Ketogenic Treatment Reduces Deleted Mitochondrial DNAs in Cultured Human Cells

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Impairment of mitochondrial energy metabolism has been associated with a wide range of human disorders. Large-scale partial deletions of mitochondrial DNA (mtDNA) cause sporadic Kearns–Sayre syndrome, a fatal multisystem disorder, in which the majority of mtDNAs in affected tissues have deletions (Δ-mtDNAs). Since most mtDNA-related diseases, including Kearns–Sayre syndrome, are recessive, only a few wild-type mtDNAs can compensate for the deleterious effects of many Δ-mtDNAs. We have developed a pharmacological approach to reduce the proportion of Δ-mtDNAs in vitro, in which we grow cells in medium containing ketone bodies, replacing glucose as the carbon source. Cells containing 100% Δ-mtDNA died after 5 days of treatment, whereas those containing 100% wild-type mtDNA survived. Furthermore, in a cloned heteroplasmic cell line, the proportion of wild-type mtDNA increased from 13% initially to approximately 22% after 5 days in ketogenic medium and was accompanied by a dramatic improvement in mitochondrial protein synthesis. We also present evidence that treatment with ketone bodies caused "heteroplasmic shifting" not only among cells (ie, intercellular selection) but also within cells (ie, intracellular selection). The demonstration that ketone bodies can distinguish between normal and respiratorily compromised cells points to the potential use of a ketogenic diet to treat patients with heteroplasmic mtDNA disorders.

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Mitochondrial encephalomyopathies are a heterogeneous group of disorders usually defined by morphological and biochemical abnormalities of the mitochondria.[1] Among these are disorders due to point mutations and partial deletions of mitochondrial DNA (mtDNA), a 16.6kb double-stranded circular molecule encoding 22 transfer RNAs, two ribosomal RNAs, and 13 polypeptides, all of which are subunits of the mitochondrial respiratory chain/oxidative phosphorylation system.[2]

Large-scale partial deletions of mtDNA (Δ-mtDNA) cause sporadic Kearns–Sayre syndrome (KSS),[3,4] chronic progressive external ophthalmoplegia,[5] and Pearson syndrome.[6] KSS is a fatal multisystem disorder defined by progressive external ophthalmoplegia, pigmentary retinopathy, and onset before age 20, and it is characterized by elevated protein in the cerebrospinal fluid, heart block, and cerebellar ataxia.[7] Muscle tissue samples from KSS patients show massive mitochondrial proliferation (ragged-red fibers) and have multiple respiratory chain enzyme defects, especially of cytochrome *c* oxidase (COX). The Δ-mtDNAs in KSS patients, who are always heteroplasmic (ie, wild-type mtDNAs [wt-mtDNAs] and Δ-mtDNAs coexist within the same cell or tissue), can be observed easily by Southern blot hybridization analysis as a single mtDNA species migrating more rapidly in electrophoretic gels than does the full-length wt-mtDNA.[4] The size and location of the deletion, and the proportion of Δ-mtDNA, differ among patients.[5] Deleted mtDNAs are transcribed into RNA but are not translated, probably because the deletions remove essential transfer RNAs that are required for protein synthesis[8,9]; thus, even genes outside the deletion are not translated.

One of the characteristics of most mtDNA-related disorders, including KSS, is that there is phenotypic expression of the pathogenic mutation only if the mutant mtDNA exceeds a certain threshold.[1] In cells and cytoplasmic hybrids (ie, cybrids) from KSS patients, the threshold is between 50 and 80% mutated mtDNAs.[10–12] Thus, most pathogenic mtDNA mutations are recessive; that is, only a relatively small amount of wt-mtDNA is able to rescue an oxidative phosphorylation deficiency because of a large amount of mutated mtDNAs. In addition, mitochondria and mtDNAs are transmitted stochastically from mother to

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daughter cells during cell division, such that the proportion of mutated mtDNAs within cells can change both in space and in time (mitotic segregation). These two features of mitochondrial genetics, the threshold effect and mitotic segregation, imply that a strategy designed to reduce the percentage of pathogenic mutation in a patient's cells or tissues below the threshold for dysfunction (termed by us "heteroplasmic shifting") might be a way to reduce the severity of the disease.

We had previously established a pharmacological system to shift heteroplasmy[13] in cells from a patient with a pathogenic mutation in the gene encoding subunit 6 of adenosine triphosphate synthase (ATP6)[14]: in growth medium containing oligomycin (a specific inhibitor of ATP synthase that binds to ATP6) and galactose (as the sole carbon source, replacing glucose[15,16]), we were able to select rapidly for the survival of cells containing wt-mtDNAs over those containing mutated mtDNAs.[13] We also found that galactose/oligomycin medium selectively killed homoplasmic cells containing the 4,977bp "common deletion" found in many KSS patients.[17,18]

We searched for other, less toxic, compounds that might mimic the ability of galactose/oligomycin to shift heteroplasmy in vitro. We have developed a novel cell culture system mimicking the conditions of a ketogenic diet that can shift heteroplasmy in cells containing Δ-mtDNAs from a patient with KSS.

Patients and Methods

Patients and Cells

We studied three different cybrid lines containing mtDNAs derived from a heteroplasmic KSS patient[19] (denoted as patient 4 in Zeviani and colleagues[4] and as patient K11 in Mita and colleagues[20]) harboring a 1.9kb partial deletion of mtDNA (the "FLP" deletion). The deletion is 1,902bp in size and removes mtDNA between nucleotide (nt) 7846 (notation of Anderson and colleagues[2]) in the COX II gene and nt 9748 in the COX III gene[20] (Fig 1A). Cybrid line FLP6a39.2 contained 100% wt-mtDNA, line FLP6a39.32 contained 100% Δ-mtDNA, and line FLP6b25.27 was heteroplasmic.

Ketogenic Treatment

We seeded 5×10^4 cells from each line in six-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 25mM glucose. The next day, the medium was changed to DMEM (lacking glucose) containing 10% fetal bovine serum and 50μg of uridine per milliliter, supplemented with either 5mM acetoacetate, lithium salt (Sigma, St. Louis, MO) (AA), 5mM DL-β-hydroxybutyrate, sodium salt (Sigma) (BHB), or a mixture of 5mM of each, for a period of 5 days, after which the medium was changed to glucose-containing DMEM, and the cells were allowed to recover for approximately 10 more days. We harvested cells from one dish for DNA isolation and maintained the rest for other studies. In some controls, L-β-hydroxybutyrate, sodium salt (Sigma) (L-BHB) was used as a nonmetabolizable source of ketone bodies.

Southern Blot Hybridization

We performed Southern blot hybridization as described previously.[4] Five micrograms of total DNA was digested with *Pvu*II at 37°C overnight, electrophoresed through a 0.6% agarose gel, transferred onto a nylon membrane, and probed with ^{32}P-deoxycytidine triphosphate–labeled probe derived from the region between the *16S* and *ND1* genes (nt 1711–4198). Quantitation of bands was performed as described previously.[4]

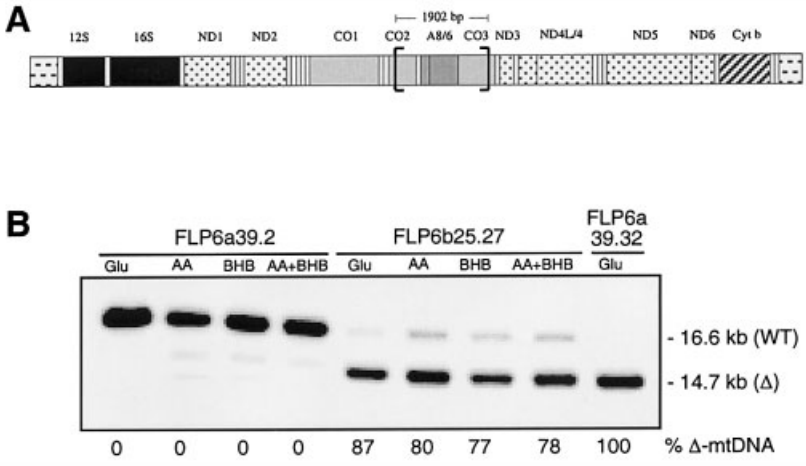

Fig 1. (A) Schematic diagram of a linearized mitochondrial genome.[2] The FLP deletion is indicated. (B) Autoradiogram of a Southern blot of lines FLP6a39.2 (100% wild-type [WT] mitochondrial DNA [mtDNA]) and FLP6b25.27 (heteroplasmic) after treatment with acetoacetate (AA), β-hydroxy butyrate (BHB), or a mixture of both, followed by 10 days of recovery in glucose-rich medium. The percentage of Δ-mtDNA is indicated below each lane (lanes 1–9, left to right). Line FLP6a39.32 (100% Δ-mtDNA), which cannot survive in ketogenic medium, was grown in glucose (Glu) during this period.

Fluorescence In Situ hybridization

We amplified mtDNA from the *ATP6* region (forward primer from nt 8528–8561 and reverse primer from nt 9208–9170) and the *ND4* region (nt 10841–10865 and nt 12092–12070) from total isolated cellular DNA. Probe fragments were nick-translated to incorporate an aminoallyl–deoxyuridine triphosphate and labeled by incubation with either AlexaFluor 488 (ND4) or AlexaFluor 594 (ATP6) using the ARES DNA labeling kit (Molecular Probes, Eugene, OR). We combined the *ATP6* (ATP594) and *ND4* (ND488) probes in a double-labeling cocktail and performed fluorescence in situ hybridization (FISH) as described previously.[21]

Time Course

We plated 5×10^4 cells in duplicate in six-well plates in DMEM containing 25mM glucose and then switched them to ketogenic medium (5mM AA). On each of five consecutive days, we collected the medium from one of the paired wells and counted the number of floating (and presumably dead) cells and then trypsinized the attached (and presumably living) cells and counted them. Coverslips in parallel wells were immunolabeled for COX II (see below) to visualize mitochondrial protein synthesis.

Immunocytochemistry

Mitochondrial morphology was visualized by incubating cells on coverslips in 10nM MitoTracker CMXRos (Molecular Probes). Cells were incubated in rabbit anti–COX II polyclonal antibody (a kind gift of R. Doolittle), followed by biotin-conjugated donkey anti–rabbit secondary antibody (Amersham) and streptavidin–fluorescein isothiocyanate (Amersham, Buckinghamshire, England).

Microscopy

Samples were viewed on an Olympus IX70 inverted system microscope. Red and green fluorescence images were captured sequentially using a SPOT RT digital camera and merged by computer using SPOT RT software (Diagnostic Instruments, Sterling Heights, MI). Subsequent image processing was performed equally on all images.

Results

We studied three cybrid cell lines containing mtDNA derived from a heteroplasmic KSS patient harboring a 1.9kb partial deletion of mtDNA[4,20] (the "FLP" deletion; see Fig 1A): line FLP6a39.2 contained 100% wt-mtDNA, line FLP6a39.32 contained 100% Δ-mtDNA, and line FLP6b25.27 was a heteroplasmic mixture of 13% wt-mtDNA and 87% Δ-mtDNA, as determined by Southern blot hybridization analysis[4] (see Fig 1B, lane 5).

Ketone Bodies Are Selectively Toxic to Homoplasmic Cells Containing Mitochondrial DNA Deletions

The cells were treated for 5 days in glucose-free medium containing 5mM AA, 5mM BHB, or a mixture of both, after which the cells were returned to medium containing glucose for approximately 10 more days. Ketone body concentrations were selected in agreement with physiological levels in patients on ketogenic diet.[22] All the homoplasmic deleted cells died, whereas the homoplasmic wild-type cells survived this treatment.

Ketogenic Medium Reduces the Proportion of Mitochondrial DNA Deletions in Heteroplasmic Cells

The heteroplasmic cells were subjected to the same ketogenic treatment protocol. The proportion of wt-mtDNA increased from 13% initially to 20–23% after 5 days of treatment in ketogenic media, followed by 10 days of recovery in glucose, as measured by Southern blot hybridization (see Fig 1B; compare lane 5 to lanes 6–8). Heteroplasmic cells grown in glucose-rich medium did not change their proportion of wt-mtDNA (13%) during the course of the experiment. We obtained essentially the same results in two other independent experiments: the proportion of wt-mtDNA increased from approximately 4% before treatment to approximately 24% after treatment.

Ketogenic Medium Increases Mitochondrial Protein Synthesis in Heteroplasmic Cells

To study the effect of "heteroplasmic shifting" on mitochondrial protein synthesis, we performed immunocytochemical analysis to detect mtDNA-encoded COX II.[23] Wild-type cells showed intense labeling of the mitochondrial network, as demonstrated by colocalization of COX II and MitoTracker Red, whereas 100% deleted cells showed no signal, as expected (Fig 2A).[8,9] Prior to ketogenic treatment of the heteroplasmic cells, 24 of 45 foci (ie, individual cells or isolated clusters of cells) examined were COX II negative (COX−), 20 had low levels of COX II labeling (COX+), and only one focus had COX II–positive cells similar in intensity to those in wild-type cells (COX++). In contrast, after 5 days in ketogenic medium, heteroplasmic cells showed a COX II signal similar to that in the wild-type cells (see Fig 2A); none of the 39 foci examined were COX−, 4 were COX+, and 35 were COX++. The uniformly high level of protein synthesis in cells harboring more than 20% wt-mtDNAs is consistent with the threshold values for function seen previously by others.[10–12] Furthermore, using MitoTracker Red staining, we observed that untreated heteroplasmic cells had a mixture of fragmented organelles (similar to those in homoplasmic deleted cells) and reticular organelles (similar to those in wild-type cells) but that treated heteroplasmic cells had only a reticular morphology (see Fig 2B). Immunolabeling and mitochondrial morphology results were identical for cells treated with AA, BHB, and AA plus BHB (data not shown).

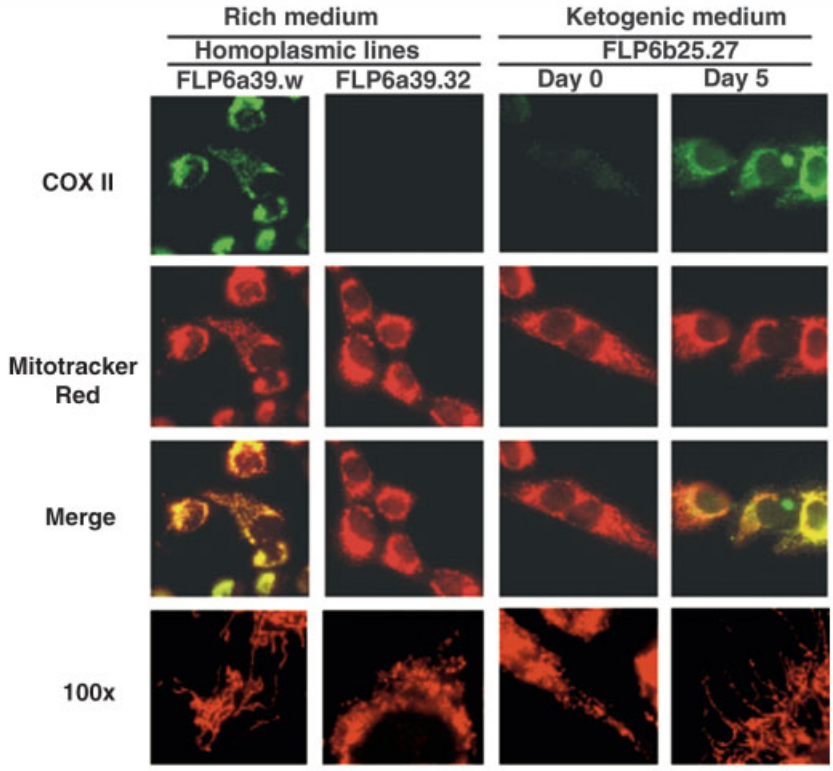

Fig 2. Mitochondrial morphology of cybrids visualized using MitoTracker Red staining and COX II immunocytochemistry. The photo of line FLP6b25.27 after 5 days in ketogenic medium (acetoacetate [AA]) was taken after a further 10 days of growth in rich medium. A closeup view at ×100 magnification is shown at bottom to highlight the mitochondrial morphology.

Evidence for Intracellular Selection in Heteroplasmic Cells

The data presented above regarding shifting in heteroplasmic cells must be viewed with caution, because although the cells began as a homogeneous clone with an overall proportion of wt-mtDNAs of 13% in each cell, there may have been a subpopulation of cells that in fact had reduced their mutant load merely by genetic drift, such that a few cells in the population now had a sufficient amount of wt-mtDNAs (eg, >20%) to be above the threshold for function.[10–12] Upon selection in ketones, the cells with high levels of mutation could have died, whereas these cells, the "jackpots," could have taken over the population. To address this concern, we performed two experiments that took advantage of the fact that cells grow poorly, if at all, in ketogenic medium, but grow rapidly when shifted to glucose medium.[13,24]

First, we studied the time course of cell growth and rescue of COX II immunocytochemical signal (Fig 3). As all three ketogenic treatments yield equivalent heteroplasmic shifts (see Fig 1), we utilized AA as a representative ketogenic treatment for simplicity. In the 5-day treatment period, wild-type cells showed robust growth and increased cell number, whereas the homoplasmic deleted cells died, as expected (see Fig 3B). The heteroplasmic cells divided very slowly, if at all, but the immunocytochemical signal for COX II showed a dramatic increase during the 5-day treatment period, with a near-complete recovery of COX II levels (to COX++) by day 3 (see Fig 3A). We note that because there were cell-to-cell differences in the intensity of the COX II immunoreactivity on day 0, the COX− cells might have been killed off due to poor mitochondrial function; however, this possibility seems unlikely. If the shift had been due solely to the death of COX− cells, the ratio of COX++ to COX+ cells should have remained constant; instead, the ratio increased 100-fold (see Fig 3B).

Second, we performed FISH[21] on the cell lines to observe the wt-mtDNAs and Δ-mtDNAs, which are organized into punctate proteinaceous assemblies termed *nucleoids*,[25] before and after ketogenic treatment (Fig 4). Both the *ND4* (see Fig 4A) and *ATP6* (not shown) probes labeled mitochondria specifically, as confirmed by colocalization of the probe with MitoTracker Red. In 100% wt-mtDNA cells, both the *ND4* and *ATP6* signals were present at high levels throughout the cells, yielding a yellow merged image, as expected (see Fig 4C). Conversely, 100% Δ-mtDNA cells had a strong *ND4* signal, but no detectable *ATP6* signal, also as expected (see Fig 4C). In the untreated

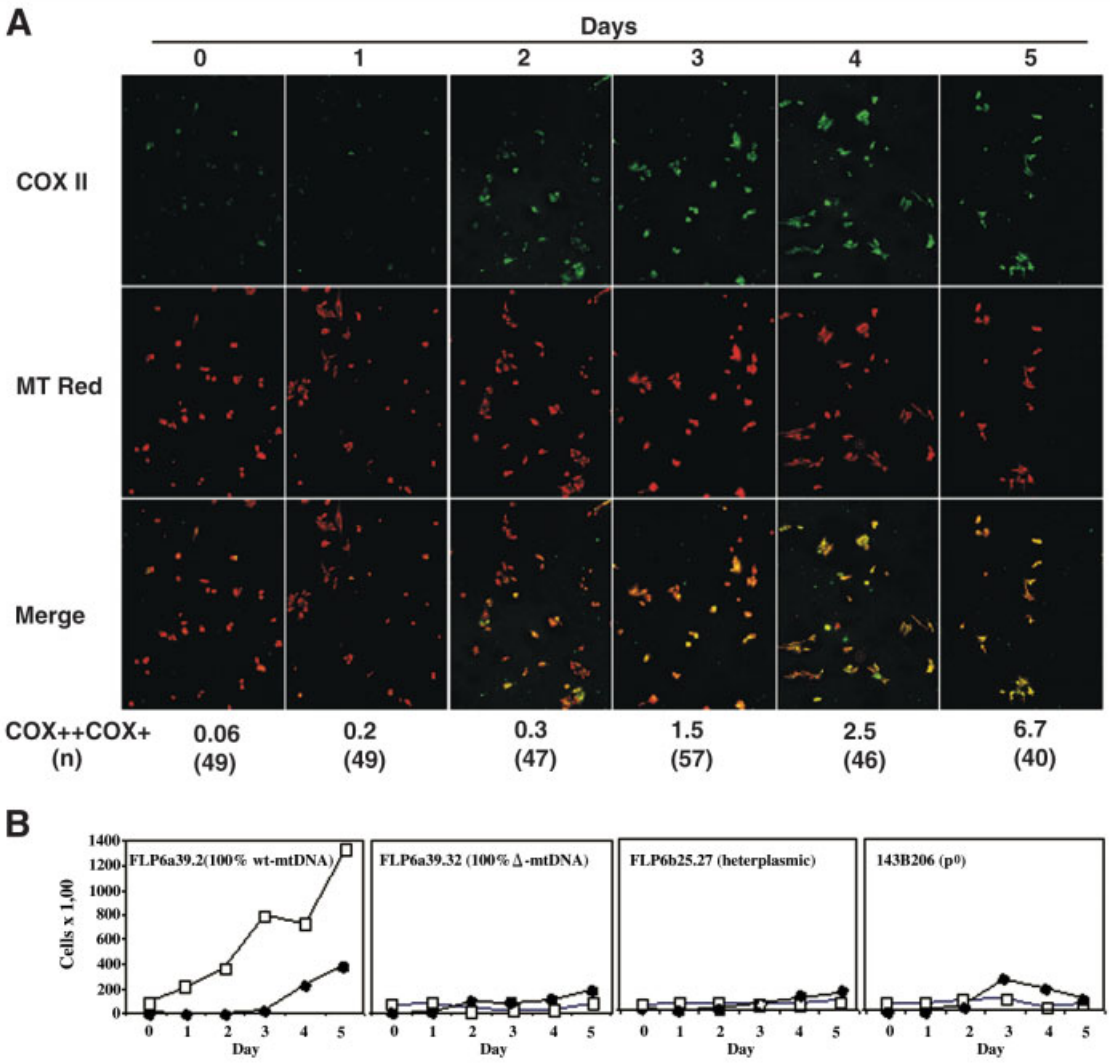

Fig 3. COX II immunocytochemistry over 5 days, at 1-day intervals. (A) Recovery of COX II immunostain in heteroplasmic line FLP6b25.27 versus time. MitoTracker Red labeled all cells. Note that the fraction of COX II–positive cells increased rapidly, such that essentially all the MitoTracker-positive cells were also COX II positive by day 5. All photos at low power (×10). The ratio of COX++ to COX+ foci (n, number of foci analyzed) is shown below each photo. (B) Cell growth during selection. The indicated cybrids were grown in 5mM ketogenic medium (AA), and the cells attached to the plate (open squares) *and floating in the medium* (solid circles) *were counted each day.*

heteroplasmic cells, in addition to the strong signal for the ND4 probe, we detected a very low level of signal for the *ATP6* probe, consistent with the presence of a low level of wt-mtDNA in these cells (presumably 13%, as determined previously by Southern blot analysis). Notably, every cell that we observed had a low level of *ATP6* hybridization, consistent with the clonality of this cybrid line (ie, essentially all the cells were heteroplasmic to the same degree). After each of the ketogenic treatments (AA, BHB, or AA plus BHB), these cells continued to show "normal" levels of *ND4* (ie, green) but had an increased signal for the red *ATP6* probe (see Fig 4C). Surprisingly, much of this increased signal was present in unusually large punctate bodies that colocalized with the *ND4* probe and was visible as bright yellow bodies in the merged image (example shown in Fig 2C, arrows), implying that these "giant" nucleoids contained high levels of wt-mtDNA; we found no such nucleoids in the homoplasmic wild-type cells after ketogenic treatment. The estimated proportion of heteroplasmic cells containing giant nucleoids was 5% before treatment (n = 61 cells) and 40% after treatment (n = 47 cells).

We note that the increased COX II signal (see Figs 2 and 3) could have been due to an upregulation of mtDNA transcription and/or translation that was independent of any shift in heteroplasmy. However, in the absence of significant cell growth (see Fig 3B), only an increase in the amount of wt-mtDNAs could explain either the increase in percentage of wt-mtDNA that we

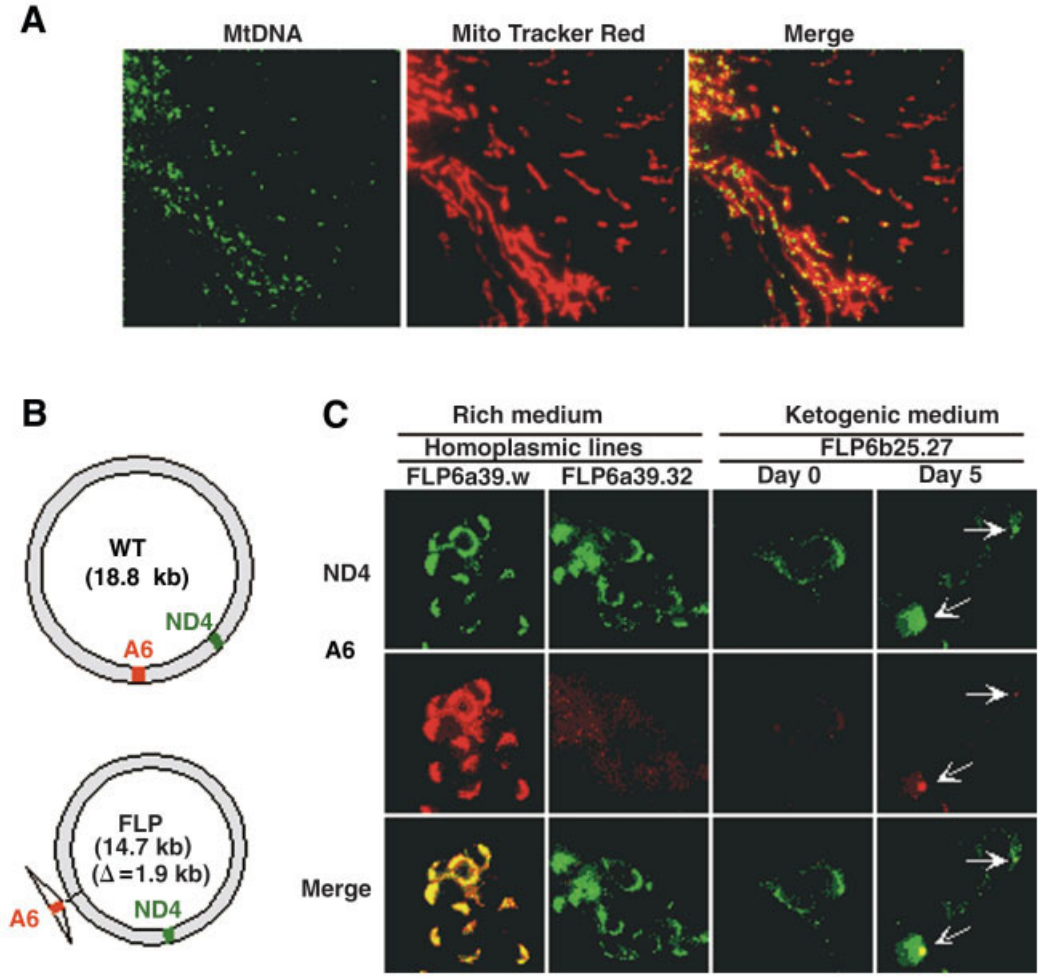

Fig 4. Fluorescence in situ hybridization (FISH) of mitochondrial DNA (mtDNA) in FLP cells. (A) FISH using ND4 probe on MitoTracker Red–stained human osteosarcoma cells. Note the punctate nucleoids within the organelles. ×100 magnification. (B) Maps of wild-type and deleted mtDNAs, showing probes to detect ATP6 (red box; inside deleted region) and ND4 (green box; outside deleted region). (C) FISH before and after selection in ketones. Notation as in Fig 2. Note the punctate pattern of the nucleoids, as well as two "giant" nucleoids found after selection (arrows). *×60 magnification.*

observed in the Southern blot (see Fig 1B) or the presence of the giant nucleoids following treatment (see Fig 4C). Taken together, our results imply that the reduction in the amount of Δ-mtDNA following ketogenic treatment was indeed due to intracellular selection.

Discussion

We have found that ketone bodies can be used to distinguish between respiratory-competent and respiratory-incompetent cells. We have also shown that ketogenic medium can shift the heteroplasmy of cells harboring a mixture of wild-type and partially deleted mtDNAs. This heteroplasmic shift was probably due to an intracellular selection for wild-type mitochondria, with a concomitant rescue of mitochondrial protein synthesis that occurred largely within 2–3 days of this treatment, and was complete by day 5. The nearly complete restoration of protein synthesis in cells harboring more than 20% wt-mtDNAs is consistent with the threshold values for function seen previously by others.[10–12]

We are quite confident that ketogenic media can cause heteroplasmic shifting *among* cells (ie, intercellularly). Moreover, the growth curves and the immunohistochemical and FISH experiments also strongly support the idea that shifting can occur *within* cells (ie, intracellularly). In the latter case, we believe that selection for wild-type homoplasmic cells within a population of heteroplasmic cells (jackpots) was unlikely, because the FISH analysis did not show any cells with wild-type levels of ATP6-positive mtDNA. Also, COX II immunolabeling during ketogenic treatment showed a widespread increase in COX II signal among all the cells analyzed, rather than a colony-based propagation of isolated foci of COX++ cells. Furthermore, the "supernucleoids" that we observed in cells after ketogenic treatment are consistent with the interpretation

that there was selection against Δ-mtDNAs within individual cells: ketogenic selection resulted in an increase in the proportion of wt-mtDNAs, diffusion of functional mitochondrial proteins throughout the organelles, and an increase in net respiratory competence. This hypothesis is also supported by our observation that the heteroplasmic cells regained a completely reticular morphology following treatment (see Fig 2).

In truth, it is unclear how a biochemical stress can generate a signal that causes only a subpopulation of mitochondria within a heteroplasmic cell to increase its proportion of wt-mtDNA above a threshold for function. It is known that the respiratory complexes are not isolated from each other but rather coalesce into a supercomplex called the respirasome,[26] in which complexes I, III, and IV are physically in contact with each other. If the mtDNA, which is packaged into discrete DNA–protein complexes as nucleoids, is also in contact with the respirasome, one could begin to imagine how selection at the level of respiratory chain function could be translated into selection at the level of the mitochondrial genome. In support of this view, at least some components of the mammalian nucleoid, namely, the lipoyl-containing E2 subunits of pyruvate dehydrogenase and branched chain α-ketoacid dehydrogenase,[27] are involved in energy metabolism, and it is conceivable that respiratory chain subunits will also be found to interact with the nucleoid.

We believe that during ketogenic selection, cells are forced to bypass glycolysis and use oxidative phosphorylation as the sole means of energy production.[22,28] Ketone bodies operate on the mitochondrion itself rather than on the glycolytic pathway (Fig 5). After import into the organelle, ketone bodies are eventually converted to acetyl-coenzyme A (CoA), which can then enter the tricarboxylic acid cycle to produce the reducing equivalents necessary to drive the respiratory chain, and hence, ATP synthesis (see Fig 5). Whereas it is experimentally difficult to determine whether ketogenic treatment per se or the absence of glucose is principally responsible for heteroplasmic shifting, ketogenic treatment provides a therapeutically tractable means of activating mitochondrial metabolism. In control experiments utilizing metabolically inactive L-BHB replacing glucose, wild-type cells survived, implying that they use whatever carbon-containing metabolites (eg, from amino acids[29,30]) are available for survival (data not shown). On the other hand, homoplasmic deleted cells died in L-BHB, highlighting the necessity of mitochondrial function for viability under carbon-stressed conditions. Thus, in glucose-free medium containing ketone bodies, only cells harboring at least some functioning respiratory chains are viable, as the mitochondria are the cells' only source of ATP.

Ketosis has been used as a means of treatment of epilepsy, and the ketogenic diet has already been established as a standard protocol for this purpose.[31–33] As is the case with mitochondrial disorders, the mechanism by which a ketogenic diet works in epilepsy is unknown but may well rely on the same underlying principles as heteroplasmic shifting. Interestingly, recent studies have shown that a ketogenic diet upregulates mitochondrial uncoupling proteins and increases mitochondrial respiratory activity in mouse brain[34] and that the use of valproic acid (a ketone body analog) to treat epilepsy causes a massive upregulation of genes whose products are targeted to mitochondria.[35]

Our results in cultured cells suggest that heteroplasmic selection with ketogenic media is a possible treatment strategy. Most mitochondrial diseases in general, and KSS in particular, are fatal, untreatable, disorders. Although it has been possible to rescue mitochondrial gene defects in cultured cells by expressing mtDNA-encoded gene products from the nucleus and targeting them to the mitochondria,[36,37] such approaches will be difficult to execute in patients in whom the entire body is affected, including relatively inaccessible tissues such as muscle and brain. A pharmacological approach to treatment would appear to be more reasonable, especially since most mitochondrial disorders show a phenotypic manifestation only when there is a large percentage of affected mitochondrial DNA. We believe

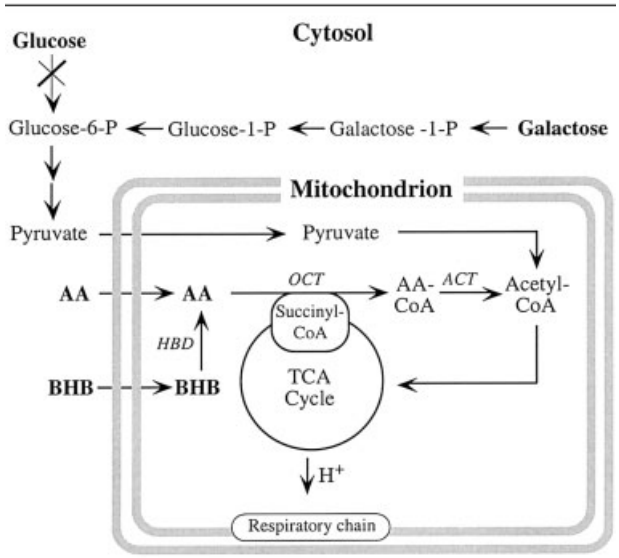

Fig 5. Ketone body metabolism in mitochondria. Acetoacetate (AA) and D-β-hydroxybutyrate (BHB) are transported into the organelle, where BHB is converted to AA by D-β-hydroxybutyrate dehydrogenase (HBD). Succinyl-coenzyme A (CoA) from the tricarboxylic acid (TCA) cycle is used by 3-oxoacid-CoA transferase (OCT) to convert AA to acetoacetyl-CoA (AA-CoA), which is then converted to acetyl-CoA by acetoacetyl-CoA thiolase (ACT). Note that ketone bodies provide oxidizable carbon to mitochondria directly, whereas galactose is only an "indirect" source.

that a modified ketogenic diet could have the potential to reduce the severity of disease in KSS patients.

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