtDNA) itself (22 transfer RNAs and 2 ribosomal RNAs). The mitochondrial genetic code (m) is similar to but not identical with the nuclear code (n), differing in four codons [AUA = Ile (n); UGA = Term (n)]. The additional 13 genes encode for subunits of the electron transport chain where carbohydrates and fats are oxidized to generate carbon dioxide, water, and ATP. Indeed, mitochondria are responsible for the majority of ATP production of a given eukaryotic cell.

Cellular Models in Mitochondrial Research

Cytoplasmic hybrid (cybrid) cells are one of the mainstays of mitochondrial research. They are created when cytoplasmic contents of two different cells are rendered coexistent within a single plasma membrane boundary. Specifically, the approach is designed so that mtDNA residing in cytosolic mitochondria of one cell type becomes perpetually incorporated within the cytoplasm of the other cell type.

When cybrids are generated, the extent of cytoplasmic material transfer has historically varied from purified mitochondria to potentially a donor cell’s entire nonnuclear content. The nucleated cell accepting the cytoplasmic donation can also vary in terms of its makeup; the acceptor cell may or may not have undergone prior modification from its native state. In cybrid studies of mitochondrial function, the most commonly (but not exclusively) used approach involves transfer of mitochondria from enucleated or nonnucleated donor cells to cells previously depleted of their own endogenous mtDNA. This strategy creates cells in which the resultant cybrid contains nuclear DNA from one cell type and the mtDNA of another. An important tenet of this strategy is that regardless of what cytoplasmic constituents are transferred, repeated divisions of the resultant cybrid cell dilutes to phenotypic irrelevancy any entity not capable of self-perpetuation. In healthy cells, the mtDNA is the best-recognized agent capable of replication and therefore perpetuation.

Cybrids created through mitochondrial transfer have been used for three distinct applications. The first application involves transfer of mitochondria containing a known mtDNA mutation (Fig. 1A). This permits phenotypic assessment of the transferred mtDNA mutation. Investigators can assess (among other things) whether the mutation alters electron transport chain activity, oxygen consumption rates, mitochondrial membrane potential, cell ATP levels, and cell growth rates. The second application involves transfer of mitochondria for which mtDNA has not already been characterized (Fig. 1B). This permits assessment of the integrity of the transferred mtDNA through biochemical and molecular assay of mitochondrial function or pathways affected by mitochondrial function. The third application relates to the use of cybrids for studies of bigenomic (mtDNA and nuclear DNA) compatibility.

The challenges faced with each type of application overlap to some degree, but some challenges uniquely relevant for one or the other applications exist. A better understanding of these challenges continues to emerge. A review of the history of cybrid research helps clarify the nature of these challenges and provides perspective on the present and future state of cybrid research.

Cytoplasmic Fusion and ρ0 Cell Development: the First Wave

Combination of enucleated cell material (cytoplasts) with nucleated cells (karyoplasts) goes back decades. One initial report from the early 1970s describes virus-mediated fusion (using inactivated Sendai virus) of enucleated mouse peritoneal macrophages and L929 murine cell cultures with the nucleated human HEp-2 cell line (96). The term “cybrid” itself entered the literature in 1974, when Bunn et al. (12) isolated cytoplasts...
Mitochondria from subject with disease

Mitochondria with a known heteroplasmic mtDNA mutation

Isolate colonies with different heteroplasmic: wild type mtDNA ratios

Compare different heteroplasmic loads to evaluate mutational consequences and threshold effects

Mitochondria from control subject

Mitochondria from subject with disease

Cybrid expresses disease subject’s mt genes

Expand Line

Cybrid expresses control subject’s mt genes

Expand Line

Mitochondria with a known heteroplasmic mtDNA mutation

rhol cell

rhol cell

Fig. 1. Two different ways to utilize cybrid cell lines. A: cells containing a known heteroplasmic mitochondrial DNA (mtDNA) mutation can be used to prepare cybrid lines containing different heteroplasmic burdens, thereby facilitating studies of threshold effects. B: cells containing unsequenced mtDNA can be used to prepare cybrid lines that vary only in the origin of their mtDNA. In this case, biochemical differences between cybrid lines presumably arise from differences between the transferred mtDNA.

From chloramphenicol-resistant mouse A9 cells and fused them with a nucleated chloramphenicol-sensitive mouse LMTK– cell line. The proposed term, a contraction of the phrase “cytoplasmic hybrid,” represented modification of an existing term, “hybrid.” The term hybrid refers specifically to the fusion product of two nucleated cells and therefore did not technically apply to the products of experiments such as these. Fusion in this early manuscript was also facilitated by exposure of cytoplasts and karyoplasts to inactivated Sendai virus. A related experiment followed in 1975, when Wallace et al. (132) fused cytoplasts from a strain of chloramphenicol-resistant HeLa cells with a strain of chloramphenicol-sensitive HeLa cells. The resultant cybrid line showed chloramphenicol resistance, demonstrating that this biochemical feature was mtDNA and not nuclear DNA mediated.

Investigators working with this procedure over the next several years found that polyethylene glycol (PEG) could also promote fusion of cytoplasts and karyoplasts to create cybrid cell lines (135). It was further found that at least in some circumstances spontaneous cytoplast-karyoplast fusion could occur. In 1982, Clark and Shay (22) reported a remarkably simple and straightforward experiment showing that particular cell types incorporate exogenous mitochondria without direct investigator-mediated assistance. In this case, the authors isolated mitochondria from a chloramphenicol-resistant cell line, and added them to a culture containing either Y-1 or PCC4 chloramphenicol-sensitive mammalian cells. The Y-1 and PCC4 cells acquired chloramphenicol resistance, presumably through mitochondrial endocytosis of the exogenous mitochondria added to the medium.

Another line of mitochondrial research unfolding through the 1970s and 1980s would ultimately converge with this mitochondrial transfer work. Investigators in several laboratories were aware that yeast mount impressive levels of mtDNA depletion when living under anaerobic conditions. This guided explorations into the ability of cultured cell lines to similarly withstand mtDNA depletion.

Such intentionally mtDNA-depleted cells were referred to as “ρ-depleted (ρ0)” cells. This nomenclature derived from the earliest report of cytoplasmic nucleic acid, from 1949, in which observed cytoplasmic nucleic acid was referred to as ρ-nucleic acid (33). ρ-Nucleic acid was not, however, identified as mtDNA until the reports of Nass and Nass in 1963 (82, 83).

Mitochondrial DNA depletion experiments in the 1970s and 1980s utilized methotrexate and ethidium bromide. The latter is a cationic mutagen capable of intercalating into DNA during replication. Because of its positive charge, it concentrates within negatively charged mitochondrial matrixes. This allows determination of cell culture concentrations that block mtDNA replication without disrupting nuclear DNA replication.

Ethidium bromide was used to completely deplete yeast lines of mtDNA to create ρ0 cells as far back as 1970 (40, 81, 105). Extending this line of research to mammalian cell lines, Wiseman and Attardi (136) accomplished extensive but not total depletion of mtDNA from the human VA2-B cell line. In 1985, Desjardins et al. (32) reported creation of ρ0 chicken embryo fibroblasts, in which mtDNA levels were reduced from ~600 copies per cell to undetectable levels. This advance capitalized on prior work showing that ethidium bromide exposure results in pyrimidine auxotrophy, which can be overcome by supplementing cells with uridine (41). Pyrimidine auxotrophy is likely a consequence of the fact that dihydroorotate dehydrogenase enzyme activity is coupled to activity of the electron transport chain. Dihydroorotate dehydrogenase, which is localized within mitochondria, catalyzes the conversion of dihydroorotic acid to orotic acid. Uridine is a pyrimidine pathway metabolite generated downstream of this synthetic step and therefore bypasses the pyrimidine synthesis block that results from elimination of electron transport chain activity.

The following year, this group succeeded in producing an immortalized ρ0 LSCC-H32 avian fibroblast cell line (31). Mitochondrial DNA from the parent cell line was taken from ~300 copies of mtDNA per untreated cell to undetectable levels. It is important to note that although these cells no longer contained detectable mitochondrial DNA (as assessed by Southern blotting), mitochondrial organelles nevertheless persisted. In fact, quantitative assessments showed proliferation of the residual mitochondrial organelles. Qualitatively, the residual mitochondrial organelles displayed swollen morphologies with disrupted cristae (78). The mitochondrial remnants from these avian ρ0 cells showed virtually no cytochrome oxidase activity, which was not surprising since 3 of the 13 protein subunits are mtDNA encoded. Cell levels of cytochrome c, on
the other hand, were increased. Subsequent investigators in the field have since labeled these mtDNA-depleted mitochondria “mitoids” (118).

Soon after this, King and Attardi (55) began efforts to deplete a human cell line of endogenous mtDNA. These authors first reported creation of human osteosarcoma ρ0 lines, which they named 143B101 and 143B206 (56). These lines were generated through chronic exposure of the parental cell line to ethidium bromide, which depleted the normal osteosarcoma mtDNA content from ~9,100 copies per cell to undetectable levels. In addition to showing uridine auxotrophy, both 143B osteosarcoma ρ0 lines were also auxotrophic for pyruvate (57). The leading explanation for pyruvate auxotrophy relates to issues of anaerobic cell redox modulation. An absence of oxidative phosphorylation deprives cells of a major NADH oxidation pathway. Theoretically, overreliance on glycolysis could shift cell NADH-to-NAD$^+$ ratios excessively toward NADH. Supplying excess pyruvate should promote regeneration of NAD$^+$ by inducing metabolism of pyruvate to lactate by lactate dehydrogenase.

In their landmark 1989 paper, King and Attardi (56) also demonstrated repopulation of their osteosarcoma ρ0 lines with exogenous mtDNA. This mtDNA “complementation” was accomplished via two approaches. One approach utilized PEG-mediated fusion of enucleated cytoplasts with ρ0 cells. The other approach involved direct microinjection of isolated mitochondria into ρ0 cells. Several years later, this same laboratory pioneered a variation of the PEG fusion method, in which platelets were used in place of enucleated cells (20). This modification capitalized on the fact that platelets do not contain nuclei but do contain mitochondria and therefore mtDNA. For this approach, platelets are essentially treated as prepackaged cytoplasts. Platelet fractions are easily prepared from whole blood samples via centrifugation, and among biological tissues, blood is extremely accessible. This rendered cybrid approaches more practical and opened them up for further experimental exploitation.

It is worth noting other modifications of the cybrid technique described by King and Attardi (56). For instance, alternative ways of generating ρ0 cells exist. Dideoxynucleoside analogs, which are clinically used to treat human immunodeficiency virus (HIV) infection, are also appropriate for accomplishing mtDNA depletion (5, 84). Expression of a dominant-negative mtDNA polymerase-γ appears capable of conferring ρ0 status to cultured cell lines (50). In cultured cell lines, rhodamine 6-G can also deplete mtDNA to levels low enough for effective production of cybrid cell lines that contain only exogenous, cytoplast-transferred mtDNA (137).

**Application of Cybrid Methodology for Basic Mitochondria and Human Disease Studies**

During the early 1990s, the cybrid technique was applied to the study of mitochondrial encephalomyopathy diseases. The mitochondrial encephalomyopathies are a relatively rare group of disorders that were among the first diseases recognized to result from specific mutations of the mtDNA. Examples include the mitochondrial encephalopathy, lactic acidosis, and strokelike episodes (MELAS) syndrome, often caused by the A3243 mutation of the mtDNA tRNA$^{\text{Leu(UUR)}}$ gene, and the myoclonic epilepsy and ragged red fiber (MERRF) syndrome, often caused by the A8344G mutation of the mtDNA tRNA$^{\text{Lys}}$ gene. The causal mtDNA mutations are often heteroplasmic, meaning that some but not all of the mtDNA copies within a cell possess the mutation.

The cybrid approach was used to screen the impact of these mutations on oxidative phosphorylation and to address the “threshold” of mutational burden needed to cause an abnormal biochemical phenotype. In osteosarcoma cybrid cells, high mutant loads encompassing >90% of cell mtDNA content were required to induce an abnormal biochemical phenotype. Further studies of the resultant cybrid lines also facilitated mechanistic explorations of how the mutations under study might actually cause mitochondrial dysfunction (20, 21, 58, 59).

During this time, the cybrid approach was also applied to studies of mtDNA mutation in Leber’s hereditary optic neuropathy (LHON). LHON can also present within a context of clear maternal inheritance, although this occurs in the minority of cases (51), and mutations in affected individuals are typically homoplasmic. The G11778A mutation of the mtDNA NADH dehydrogenase (ND)4 gene was among the earliest demonstrated disease-related mtDNA mutations (133). Two studies using osteosarcoma ρ0 cell lines to generate cybrids expressing the G11778A mutation found on polarographic assessment a reduction of NADH-Q oxidoreductase activity (complex I of the electron transport chain) (45, 130). However, neither study detected any decrease in complex I activity when measured by direct spectrophotometric assay of sonicated mitochondria. These results were similar to those previously obtained from direct analyses of LHON subject mitochondrial function (65, 72). Subsequent studies of other LHON-causative ND gene mutations have, however, used spectrophotometry to demonstrate complex I $V_{\text{max}}$ activity reductions (23, 52). Later cybrid studies of the G11778A mutation, performed with a non-osteosarcoma ρ0 cell line, also found a spectrophotometric reduction in complex I $V_{\text{max}}$ activity (11).

Starting in the mid 1990s, investigators began using cybrids to assess the impact of mtDNA on electron transport chain enzyme function in persons with sporadic neurodegenerative diseases. The rationalization for this was that specific defects of the mitochondrial electron transfer chain were by this time recognized to occur in both Parkinson disease (complex I) and Alzheimer disease (complex IV) (119). In addition to occurring in the brains of affected patients, these enzymatic defects were also present in platelets. As mtDNA theoretically could either account for or contribute to the enzymatic lesions and platelets are quite accessible, it seemed reasonable to transfer platelet mtDNA from persons with these diseases to ρ0 cells. The key conceptual distinction between this type of experiment and those already described is that in this case the nucleotide sequence of the transferred mtDNA was unknown. In this situation, biochemical assessments of cybrid cell lines would be used to screen the integrity and nature of the mtDNA used to restore the ρ0 cells to aerobic status.

A comprehensive discussion of sporadic neurodegenerative disease cybrid studies is beyond the scope of this review; those interested in a more detailed discussion of some of these data may wish to consult other reviews dealing with this subject (38, 90, 107, 113, 119). To nevertheless summarize the available data, a large number of fairly large studies using either human neuroblastoma (SH-SY5Y) or human teratocarcinoma.
Table 1. Cybrid study variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Nuclear background of the ( \rho_0 ) cell line</td>
<td>Some ( \rho_0 ) parental cell lines are more aerobically active than others. Polymorphic variability between nuclear electron transport chain genes may exist between cell lines. Aneuploidy can vary between ( \rho_0 ) lines. ( \rho_0 ) Cell lines derived from different tissues may express different isoforms of nuclear encoded electron transport chain genes.</td>
</tr>
<tr>
<td>Method of mtDNA depletion used to create ( \rho_0 ) cell</td>
<td>Possibility that nuclear DNA is affected by the mtDNA depletion procedure requires consideration.</td>
</tr>
<tr>
<td>Source and handling of mtDNA used to repopulate ( \rho_0 ) cells</td>
<td>Mitochondrial viability and stability may influence experimental outcomes. Cell medium or additives can minimize or maximize mitochondrial functional differences.</td>
</tr>
<tr>
<td>Cell culture variables</td>
<td>Useful when transferring mitochondria from one tumor cell line to another, since cytoplasmic enucleation failures will cause contamination. Do not appear necessary when transferring mitochondria from a nontumor source. Insensitivity of assay methodology can cause type I error. Inadequate sample sizes could lead to type I or type II error. Choice of statistical methods could influence chances of type I or type II error.</td>
</tr>
<tr>
<td>Presence or absence of genes enabling nuclear selection</td>
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<tr>
<td>Assay end points and methodology</td>
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<td>Sample size and data analysis</td>
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(NT2) \( \rho_0 \) cell backgrounds found that transfer of mtDNA from persons with Alzheimer disease generated cybrid cell lines manifesting reduced complex IV activity, increased oxidative stress, altered calcium homeostasis, mitochondrial depolarization, increased \( \beta \)-amyloid production, increased cytoplasmic cytochrome c levels, caspase 3 activation, altered intracellular stress responses, decreased mitochondrial movement, and enhanced susceptibility to exogenous \( \beta \)-amyloid exposure (10, 13, 17, 27, 29, 38, 54, 86, 88, 100, 115, 122, 125, 127, 128). However, a single small study of Alzheimer disease cybrids that used a HeLa \( \rho_0 \) cell background did not reveal any deficiency of oxidative phosphorylation (49).

Similarly, multiple studies using either SH-SY5Y or the human lung carcinoma A549 \( \rho_0 \) lines found that transfer of mtDNA from persons with Parkinson disease generated cybrid cell lines manifesting reduced complex I activity, increased oxidative stress, altered calcium homeostasis, mitochondrial depolarization, \( \alpha \)-synuclein aggregation, altered mitochondrial movement, and altered intracellular stress responses (15, 16, 38, 42, 87, 89, 101, 103, 117, 118, 126, 128, 129). However, a single small study of Parkinson disease cybrids that used a HeLa \( \rho_0 \) cell background did not reveal any deficiency of oxidative phosphorylation (4).

In addition to this Alzheimer disease and Parkinson disease cybrid work, a cybrid study in which platelet mtDNA from persons with amyotrophic lateral sclerosis was used to repopulate SH-SY5Y \( \rho_0 \) cells found evidence of electron transport chain dysfunction (especially in complex I), increased oxidative stress, and altered calcium homeostasis (116). Another study using the osteosarcoma \( \rho_0 \) cell background did not, however, find any alterations in electron transport chain function (37). Studies of cybrids in which SH-SY5Y receive and express mtDNA from persons with progressive supranuclear palsy demonstrate reduced complex I activity and increased oxidative stress (1, 114).

A preponderance of published cybrid data therefore suggests that mtDNA aberration occurs in at least some late-onset, sporadic neurodegenerative diseases (Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and progressive supranuclear palsy). Many investigators, however, prefer not to accept this interpretation pending demonstration of actual specific mtDNA mutations. This undertaking has proven quite complicated for several reasons. First, the sporadic nature of these diseases makes it difficult to link causality to a particular sequence deviation. Second, mtDNA is intrinsically variable. It is uncommon for individuals not maternally related to carry identical mtDNA sequences. Attributing phenotype causality to this variability is exceptionally difficult. Third, a particular mtDNA sequence deviation may only prove phenotypically relevant when expressed against a particular nuclear DNA context. Fourth, it has recently become clear that microheteroplasmoses are rampant throughout an individual’s mtDNA (25, 69, 75, 104, 106, 107). Important questions about this molecular phenomenon remain unanswered, such as whether these micro heteroplasmoses are distributed evenly between the cells of a tissue or concentrated within a limited number of cells in a tissue and whether these mutations are inherited or somatically acquired (for review see Ref. 107).

Cybrid approaches have also proven useful in studies of mtDNA-nuclear DNA compatibility. The mitochondrial electron transport chain enzymes are large multimeric complexes that contain a mix of mtDNA and nuclear DNA-encoded subunits. As differences in electron transport chain-encoding genes have evolved over the course of evolution, selective pressure to keep all these bigenomically produced proteins fitting and working together has existed within but not between species. Theoretically, with increasing mtDNA-nuclear DNA evolutionary divergence, increasingly feeble electron transport chain complexes should result. Kenyon and Moraes (53) applied a cybrid strategy to experimentally evaluate this prediction. The authors transferred chimpanzee, gorilla, and orangutan mitochondria to osteosarcoma \( \rho_0 \) cells, thereby creating “xenomitochondrial cybrids.” Xenomitochondrial cybrids produced via transfer of orangutan mitochondria to human osteosarcoma \( \rho_0 \) cells were not viable. For the viable xenomitochondrial cybrids (containing chimpanzee and gorilla mtDNA), evolutionary divergence from humans was associated with reduced complex I activity (7).

Cybrid Studies: Lessons Learned to Date

When it comes to cybrid work, no one standard approach exists. Technical variables figure prominently between laboratories. A partial list of these variables is provided in Table 1.
It consistently appears that whether a certain mtDNA species produces an altered electron transport or electron transport-related measure within a cybrid line depends on the background of the p0 line used to produce it. In general, it appears that the more glycolytic the background of the utilized p0 line, the less likely it is that its derivative cybrids will manifest oxidative deficits. It is therefore dangerous to assume that simply removing mitochondria from glycolytic cells for use in biochemical assays somehow frees them of a glycolytic origin. On the contrary, it is already known that mitochondria isolated from different tissues behave differently (64). It is also possible that mitigating nuclear factors render some cell lines less likely to manifest mitochondrial deficits (44). For these reasons, some p0 lines may prove less effective than others for cybrid applications requiring detection of potentially subtle biochemical changes.

On a positive note, variation in the field will ultimately enhance our knowledge of mitochondrial biology and strengthen the veracity of cybrid experiment interpretations. On a negative note, in the short term these differences have perhaps generated confusion within the field and limited the impact of potentially important observations. At this time, it seems prudent to assume that particular cybrid studies reaching different conclusions do not actually refute each other unless equivalent methods are utilized. Also, it is necessary to keep in mind that even though immortalized cell lines are aerobically active, such cells derive from neoplastics and in terms of energy metabolism are exceedingly glycolysis dependent. Therefore, it is probably wise to use caution when extrapolating from studies of immortalized cybrid cell lines to differentiated or nonimmortalized, heavily aerobic cell models. These methodological concerns also affect the concept of phenotypic threshold, where the level of a heteroplastic mutation must be high to produce a measurable effect. If true, threshold may only be pertinent to the model in question and may not be expandable to mitochondrial physiology in general.

Finally, as utilized to date for studies of mtDNA, one limitation of the cybrid technique is that mtDNA repopulation of p0 cells is accomplished as part of a greater cytoplasm or mitochondrial transfer. Techniques allowing for repopulation of p0 cells with mtDNA isolates could substantially advance the state of the art of cybrid research.

Animal Models of mtDNA Biology

Animal models of mtDNA function and mutation are of great importance in elucidating mitochondrial biology. Because of the perceived technical difficulties of introducing mtDNA into cells, however, only a few such models exist.

The only known animal model possessing a homoplasmic mtDNA mutation was recently described by Li et al. (68). They identified two separate canine families, Australian cattle dogs and Shetland sheepdogs, with progressive neurological degeneration characterized by a spongiform leukencephalomyelopathy produced as a consequence of a G to A transition at 14,474 of dog mtDNA. This produces a valine to methionine (V98M) amino acid change in canine cytochrome b, for which there is no reported human equivalent. Furthermore, the spongiform degeneration is unique in that no human homoplasmic mtDNA disease has been so phenotypically characterized. The naturally occurring canine model further highlights the variability and penetrance of phenotypes produced from mtDNA mutations and points to the necessity of guarded conclusions when interpreting mtDNA disease. A possible framework, which the authors suggest, is that mtDNA mutations may predispose to certain phenotypes and not necessarily be the direct cause. This interpretative framework may explain the inherent phenotypic variability seen in mtDNA disease where penetrance and clinical phenotype are not simply explained by the nature of the mtDNA mutation alone.

Li et al. (68) further observed that common disease causing mutations in human mtDNA can be found in a variety of species as neutral polymorphisms. Thus mtDNAs of closely related species can vary widely even to the point of one species possessing a wild-type mtDNA sequence that is a disease causing mutation in the sibling species, as is the case for great apes and humans (28). These features highlight the variability of phenotypes produced from mtDNA mutations and meshes with the observation in human mitochondrial clinical work that a single mtDNA mutation may produce a number of different phenotypes, depending on the nuclear background and the level of heteroplasmy. Interpretation of mitochondrial phenotypes must therefore take into account many variables beyond the mitochondrion itself.

The lack of a simple method by which to transfer mtDNA alone to generate homoplasmic mtDNA animal models and the seemingly confounding animal mtDNA sequences have helped shift focus away from animal models of homoplasmic mutations. These technical difficulties have given rise to clever approaches to manipulate the mtDNA of animals. These manipulations include 1) introducing exogenous mitochondria and thus their mtDNA into ova and embryonic stem (ES) cells, 2) altering nuclear genes to affect rates of mtDNA mutation accumulation, 3) expression of a restriction enzyme targeted to mitochondria to induce mtDNA deletions, and 4) repeated backcrossing to study mtDNA within the context of differing nuclear genetic backgrounds. These approaches would be unnecessary if an mtDNA vector capable of transfecting mitochondria in the germ line or in vivo existed.

**Cloned animals.** Cloning of mammals is achieved by the fusion of an enucleated ovum with a somatic cell. This procedure introduces a small amount of somatic cell mitochondria to the zygote and has been successfully applied to mice (47) and cattle (112). The introduced mtDNA can be detected in various tissues in mice and remains stable during embryonic development. However, during adult life there is evidence of nonneutral segregation of introduced mtDNA, which achieves the highest concentrations in the liver, up to 14% (average 1.7%) (47).

The cybrid technique has been utilized on mouse ES cell lines in the hope of creating mtDNA transgenic mice (95). This approach has proven successful despite significant technical hurdles and low efficiencies of viable births (74).

**Mitochondrial Psrl mice.** Intramitochondrial delivery of restriction enzymes has been used to effect changes in the heteroplasmic frequency of mtDNA variants in cell culture (111, 121) and in an animal model (8). The animal model uses Psrl, which has two restriction sites in murine mtDNA. Expression of Psrl under the control of a skeletal muscle promoter leads to a mosaic pattern of Psrl activity in muscle, with myopathy occurring at ~6 mo of age, generation of DNA double-strand breaks, and accumulation of multiple mtDNA
deletions. The sites of the deletions frequently involve the \textit{PstI} sites as well as the end of the D loop, which is similar to many naturally occurring mtDNA deletions. This implies that double-strand breaks may be involved in the generation of multiple-deletion syndromes.

\textit{\Delta mtDNA mice.} A technique analogous to cloning has been used to generate \textit{\Delta mtDNA} mice (46). Mouse \textit{p0} cells were first used to generate cybrid clones with mtDNA isolated from adult mouse synaptosomes and subsequently analyzed for the presence of deletions. A clone stably expressing a large deletion, \textit{\Delta mt4696}, was selected for further work. The cybrid cells were enucleated and fused with mouse zygotes. The mitochondrial-transgenic mice derived from these zygotes inherited various levels of the \textit{\Delta mt4696} DNA (5–90%) and demonstrated a pathological phenotype correlating with the level of mutated mtDNA.

\textit{mtDNA hypermutator mice.} Polymerase-\gamma is the enzyme exclusively responsible for the replication of mtDNA. Hypermutator polymerase-\gamma mice are created by the introduction of a proofreading-deficient form of polymerase-\gamma into mitochondria, which results in an accelerated accumulation of randomly dispersed point mutations and large deletions. Polymerase-\gamma mutations have been previously associated with Alpers syndrome, Parkinsonism, and accumulation of multiple mitochondrial deletions in humans (71).

The first of these mice was generated by Zhang and coworkers (79) with the enzyme overexpressed under the control of the cardiac-specific promoter of the \textit{\alpha}-myosin heavy chain and without removing the wild-type polymerase gene. As a result, there is a very high level of mutant polymerase in the heart, associated with downregulation of the nonmutant enzyme. The mice accumulate both point mutations and large deletions, with the former reaching a frequency of $1.4 \times 10^{-4}$ at 1 mo of age, compared with $0.06 \times 10^{-4}$ in control mice. This high mutation frequency corresponds to approximately two mutations per genome, which is comparable to the microheteroplasmic mutation levels in aged humans (106). At 12 wk of age the microheteroplasmic level stabilizes at $2 \times 10^{-4}$. These genomic changes are not associated with impairment of oxidative phosphorylation, oxygen consumption, changes in the amount of mtDNA or mtRNA in the tissue, mitochondrial biogenesis, upregulation of glycolysis, or increased oxidative stress and antioxidant responses. Genes involved in cardiac extracellular matrix remodeling as well as the antiapoptotic factors Bcl-2, heat shock protein 27, Bcl-xL, and Bfl1 are activated (139). Phenotypically, the mice develop a dilated cardiomyopathy, which is first observed at 4 wk after birth and is fatal within 18 mo. Cardiomyopathy is associated with a wave of myocyte apoptosis and the release of cytochrome \textit{c}. Administration of cyclosporin \textit{A}, a potent antiapoptotic drug that specifically inhibits the mitochondrial transition pore opening, which initiates apoptosis, protects mice from cardiomyopathy without changing the levels of apoptosis (80). Interestingly, levels of apoptosis decline to near normal values at 8–10 wk of age, without resolution of cardiomyopathy or attenuation of the antiapoptotic gene expression.

The second polymerase-\gamma hypermutator model was generated by Trifunovic et al. (124) by knock-in (i.e., replacement) of the wild-type polymerase with a proofreading-deficient version mutated at a conserved residue, without changes in the gene expression control mechanisms. This resulted in ubiquitous, pan-tissue expression of only the mutated enzyme. The phenotype began to manifest at $\sim$25 wk of age with appearance of kyphosis, alopecia, osteopenia, loss of body mass, anemia, lipodystrophy, cardiomyopathy, and reduced fertility. The median life span was $\sim$48 wk. On the genome level, there was accumulation of a linear, deleted form of mtDNA, reduction in the copy number of full-length mtDNA, and accumulation of point mutations. The levels of mutations were not statistically higher in heterozygotes, although the overall number of observed animals may have been too small for detection of modest increases. In the homozygote, the absolute numbers of mutations were much higher than in the cardiac hypermutator mice, with $1.5 \times 10^{-4}$ reported in the wild type at embryonic age 13.5 days (compare to $0.06 \times 10^{-4}$) and $7.8 \times 10^{-4}$ in the mutant mouse (compare to $1.4 \times 10^{-4}$). There is a further accumulation of mutations in both groups, reaching $15.7 \times 10^{-4}$ at 40 wk in the mutator. In contrast to the cardiac hypermutator model, there was a significant and progressive decline in the activities of the electron transport chain, specifically in the complexes dependent on mitochondrial gene products, as well as a reduction in the mitochondrial ATP production rates, indicating a global dysfunction of oxidative phosphorylation. In embryonic fibroblasts from mutant mice, respiration is depressed to below 5% of normal, and substantial activation of glycolysis is observed. Levels of oxidative stress and antioxidant defenses were not elevated, in agreement with the cardiac hypermutator model.

The third hypermutator mouse was generated by Kujoth et al. (63), using an approach identical to the Trifunovic et al. mouse, although the conserved residue altered here was different. The phenotype developed at $\sim$9 mo of age and consisted of hair loss, graying, kyphosis, thymic involution, testicular atrophy associated with the depletion of spermatogonia, osteopenia, loss of intestinal crypts, progressive decrease in circulating red blood cells, sarcopenia, hearing loss, and weight loss. The mean life span of these mutator mice was 416 days, compared with $>$850 days for wild type. On the genomic level, sequencing revealed that the frequency of mtDNA mutations in the mutant mice was three to eight times that in wild-type mice for most tissues examined. The absolute frequency of mtDNA mutations in 5-mo-old wild-type mice was as high as $2.1 \times 10^{-4}$ in a protein coding gene and $5.4 \times 10^{-4}$ in the D loop, in line with the Trifunovic et al. mutator model. Biochemically, there was no elevation of oxidative stress, but there was an increase in the number of TUNEL-positive cells, indicating increased apoptosis, similar to what was observed with the other mutator models. Mitochondrial respiratory function and ATP generation were not assayed.

Polymerase-\gamma mutations in humans result in a wide array of distinctly different conditions: Alpers-Huttenlocher syndrome, characterized by early-onset cerebral and hepatic dysfunction, progressive external ophthalmoplegia (PEO); familial Parkinson disease; sensory ataxic neuropathy, dysautonomia, and ophthalmoplearesis (SANDO); and male infertility, as well as presentations resembling MNGIE and MERRF (71). Multiple mitochondrial deletions are known to accumulate in some of these syndromes, but at least in some cases there is no accumulation of point mutations. This implies that the phenotypic expression and, presumably, mitochondrial mutational spec-
Elevated oxidative stress has been reported in patients with PEO (93) and is one of the hallmarks of normal human aging. It is known that many specific mtDNA mutations increase reactive oxygen species (ROS) production, and therefore random mtDNA mutations were predicted to have the same effect by increasing the electron leak from the electron transport chain. It is therefore difficult to interpret the absence of elevation in oxidative stress in the hypermutator mice as pertinent to either aging or classic mitochondrial disease. A possible way of reconciling these observations is that the accumulation of very high levels of both point mutations and deletions (much higher than in normal aging) results in the activation of signaling pathways that lead to a suppression of respiratory function by an ROS-independent mechanism. It is known that many forms of severe respiratory depression [e.g., due to mtDNA depletion in mitochondrial transcription factor (TFAM)-knockout mice; Ref. 66] lead to the dissipation of the mitochondrial membrane potential and low levels of ROS. Therefore, even if many of the random mutations in mutator mice are capable of increasing ROS production individually, their combined effect on ROS may be the opposite—a global suppression of respiration sufficiently severe to cause cellular dysfunction without increased ROS production. This scenario is supported by the observation of diminished respiration in the Trifunovic et al. mouse but is called into question by the Zhang et al. mouse.

**Backcrossed mtDNA mouse strains.** To study the effects of mtDNA on cognition Roubertoux et al. (98) took advantage of the maternal inheritance of mtDNA to exchange mtDNA between two closely related mouse strains (N and H). Roubertoux et al. spent 2 years crossing females of one strain with males of another strain, followed by 20 generations of backcrossing to males of the paternal strain to ensure little or no nuclear genetic influence. The two mtDNA-substituted strains (NmtDNAH = N nuclear background + H mtDNA, and HmtDNAN = H nuclear DNA + N mtDNA) were compared with each other and with the parental strains on a number of cognitive tasks.

Cognitive capabilities, such as learning and memory, were found to be significantly influenced by mtDNA, while exploratory activity exhibited significant interactions between nuclear and mitochondrial influences. For example, the least intelligent strain, N, showed a quantitative, absolute improvement of 50% in the radial maze score (a test of learning) after receiving the H strain mtDNA, indicating a substantial cognitive boost provided by mtDNA. Interestingly, the magnitude of the mtDNA impact on these measures increased with advancing age of the mice. As the mtDNA transferred between strains differed only in polymorphisms believed to be neutral, the apparent impact of such polymorphisms on cognitive functioning suggests they were not neutral after all.

**Summary: lessons from animal models of mitochondrial mutations.** Available animal models of mtDNA mutation argue the importance of mtDNA to aging and disease. At the same time, they temper mechanistic interpretations of how mtDNA induces phenotypically relevant phenomena in these situations. Substantially improved knowledge of the full mtDNA mutational spectrum residing within tissues, coupled with better techniques of mtDNA transfer, will be necessary for further progress.

**Mitochondrial Membranes and DNA Transfection**

Although mitochondria were known to possess a separate genome, it was not until 1980 that the sequence of the human mitochondrial genome was published. Shortly thereafter, Clark and Shay (22) were the first to successfully transfect the mitochondria of animal cells. This first demonstration of successful transfection, however, would come to be ignored as the need for transfecting mitochondria seemed superfluous at the time. In fact, it was commonly believed that mutations in mtDNA were incompatible with life since mtDNA encoded for proteins crucial to oxidative metabolism and thus unlikely to have meaningful biological sequelae. This was believed despite the fact that many decades earlier Otto Warburg and others had shown that many tumors lack any oxidative metabolism to speak of. Not until the first descriptions of mutations in mtDNA associated with human disease did the need for mtDNA transfection become apparent. By then the efforts of Clark and Shay were all but forgotten. To enable the study of mtDNA mutations associated with human disease, cybrid technology was developed as it became generally accepted that mitochondria of animal cells were not amenable to transfection.

Mitochondria traditionally have been described as organelles possessing a double membrane with little permeability but the capacity to import macromolecules through defined pores. Generally, these import activities are energy dependent and the macromolecules limited to proteins, metabolites, and lipids. The view of mitochondria as organelles impenetrable to nucleic acids has been so generally accepted as to frustrate efforts to deliver nucleic acids to the mitochondrial matrix. Since the only entry point to the mitochondrial matrix is through macromolecular pores, delivering full-length mitochondrial genomes is akin to a camel passing through the eye of a needle.

Informed by this view, investigators designed clever attempts to utilize the mitochondrial protein import machinery for manipulating mitochondrial genes. These efforts have been broadly divided into two separate categories, direct and indirect (26). The first is delivery of DNA directly into mitochondria by utilizing the translocases of the outer and inner mitochondrial membranes (TOM and TIM), termed “direct mitochondrial gene therapy.” The second involves expression of mitochondrial genes in the nucleus and subsequent targeting of the gene product to mitochondria for import through the same translocases. This approach has been termed “indirect mitochondrial gene therapy.”

In the direct approach, mitochondrial leader sequences (MLS), peptide motifs involved in mitochondrial import of many cytoplasmically translated proteins, are utilized. These MLS-containing peptides are conjugated to nucleic acids (134). This includes peptide nucleic acids (PNAs) (19) and oligonucleotides (34, 60). However, no long DNA molecules, much less full-length mtDNA, have yet been delivered to mitochondria in mammalian cells by these methods.

In the indirect approach, also termed allotopic expression, mtDNA recoded for cytoplasmic translation is targeted to the nucleus and directs the production of proteins that are then imported to the mitochondria with TOM/TIM import machinery.
ery. Possibly because of their high hydrophobicity, to date only four mitochondrial genes have been corrected in this manner, in plants (94) and yeast (99) and in cell culture (43, 85, 140). This approach is limited by the general difficulties of nuclear gene therapy, as well as problems with aggregation of some of the allotopic proteins (85). Additionally, many pathological mitochondrial mutations are in mtDNA (transfer and ribosomal) genes and therefore cannot be corrected with allotopic expression alone. This approach is further confounded by the complex issue of controlling expression of allotopic mitochondrial genes. Finally, since the mutated gene is not removed from mitochondria, its product may still cause damage through toxic gain-of-function mechanisms, such as generation of ROS after incorporation into the electron transport chain.

In a hopeful turn, the indirect approach has been used to target proteins not normally present in mitochondria, such as restriction enzymes. In a small subset of mitochondrial disease patients, the causative mutation produces a new restriction site in the mitochondrial genome. Genomes with such abnormal restriction sites are specifically degraded by the appropriate restriction enzyme while wild-type mtDNA present in the same cell is left intact. In two cell culture studies this strategy allowed shifting of heteroplasmic ratios toward the wild-type (111, 121). However, this approach is in principle limited to patients with abnormal restriction sites but has shown promise in vivo (8).

Methods in Mitochondrial Transfection

Delivery of mtDNA to the mitochondria of yeast was achieved with a biological ballistic (biolistic) gun (3), but it has thus far not been reported in mammalian cells. Full-length mtDNA has been delivered to mammalian ova by injection of cytoplasm and used in an assistive reproductive technology in humans (67); however, this method cannot be adapted for therapeutic use in patients with preexisting disease. Nevertheless, experiments using similar approaches (cytoplasmic fusion) have shown that the proportion of mutated to normal mtDNA influences the phenotype. Fewer abnormalities are seen as the proportion of normal mtDNA is decreased. Cells with less mutant mtDNA have more resistance to UV light and certain poisons, lower risk of becoming cancerous, lower ROS output, and better respiratory function (14, 18, 70, 92). This indicates that the addition of normal mtDNA could shift abnormal cells toward a healthier phenotype, as evidenced by the indirect restriction enzyme strategy described above (8).

Mitochondria share genetic information through fusion and fission processes, suggesting that complementation occurs within cells. As Clark and Shay previously showed that exogenous mitochondria added to cells could be imported via an endocytic route, the ability of mitochondria to disperse their genes may suggest a novel strategy for transfection of mtDNA. Recently, Spees et al. (110) showed that ϕ0 cells could be rescued by the simple addition of cells possessing mtDNA. Their observations supported sharing of mitochondria and mtDNA between cells through active uptake processes yet to be defined. This in vitro result may point to a novel in vivo approach to treating mtDNA disease through the transfection of progenitor cells such as human bone marrow stem cells. Unfortunately, preliminary in vivo work utilizing this strategy seems to suggest that although amelioration of mortality can be achieved this is not mediated through transfer of mtDNA (48).

Shrinkage of the mitochondrial genome requires a mechanism(s) for gene transfer to the nucleus. How this occurs remains unclear, but increasing evidence supports the possibility of direct nucleic acid passage across the mitochondrial membrane through porin channels (62). Investigators, recognizing that mitochondria have a bacterial origin, have utilized both electroporation and bacterial conjugation to successfully deliver mtDNA, presumably through porin channels as happens in bacteria (24, 138). Mitochondria can also acquire genetic material, potentially through a similar mechanism. For instance, HIV-1 RNA can accumulate rapidly in mitochondria of infected lymphocytes, within hours of stimulation of viral expression (109). The mitochondria of plants and protozoa contain nuclear encoded tRNAs (39), and mammalian mitochondria contain the nuclear encoded RNA subunit of a mtRNA-processing endonuclease (97). Beef heart mitochondrial porin incorporated into a planar bilayer membrane was shown to allow passage of double-stranded DNA across an electrical field gradient (120), and plant mitochondria import DNA through porin in a way that involves mitochondrial permeability transition (61). This provides both a mechanism for DNA entry into mitochondria and a potential vulnerability of mitochondria to transfection by foreign DNA.

The striking similarities between mitochondria and bacteria enable the curious possibility that mitochondria may yet be amenable to bacteriophage virus-mediated transfection. In fact, contemporary α-proteobacteria demonstrate the consequences of phage-mediated alterations. A recent comparison of the genomes of Brucella suis with B. melitensis revealed a finite set of differences that help explain virulence patterns and that arose by phage mechanisms (91). mtRNA polymerase more closely resembles phage than bacterial RNA polymerases (123), suggesting that such an infection event took place in the past, with subsequent transfer of the mtRNA polymerase gene to the host nucleus. It is unclear whether mammalian mitochondria as α-proteobacterial remnants retain the capacity for phage-mediated genetic alterations, but the hypothesis that bacteriophage could alter mitochondrial genomic content and provide a mechanism for expression of novel genes in mitochondria remains tantalizing.

Bacteriophage may not be the only virus capable of transfection mitochondria. Flock house virus has been shown to target mitochondria for replication (77). This positive-stranded, nonenveloped RNA virus possesses arginine-rich transduction domains in its capsid proteins that enable uptake of the virion into a variety of host cells, from plant and yeast to insect, as well as mammalian (35, 36). Viral replication is conducted via the RNA-dependent RNA polymerase protein A, which targets and anchors replication complexes to the inner leaflet of the outer mitochondrial membrane via an amino-terminal sequence that contains a transmembrane domain (76). Strikingly, Droso- sophila cells infected with the virus produce active virions in membrane-bound spherules within the mitochondrial intermembrane space (77). Not surprisingly, domains similar to the flock house virus arginine-rich transduction domain (protein transduction domains or PTDs) have been used successfully to target proteins to mitochondria (30, 102).

PTDs are short, amphipathic helices consisting of highly basic amino acids, such as lysine and arginine. PTDs added to
proteins confer the ability to enter cells. There is extensive literature on the delivery of proteins containing a PTD through the blood-brain barrier, placenta, and other barriers after intravenous injection (6). Such proteins are taken up by many cell types, dividing and nondividing, leading to very efficient systemic delivery and high bioavailability to tissues throughout a living animal. As DelGaizo and colleagues (30) have shown, reporter proteins such as green fluorescent protein with a PTD and an MLS can cross the blood-brain barrier and other tissue barriers and accumulate in large amounts within mitochondria in vivo. The exact mechanism of import into the cytosol is unclear, but data from different investigators converge on lipid rafts as a possible mediator. Interestingly, import into mitochondria in a recent study was also independent of membrane potential and the translocase machinery suggests that a novel mechanism through which mitochondria import macromolecules exists (62). In fact, these recent studies suggest that mitochondrial membranes are not impenetrable barriers and that the mitochondrial and plasma membranes exchange components. Mitochondrial membranes, therefore, appear more dynamic than previously thought. Summary: mtDNA Gene Therapy

For the successful transport of DNA to mitochondria in living cells, the delivery system must 1) bind the DNA; 2) neutralize the negative charge of the DNA; 3) protect the DNA from proteolytic degradation; 4) mediate the cellular uptake of DNA across lipid membranes; 5) transport the DNA to mitochondria; 6) cross the double mitochondrial membrane; and 7) present the DNA to the mitochondrial matrix in a form suitable for replication and transcription. Binding of DNA to protect against degradation and mediate intracellular uptake has to date been accomplished by a variety of substances, from lipids and polymers to viruses and recombining proteins such as histones. Some of these approaches are currently in clinical trials for nuclear gene therapy. The challenge of how to transport DNA through the cytosol and specifically into mitochondria of living animals, however, has not been satisfactorily addressed. This hurdle must be overcome if clinically effective mitochondrial gene therapy approaches are to be realized.

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### Invited Review

C668 CELL AND ANIMAL MODELS OF mtDNA BIOLOGY

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