Unmasking the causes of multifactorial disorders: OXPHOS differences between mitochondrial haplogroups

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Many epidemiologic studies have associated human mitochondrial haplogroups to rare mitochondrial diseases like Leber’s hereditary optic neuropathy or to more common age-linked disorders such as Parkinson’s disease. However, cellular, biochemical and molecular-genetic evidence that is able to explain these associations is very scarce. The etiology of multifactorial diseases is very difficult to sort out because such diseases are due to a combination of genetic and environmental factors that individually only contribute in small part to the development of the illness. Thus, the haplogroup-defining mutations might behave as susceptibility factors, but they could have only a small effect on oxidative phosphorylation (OXPHOS) function. Moreover, these effects would be highly dependent on the ‘context’ in which the genetic variant is acting. To homogenize this ‘context’ for mitochondrial DNA (mtDNA) mutations, a cellular approach is available that involves the use of what is known as ‘cybrids’. By using this model, we demonstrate that mtDNA and mtRNA levels, mitochondrial protein synthesis, cytochrome oxidase activity and amount, normalized oxygen consumption, mitochondrial inner membrane potential and growth capacity are different in cybrids from the haplogroup H when compared with those of the haplogroup Uk. Thus, these inherited basal differences in OXPHOS capacity can help to explain why some individuals more quickly reach the bioenergetic threshold below which tissue symptoms appear and progress toward multifactorial disorders. Hence, some population genetic variants in mtDNA contribute to the genetic component of complex disorders. The existence of mtDNA-based OXPHOS differences opens possibilities for the existence of a new field, mitochondrial pharmacogenomics.


INTRODUCTION

Mitochondrial DNA (mtDNA) accumulates mutations much faster than nuclear DNA (nDNA) (1). Very severe mtDNA mutations are rapidly removed from the female germline by purifying selection, thereby minimizing their impact on population fitness. Most of the mtDNA mutations that cause disease are moderately deleterious and persist in human populations for a small number of generations (2). On the other hand, the population survival of functionally neutral mutations is a matter of chance. Mutations that lie elsewhere on the spectrum with mild phenotypic effects will increase their population frequencies depending on a combination of randomness and selective advantage (3). These mutations could be advantageous in some environments but detrimental in other conditions (4). Thus, according to the common disease–common variant hypothesis (5), mutations of the latter category can be part of the genetic basis underlying complex disorders, such as age-linked diseases.
mtDNA encodes very important subunits of the oxidative phosphorylation (OXPHOS) system and the RNAs required for their expression. Through this system, cells obtain most of the ATP necessary to live. The importance of mitochondrial energy makes mtDNA an interesting candidate to study in relation to multifactorial diseases. In fact, many disease phenotypes have been related to groups of phylogenetically related mtDNA genotypes or haplogroups (Supplementary Material, Table S1), and biochemical evidence obtained from individuals with these associations has been provided in epidemiologic studies (6). However, progress in this area has been limited because the phenotypic effects produced by variation in mtDNA are difficult to isolate due to confounding variations of the nuclear genome and to environmental factors. Nevertheless, by using rat or mouse conplastic strains, with identical nuclear genomes but divergent mtDNA genomes, it has been shown that natural mtDNA variation can promote OXPHOS differences that are relevant to the pathogenesis of common diseases (7–10). Obviously, this model cannot be applied in human beings. However, phenotypic differences due to mtDNA variation have also been confirmed by using a different experimental model, mouse trans-mitochondrial cell lines or cybrids (11). These cybrids share the same nuclear genetic background and environmental conditions but differ in their mtDNA.

Thus, before one can seriously consider the role of human mtDNA haplogroups in terms of clinical applications, evidence of cellular, biochemical and molecular-genetic differences between the groups must be obtained. Because the haplogroup Uk has been found to be under-represented in patients with Parkinson’s or Alzheimer’s diseases, thus behaving as a resistance factor, and the haplogroup H has been found to be over-represented in individuals with neurodegenerative diseases, thus being a potential susceptibility factor (Supplementary Material, Table S2), we used the cybrid model to compare the OXPHOS function of these haplogroups. Our results showed that the mtDNA haplogroup differentially contributes to OXPHOS functionality and can therefore be a risk factor that contributes to the development of some of these age-linked diseases.

RESULTS

Nuclear- and mitochondrially convenient cybrids for analysis of mtDNA population genetic variants

To compare the OXPHOS function between the haplogroups H and Uk, we built cybrids with an osteosarcoma 143B TK− nuclear background. Before we used this cell line in our experiments, we considered several aspects of the model. It is known that this cell line is aneuploid, and it was previously shown that cybrids from this background could have different chromosomal numbers (12). Therefore, to rule out major nuclear influences, we took two approaches. First, to avoid clonal effects, we constructed five cybrid cell lines per haplogroup and confirmed that we had introduced the correct mtDNAs (Supplementary Material, Fig. S1). Second, we karyotyped all our cybrids and observed that the chromosomal number was not significantly different between them [H, 66.3 ± 3.8 (5); Uk, 64.6 ± 2.1 (5)] (Fig. 1A and Supplementary Material, Table S3).

Because we were interested in the functional effects of haplogroup-defining polymorphisms, we sought to discard the existence of non-haplogroup-defining mutations with potential phenotypic effects, so we built our cybrids (9 out of 10) from donors in their 20s or 30s, well-below the age when somatic mtDNA mutations tend to accumulate (13). Moreover, although this cell line is thymidine kinase negative (TK−), cells also contain a mitochondrial TK (TK2). Thus, it could be possible that during the selection of the cybrids with 5-bromo-2′-deoxyuridine, the mtDNA may accumulate mutations (14). Therefore, we sequenced the entire mtDNA from the cybrids after selection instead of directly from the donors. In addition, it was recently shown that cybrids could accumulate mtDNA mutations during culture (15). To have a gross estimation of the culture mutation rate, we compared control region sequences that consisted of the mtDNA region with a higher mutational rate between cybrids and donor blood, and we did not find any sequence differences between them.

These trans-mitochondrial cell lines included haplotypes from two subhaplogroups of Uk (Uk1, 2 cybrids; Uk2, 3 cybrids) and three subhaplogroups of H (H1, H5 and H13 with 3, 1 and 1 cybrids, respectively) (Fig. 1B). To exclude private variants (those occurring at the tips of individual branches within the phylogenetic tree) with a possible phenotypic effect, mtDNA mutations were analyzed in a large mtDNA database that included more than 3000 human sequences (16). We found 43 mutations (16 in the control region, 14 synonymous and 5 non-synonymous in protein genes and 8 in RNA genes), but 42 had already been described. Only one, the m.10428A > G/MT-TR mutation in the cybrid 110K, had not been reported previously. This mutation was a heteroplasmic transition (Supplementary Material, Fig. S2A), a condition frequently associated with pathologic mutations, and it broke a Watson–Crick base pair in the tRNA-Arg anticodon stem (Supplementary Material, Fig. S2B). In this cell line, however, a posterior analysis of mitochondrial protein synthesis and other OXPHOS parameters did not show any significant difference with cybrids from the same haplogroup (Supplementary Material, Fig. S2C).

Therefore, these results allowed us to consider these 10 cybrid cell lines nuclear and mitochondrially convenient for posterior analysis of the functional effect of haplogroup-defining polymorphisms.

mtDNA levels were lower in cybrids from the haplogroup Uk

OXPHOS complex activities are under tight control by mtDNA levels (17). To be sure that these levels had been recovered after the cybridization process, we determined the mtDNA amount by qRT-PCR in different culture passages and observed that at least 20 passages were necessary to get the steady-state levels. Therefore, all the experiments were performed in cybrids with a passage number higher than 20. When we compared the mtDNA levels between cybrids H and Uk, we found that cybrids Uk had 7.3% less mtDNA than those in the haplogroup H and this difference was significant (Fig. 2A). The existence of differences in mtDNA levels was also recently shown from cybrids from
the haplogroups H and J (18). These differences were apparently due to a single-nucleotide polymorphism (SNP) in a sequence of the mtDNA control region that was important for its replication. However, our cybrids did not have SNPs in sequences involved in mtDNA replication (Fig. 1B). Consequently, mtDNA levels had to be explained by another functional difference that is due to mitochondrial genotype. Another explanation could involve the fact that it has been reported that high reactive oxygen species (ROS) levels enhance mtDNA replication (19).

Because high ROS levels due to mtDNA pathologic mutations could affect aconitase activity (20) or trigger antioxidant responses, such as manganese superoxide dismutase (MnSOD) overexpression (21), we measured these parameters but did not find any significant difference between both haplogroups. Moreover, we were not able to find differences in either cellular hydrogen peroxide or mitochondrial superoxide anion levels (Supplementary Material, Fig. S3). This could be because our cybrids did not have pathologic mutations but only had population SNPs.

Figure 1. Nuclear and mitochondrial genome analysis in cybrids H and Uk. (A) Spectral karyotyping (SKY) analysis of the cybrids. Metaphase after DAPI stain (a.1), SKY hybridization (a.2) or classified colors (a.3). In this karyotype of one cybrid cell 48K (a.4), structurally rearranged chromosomes are grouped according to the chromosomal type of their major component. Every chromosome from pictures a.1 to a.3 is aligned in this karyotype. (B) Phylogenetic tree of the cybrids’ mtDNA. Black, green, red and blue colors define control region, protein synonymous, protein non-synonymous and RNA mutations, respectively. The affected mtDNA gene or sequence is showed in parenthesis. For non-synonymous mutations, amino acid substitution and position in the protein is also indicated. rCRS means revised Cambridge reference sequence (45).
ATP6-ATP8: H, 100.0 %; H, 100.0 + 2.6% (5); H (NAC), 79.8 ± 7.0% (5); Uk, 92.7 ± 3.6% (5); Uk (NAC), 85.0 ± 5.2 (5). *P ≤ 0.026. Striped bars represent the mtDNA levels after treatment with 5 mM NAC. (B) mtRNA levels. The mean value for cybrids H has been set to 100%. 12S: H, 100.0 ± 7.8% (5); Uk, 89.9 ± 5.0% (5); 16S: H, 100.0 ± 7.2% (5); Uk, 86.9 ± 2.9% (5); ND4-ND4L: H, 100.0 ± 10.2% (5); Uk, 95.5 ± 12.6% (5); ND6: H, 100.0 ± 9.4% (5); Uk, 84.3 ± 17.5% (5); Cytb: H, 100.0 ± 7.9% (5); Uk, 85.9 ± 8.9% (5); COI: H, 100.0 ± 7.9% (5); Uk, 96.0 ± 5.9% (5); COII: H, 100.0 ± 9.0% (5); Uk, 87.2 ± 9.4% (5); COIII: H, 100.0 ± 9.2% (5); Uk, 94.0 ± 9.0% (5); ATP6-ATP8: H, 100.0 ± 13.9% (5); Uk, 99.2 ± 6.7% (5). *P ≤ 0.041.

On the other hand, in a recent study, it was shown that mouse cybrids producing more ROS had higher mtDNA levels, which decreased after treatment with the antioxidant N-acetyl-cysteine (NAC). However, the mtDNA amount was unaffected in those cybrids producing less ROS (11). Similarly, although both cybrids H and Uk decreased their mtDNA levels after the NAC treatment, the effect was larger for cybrids H (20.2% versus 8.3%). Very interestingly, the mtDNA levels after treatment were not significantly different between both haplogroups (Fig. 2A), as though they reached a basal level.

**Mt-rRNA levels were lower in cybrids from the haplogroup Uk**

To check whether the mtRNA levels were related to mtDNA amount, we studied mtDNA gene expression by qRT-PCR and found that, despite the lower mtDNA levels in cybrids Uk compared with cybrids H, there were no significant differences in the RNA levels of mtDNA-encoded complex I (Cl), IV (CIV) and V (CV) subunits (Fig. 2B). Because most of the mt-mRNAs (except that for p.MT-ND6) are part of the same polycistronic transcript, the lower levels of cytochrome b mRNA found (Fig. 2B) could be related to differences in the molecule’s half-life or in the hybridization process due to the presence of particular MT-CYB SNPs in the mtDNA Uk (Fig. 1B). On the other hand, the levels of the rRNAs were significantly lower in the cybrids Uk (Fig. 2B). Given that there were no genetic differences between the mtDNA sequences of both haplogroups that related to the control of transcription process (Fig. 1B), these lower RNA levels are likely due to other mtDNA-related factors, one of which might be the mitochondrial ATP amount.

Human mtRNA synthesis starts at three different locations, one for the L-strand (L) and two for the H-strand (H1 and H2). Mt-rRNAs are mainly synthesized when transcription starts at H1, whereas most of the mRNAs are produced when H2 transcription begins (22). It was shown that the pattern of mtRNA synthesis changes dramatically depending upon the level of ATP available. mRNA synthesis was stimulated at low ATP levels, whereas at high intra-mitochondrial ATP levels, rRNA synthesis and L-strand transcription were strongly stimulated (23). Because the decrease in the amount of mtRNA from cybrids Uk was larger for L and H1 than for H2 transcripts (14.1%, 11.6% and 7.0% for L, H1 and H2, respectively), and because it was recently reported that ATP levels were higher in CD4+ cells from haplogroup H versus non-H patients suffering from Huntington disease (24), we measured the levels of ATP in our cybrids. We found that these levels were significantly higher in cybrids Uk growing in glucose (Fig. 3). If ATP levels mirror cell energy requirements, then cybrids Uk had higher ATP necessities. By growing the cells with 2-deoxy-glucose, a glycolytic inhibitor, and pyruvate, a respiratory substrate, we observed that cybrids H and Uk produced the same ATP amount, although this level was around 30% lower than that obtained by growth in glucose (Fig. 3). This is probably because these are very glycolytic cells and by inhibiting the OXPHOS function with oligomycin, we found that the difference previously described was due to ATP produced by glycolysis (Fig. 3). Therefore, because it was not the intra-mitochondrial ATP, another mtDNA-related functional parameter likely accounts for the difference in the RNA levels.

**Figure 2.** Nucleic acid levels in cybrids H and Uk. White and black bars represent mean values for cybrids H and Uk, respectively. (A) mtDNA levels. The mean value for cybrids H without NAC has been set to 100%. H, 100.0 ± 2.6% (5); H (NAC), 79.8 ± 7.0% (5); Uk, 92.7 ± 3.6% (5); Uk (NAC), 85.0 ± 5.2 (5). *P ≤ 0.026. Striped bars represent the mtDNA levels after treatment with 5 mM NAC. (B) mtRNA levels. The mean value for cybrids H has been set to 100%. 12S: H, 100.0 ± 7.8% (5); Uk, 89.9 ± 5.0% (5); 16S: H, 100.0 ± 7.2% (5); Uk, 86.9 ± 2.9% (5); ND4-ND4L: H, 100.0 ± 10.2% (5); Uk, 95.5 ± 12.6% (5); ND6: H, 100.0 ± 9.4% (5); Uk, 84.3 ± 17.5% (5); Cytb: H, 100.0 ± 7.9% (5); Uk, 85.9 ± 8.9% (5); COI: H, 100.0 ± 7.9% (5); Uk, 96.0 ± 5.9% (5); COII: H, 100.0 ± 9.0% (5); Uk, 87.2 ± 9.4% (5); COIII: H, 100.0 ± 9.2% (5); Uk, 94.0 ± 9.0% (5); ATP6-ATP8: H, 100.0 ± 13.9% (5); Uk, 99.2 ± 6.7% (5). *P ≤ 0.041.

**Figure 3.** ATP levels in cybrids H and Uk. G, DGP, DGPO and GO are abbreviations for glucose, 2-deoxy-glucose plus 1 mM pyruvate, 2-deoxy-glucose plus 1 mM pyruvate and 2.5 µg/ml oligomycin and glucose plus 2.5 µg/ml oligomycin, respectively. The mean value for cybrids H in glucose has been set to 100%. G: H, 100.0 ± 8.3% (5); Uk, 132.9 ± 18.3% (5); DGP: H, 71.1 ± 33.2% (5); Uk, 66.2 ± 10.9% (5); DGPO: H, 5.9 ± 2.6% (5); Uk, 4.3 ± 0.6% (5); GO: H, 96.2 ± 10.7% (5); Uk, 138.3 ± 22.8% (5). *P = 0.009. White and black bars represent the mean values for cybrids H and Uk, respectively.
Mitochondrial protein synthesis and respiratory complex IV levels and activities were lower in cybrids from the haplogroup Uk

To test whether different mtRNA levels could affect OXPHOS function, we determined the enzymatic activities of CII (nDNA-encoded) and CIV (nDNA&mtDNA-encoded) as electron transport chain (ETC) markers and normalized these values for citrate synthase (CS) enzymatic activity, a matrix enzyme (nDNA-encoded) that reflects the mitochondrial number or volume. The results showed that there were no significant differences for CS and CII/CS. However, the CIV/CS ratio was significantly lower in the Uk cybrids (Fig.4A). Except for the synonymous polymorphism m.7028C>T, there were no other SNPs in mitochondrial CIV genes that differed between these haplogroups (Fig. 1B). To explain this lesser activity, we determined the CIV levels and found that they were significantly lower in cybrids Uk (Fig.4B). Moreover, there was a statistically significant correlation between CIV activities and levels (Fig.4C). Therefore, the reduction in CIV activity was due to a lower CIV quantity.

The decline in the CIV amount could be due to decreased synthesis efficiency because of lesser rRNA levels or SNPs in mtDNA protein synthesis genes. There were two polymorphisms in the MT-RNR2 (m.1811A>G and m.2706A>G) gene and one in the MT-TL2 (m.12308A>G) gene that could be responsible for these differences (Fig.1B). Very interestingly, the analysis of mitochondrial translation products showed a decrease in mitochondrial protein synthesis in cybrids Uk (Fig. 4D and E).

The 29.4% decline in CIV levels in cybrids Uk was accompanied by a 29.0% reduction in mitochondrial protein synthesis.

Oxygen consumption and mitochondrial inner membrane potential were different in cybrids from haplogroups H and Uk

Subsequently, we measured oxygen consumption by using high-resolution respirometry and we did not find significant differences in the endogenous, leaking or uncoupled respiration between cybrids H and Uk when expressed as fmole/min/cell (data not shown). However, oxygen consumption rate, when expressed relative to cell number, tends to decrease with increasing cell density due to a decrease in the size of the cells (25) and, probably, in the number of mitochondria. To avoid this problem, we measured oxygen consumption again and related it to the CIV/CS ratio in the cybrid cell lines, as a surrogate of the oxygen consumption per ETC unit (26). We showed that endogenous, leaking and uncoupled respiration was significantly higher in cybrids Uk (Fig.5A).

Oxygen consumption rate is inversely related to the mitochondrial inner membrane potential (MIMP) (27). Thus, our results on oxygen consumption suggested that cybrids Uk had lower MIMP than cybrids H. The determination of MIMP showed that this potential was significantly lower in the cybrids Uk (Fig. 5B). To rule out the differences in the mitochondrial inner membrane surface (MIMS), we used a
probe with high affinity for cardiolipin, and we found no differences in MIMS (Fig. 5B).

The cybrids Uk grew more slowly than the cybrids H To analyze how these OXPHOS differences finally affect cell performance, we analyzed cybrid viability and growth capability. Cell viability was not different between cybrids (Supplementary Material, Fig. S4A). Growth capability was measured in two ways. First, we analyzed the cell doubling times (DTs) in glucose or galactose medium, and we observed that the growth capability in glucose was the same for cybrids of both haplogroups but that the growth in galactose was slower for cybrids Uk, though not significantly (Supplementary Material, Fig. S4B). Secondly, we used competitive mix experiments to estimate cell growth and found that there were significant deviations in the percentage of each genotype H and Uk. After 10 days, the percentage of the genotype H was significantly higher than that at the initial mix, in both glucose and galactose medium. Moreover, there was a significant difference in the percentage of genotype H of cells growing in galactose versus those growing in glucose (Fig. 6), thus suggesting a growth advantage for this haplogroup.

DISCUSSION

A specific combination of diverse genetic (including nuclear and mitochondrial genetic variants) and environmental factors may be involved in a multifactorial disorder, but most of the factors involved are often still unknown. Different combinations of these factors can hamper the analysis of the contribution of any particular factor. mtDNA haplogroups have been epidemiologically associated with different diseases. To analyze the phenotypic effects of human variation in mtDNA and to remove confounding nuclear and environmental influences, a cybrid approach is necessary. Because individuals from the haplogroup T had been found to be over-represented in moderate asthenozoospermia and other phenotypes, whereas those from the haplogroup H were over-represented in the normal sperm motility group (6), cybrids were used to investigate mitochondrial function in mtDNA haplogroups H and T (28). Researchers did not find differences between cybrids H and T in terms of the percentage of basal, leaking and uncoupled respiration (28). We also did not find differences in endogenous, leaking and uncoupled respiration between cybrids H and Uk when expressed as fmole/min/cell. However, we observed that cybrids Uk had lower mtDNA and mt-rRNA levels. These levels were accompanied by a decrease in mitochondrial protein synthesis and CIV activities and levels. Therefore, lower CIV levels per cell or per mitochondrion means that the same level of cell oxygen consumption was carried out by a lower amount of ETC. Thus, each ETC unit consumed more oxygen. Because normalized oxygen consumption was significantly different between uncoupled cybrids from both haplogroups, meaning that the proton gradient was not affecting this rate, the electron transport rate through the ETC should be higher in the cybrids
Uk. Moreover, by using oligomycin to measure leaking respiration, ATP synthase is inhibited, the proton gradient increases and the oxygen consumption decreases. Under these conditions, the higher leaking respiration rate might indicate a lower MIMP, as was shown for cybrids Uk, due to proton leakage or inefficiency in the proton pumping of cybrids Uk. The faster electron flow and lower MIMP in cybrids Uk probably means that electron slippering was happening (i.e. non-coupled or decoupling respiration in which electrons are transported without formation of a potential) (29).

Because only two non-synonymous SNPs (m.14766C>T and m.14798T>C) were present in OXPHOS subunits that are involved in electron flow and proton pumping (Fig. 1B), only these could be responsible for the differences in oxygen consumption and inner membrane potential. The first (p.MT-CYB:Thr7Ile) defines the cluster HV, and the Thr7 is only conserved in 4 of 276 mammalian species (conservation index, CI = 1.4%), but it has been hypothesized that this substitution has an impact on the efficiency of the CI/III C cycle (30). The second one (p.MT-CYB:Phe18Leu) was found twice at internal branches of an mtDNA phylogenetic tree built with more than 3000 complete mtDNA sequences (16) and defines genetic backgrounds Jc and Uk. Interestingly, both haplogroups were found to be over-represented in centenarians and LHON patients and under-represented in patients with Parkinson’s disease. Phe18 is conserved in 220 of 276 mammalian species (CI = 79.7%), thus hinting at its functional importance. This position was located <3.5 A˚ from the inner ubiquinone binding (Qi) site (31), and it was shown that a similar change alters the susceptibility to diuron in yeast, an ETC inhibitor (32). Moreover, this position was situated in a helical region parallel to the plane of the membrane and might participate in relaying conformational information between the cytochrome b monomers (33).

ETC is a metabolic pathway involved in many cell functions. The proton gradient that originates from electrons passing through the ETC complexes is used for many different purposes such as protein and substrate import toward the mitochondria, thermogenesis, apoptosis, maintenance of the cytosolic calcium levels and production of ROS and ATP. Moreover, OXPHOS is important for adaptation to the environment. In fact, external signals, in the form of nutrients and oxygen, interact at the OXPHOS level and trigger intracellular retrograde responses mediated by second messengers such as cAMP or ROS and Ca2+. We did not find differences between cybrids H and Uk in ROS production. In fact, our results were similar to those obtained with the mtDNA mutator mice that accumulated mtDNA mutations and suffered premature aging phenotypes. In these mice, the amount of ROS was normal and the aconitate activity or expression levels of antioxidant enzymes indicated no oxidative stress in their tissues (34). However, we observed that the antioxidant NAC decreased mtDNA levels. Thus, it is possible either that we experienced methodological problems from the fluorescent dyes being unable to distinguish small differences in ROS levels (35) or that NAC affected another process that is quantitatively different in cybrids H and Uk (36). In any case, if differences are not a result of ROS, another difference in cybrids H and Uk due to distinct OXPHOS capacities must be responsible for the mitochondrial phenotypes that we observed. Considering this, it has been shown that mitochondrial matrix pH and intracellular calcium dynamics were different in cybrids from mtDNA macrohaplogroups N and non-N (37). ROS, calcium or other second messengers can modify the expression of many nuclear and mitochondrial genes. HSP60 mRNA and protein levels have also been found to be different in cybrids H (38). These nuclear compensations might hide true differences in OXPHOS function (28), as has recently been shown in mouse cybrids (11). Thus, the significant surplus of glycolytic ATP observed in cybrids Uk might be an attempt to perform nuclear compensation for their lower MIMP. Curiously, it had been previously shown that osteosarcoma 143B.TK+ cells treated with dinitrophenol for 3 days did not change in oxidative capacity but increased their glycolytic metabolism. It was suggested that glycolytic ATP in these cells supplied energy for maintaining mitochondrial membrane potential (39,40).

Thus, along with time and with the cumulative effects of mtDNA somatic mutations and other nuclear and environmental factors, the mtDNA inherited basal differences in OXPHOS capacity reported here can help to explain why some individuals take longer to reach a certain threshold below which tissue symptoms appear and progress toward multifactorial disorders (41). This finding could aid in understanding the overrepresentation of haplogroup Uk in individuals who become centenarians and the underrepresentation in individuals with age-related neurodegenerative disorders such as Parkinson’s and Alzheimer’s diseases. Moreover, slight modifications of the cybrid model can contribute to an unraveling of the particular combination of nuclear, mitochondrial and environmental factors that cause a particular multifactorial disease.

MATERIALS AND METHODS

Biological samples

After winning the approval of the Ethical Committee of the Government of Aragon (Acta nº17/2008) and securing signed informed consent, blood from 165 healthy volunteers was obtained. To homogenize nuclear and environmental factors, we used trans-mitochondrial cell lines or cybrids with the osteosarcoma 143B rho0 nuclear background (42). Ten cybrids (five from mtDNA haplogroup H and five from Uk) were built by fusing platelets from selected individuals with this rho0 cell line (43).

Growth conditions, DTs and cell mix experiments

Most of the experiments were performed with cell lines grown in Dulbecco’s modified eagle medium (DMEM) containing glucose (4.5 g/l), pyruvate (0.11 g/l) and fetal bovine serum (FBS) (5%). When cells were grown with an antioxidant, 5 mM of NAC was used in the culture medium (11). To avoid undesired phenotypic effects, we grew our cybrid cell lines without any antibiotics. DTs of 10 cybrid cell lines growing in DMEM as previously reported or DMEM with galactose (0.9 g/l), pyruvate (0.11 g/l) and FBS (5%) were determined by using the Z2 Beckman Coulter. Initially, 1.5 × 10^5 cells were plated. Three to six growth curves were performed for every cell line, and each time point (0, 24,
48, 72 and 96 h) was counted in triplicate. Only those curves with $R^2 ≥ 0.9$ were considered.

To perform the competitive mix experiments, we combined each cybrid cell line from the mitochondrial haplogroup H with each cybrid cell line from the mitochondrial haplogroup Uk. We grew them in galactose or glucose medium for 10 days, and then we estimated the percentage of every genotype (H and Uk) by qRT-PCR at the final and initial (just after the mix) time points.

Genetics analysis

Samples from the volunteers were genetically characterized by performing PCR-RFLP for mitochondrial haplogroup-defining SNPs in the coding region and sequencing the hypervariable regions I and II (HVRI and HVRII) (6,44).

For molecular cytogenetic analysis of cybrids, cells were exposed to colchicines (0.5 μg/ml) for 4 h at 37°C and harvested routinely. Metaphases were prepared from the cybrids following a conventional cytogenetic protocol for methanol-acetic acid (3:1)-fixed cells. Slides were prepared from the fixed material and hybridized using the SKY method according to the manufacturer’s protocol (Applied Spectral Imaging, Migdal Ha’Emek, Israel). Images were acquired with an SD300 Spectra Cube (Applied Spectral Imaging) mounted on a Zeiss Axioplan microscope using a custom-designed optical filter, SKY-1 (Chroma Technology, Brattleboro, VT, USA). Around 20 metaphase cells were captured and analyzed for each cell line.

The mtDNA sequence was obtained by using the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Rockville, MD, USA) and an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). To locate mutations, the human revised Cambridge reference sequence (NC_012920) (45). The mtDNA content was measured by the qRT-PCR method using an Applied Biosystems StepOne™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems), as described elsewhere (46). The mtDNA levels were determined in triplicate in three to five independent experiments.

The genome shifting quantification in the mix experiments was performed by qRT-PCR, using TaqMan reagents. It includes two specific primers around the m.7028 position and two probes: one labeled with the fluorophore VIC that is specific for m.7028C; and another labeled with the fluorophore FAM that is specific for m.7028T. DNA was amplified in a final volume of 25 μl, using 12.5 μl of TaqMan Gene Expression Master Mix (Applied Biosystems), a final concentration of 0.9 μM of each primer, a final concentration of 0.2 μM of each probe and 10 ng of total DNA. The amplification was performed under universal conditions.

To assess the mtRNA levels, total RNA was isolated from exponentially growing cells using a RNA isolation kit (NucleoSpin RNA II) from Macherey-Nagel according to the manufacturer’s protocol; 2.5 μg of total RNA was reverse-transcribed into cDNA with the High capacity cDNA reverse transcription kit (Applied Biosystems), using the manufacturer’s conditions. The levels of MnSOD mRNA and mtRNAs were determined in triplicate in two independent experiments by qRT-PCR using the One-Step Real-Time system (Applied Biosystems). The expression levels were normalized using the 18S rRNA. The comparative $C_t$ method was used for relative quantification of gene expression as described by the real-time PCR machine manual. Differences in the $C_t$ values (dCt) of the transcript of interest and the reference gene were used to determine the relative expression of the gene in each sample. The dCt method was used to calculate fold expression. StepOne software version 2.0 (Applied Biosystems) was used for data analysis.

Measurement of ROS production

The production of the mitochondrial superoxide anion was measured in triplicate with a Cytomics FC 500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) by using MitoSOX Red® (Invitrogen, Carlsbad, CA, USA) as described previously (47), with slight modifications. The production of cell hydrogen peroxide was measured in triplicate in 4–5 independent experiments with the same flow cytometer by using 2’,7’,dichlorodihydrofluorescein diacetate (2,7-DCFH-DA) (Invitrogen) as described previously (48), with slight modifications. Aconitase activity was measured in triplicate in 3–5 independent experiments as described previously (49,50), with slight modifications. The values were expressed as nU/mg protein.

Determination of ATP levels

ATP levels were measured four times in three independent experiments as described previously (51), with some modifications, using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions. Briefly, 20 000 cells/well were seeded 10–12 h before measurement. Then, cells were washed twice with PBS and incubated for 6 h in record solution with either 5 mM glucose, 5 mM glucose plus 2.5 μg/ml oligomycin (glycolytic ATP generation), 5 mM 2-deoxy-d-glucose plus 1 mM pyruvate (oxidative ATP production) or 5 mM 2-deoxy-d-glucose plus 1 mM pyruvate plus 2.5 μg/ml oligomycin. Cells were lysed, and lysates were incubated with the luciferin/luciferase reagents. Samples were measured using a NovoStar MBG Labtech microplate luminometer, and the results referred to the protein quantity.

Oxygen consumption and respiratory complex activities and levels

Oxygen consumption was analyzed using the high-resolution oxygraph OROBOROS®. Exponentially growing cells were collected by trypsinization, washed, counted and resuspended at 1.5 × 10⁶ cells/ml. Endogenous, leaking (with oligomycin added at 49 μM) and uncoupled (with FCCP added at 1.2 μM) respiration analyses were performed. To correct for the oxygen consumption that is not due to the ETC, respiration inhibition by KCN was performed. Each cell line was measured three to four times in DMEM glucose. Respiration was measured at 37°C with chamber volumes set at 2 ml. The software DatLab (Oroboros Instrument, Innsbruck, Austria) was used for data acquisition (1 s time intervals) and analysis (52).
The enzymatic activities of OXPHOS CII and CIV and CS were assayed following previously described protocols (53–55) in a Unicam UV 500 spectrometer (Unicam Instruments, Cambridge, UK). Mitoprofile® Human Complex IV Activity and Quantity from Mitosciences (Invitrogen) was used according to the manufacturer’s instructions for the determination of CIV activity and levels. A NovoStar MBG Labtech microplate instrument was used for analysis.

**Determination of MIMP and MIMS and cell viability**

The determination of the MIMP was done in triplicate in three independent experiments using 3,3’-dihexyloxacarbocyanine [DiOC\(_6(3)\)] as published previously (56,57). The MIMS was measured, based on the quantity of cardiolipin, four times in three independent experiments by using NAO (nonyl-acridine-orange) (58). The cell viability was measured in triplicate in two independent experiments by using propidium iodide (PI) as described previously (59). A Beckman Coulter Cytomics FC500 cytometer was used for measurements of intracellular fluorescence.

**MtDNA-encoded protein synthesis**

The mitochondrial protein synthesis was analyzed as described previously (60) with minor modifications. Electrophoresis was performed with a Protean II xi system (BIORAD). As a load control, we dyed the gel for 15 min with fixing solution (30% methanol, 10% acetic acid) plus 0.025% of Brilliant Blue R (Coomassie Blue) (Sigma). Then, the gel was washed several times with a 50% methanol, 10% acetic acid solution and left overnight in fixing solution. Finally, it was treated for 20 min with Amplify solution (AMERSHAM), dried and used for autoradiography. The band intensities from appropriate exposures of the fluorograms from two independent gels were quantified by densitometric analysis with the Gelpro analyzer v 4.0. Three bands, corresponding to p.MT-ND5 (upper part of the gel), p.MT-ND1 (middle part of the gel) and p.MT-ND3 (lower part of the gel) polypeptides were selected for quantification.

**Statistics analysis**

The statistical package StatView 6.0 was used to perform all the statistics. Data for mean, standard deviation and sample size \([M \pm SD (N)]\) are presented. The normal distribution was checked by the Kolmogorov–Smirnov test. For those normal variables, the unpaired two-tailed t-test was used to compare parameters. Those variables that were not normally distributed (ATP in glucose and in glucose plus oligomycin; genotype shifting) were analyzed by the non-parametric Mann–Whitney U-test (ATP in glucose and in glucose plus oligomycin) or the Wilcoxon signed-rank test (genotype shifting). \(P\)-values of \(<0.05\) were considered statistically significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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