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Xenomitochondrial Mice: Investigation into Mitochondrial Compensatory Mechanisms

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Abstract

Xenomitochondrial mice, harboring evolutionarily divergent *Mus terricolor* mitochondrial DNA (mtDNA) on a *Mus musculus domesticus* nuclear background (B6NTac(129S6)-mt^{*M. terricolor*}/Capt; line D7), were subjected to molecular and phenotypic analyses. No overt *in vivo* phenotype was identified in contrast to *in vitro* xenomitochondrial cybrid studies. Microarray analyses revealed differentially expressed genes in xenomitochondrial mice, though none were directly involved in mitochondrial function. qRT-PCR revealed upregulation of mt-Co2 in xenomitochondrial mice. These results illustrate that cellular compensatory mechanisms for mild mitochondrial dysfunction alter mtDNA gene expression at a proteomic and/or translational level. Understanding these mechanisms will facilitate development of therapeutics for mitochondrial disorders.

Keywords

mitochondria; transmitochondrial mice; xenomitochondrial mice; mtDNA compensation; gene expression

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Introduction

Because of its cellular metabolic role, mitochondrial function is an important factor in metabolic and developmental disease states including Leber's Hereditary Optic Neuropathy (LHON), Leigh syndrome, Alzheimer's disease, and many others (Wallace 1999; Rossignol et al. 2003). Accordingly, it is vital to understand the exact role of mitochondrial biology in pathogenesis. The human mitochondrial genome, which codes for 13 proteins, 22 tRNAs and two rRNAs, may harbor mutations or polymorphisms known to be either directly causative or associated with the severity of several diseases (Wallace 1999). Animal models are needed to understand how specific mitochondrial DNA (mtDNA) mutations and polymorphisms affect the pathology of metabolic disorders and related disease. Unfortunately, production of animals harboring engineered mutations in mtDNA has been limited. Biological and biotechnological obstacles that must be overcome in order to affect directed alterations in the mitochondrial genome include:

1. The apparent lack of recombination within the mitochondrial matrix (Howell 1997; Eyre-Walker and Awadalla 2001).
2. The challenge of targeting alterations to the hundreds or thousands of mitochondrial genomes within a cell (Wallace 1999).
3. Designing a system capable of penetrating the double membranes of mitochondria efficiently (Khan et al. 2007).
4. Ensuring that selection pressure does not eliminate the altered mtDNA population (Pinkert and Trounce 2002).

As targeted alteration of the mtDNA is not currently possible, other approaches were used to study the association of mtDNA mutations with disease (Cannon et al. 2004). One such approach produced a model referred to as the CAP^r mouse model (Bunn et al. 1974; Wallace 1999). This model was produced from cells found to have a cytoplasmically conferred resistance to the antibiotic chloramphenicol, attributed to a mtDNA mutation in the 16S rRNA gene. mtDNA mutant cell lines were ultimately used to produce a mouse model exhibiting mitochondrial dysfunction (Levy et al. 1999; Sligh et al. 2000).

Another method for modeling mitochondrial disease involves the use of cybrid cell lines. A cybrid cell is produced by fusing two cells, one of which is enucleated, to ultimately give rise to a cybrid cell line. This is usually done using a cancer cell line nuclear background to allow for easy propagation (Khan et al. 2007; Trounce and Pinkert 2007). The fusion of mitochondria-containing cytoplasm from human patients with cancer cell lines allows characterization of the biochemical phenotype of numerous cell lines containing specific mtDNA mutations. While this strategy did provide insight into the cellular and biochemical pathology of mitochondrial diseases, it did not provide a model for the study of disease progression at the tissue or organismal level (Khan et al. 2007).

Cybrids containing a *Mus musculus domesticus* nuclear contribution and *Mus terricolor* mtDNA were hypothesized to exhibit mitochondrial dysfunction due to evolutionary changes in mtDNA between the two murine species (Pinkert and Trounce 2002; Pogozelski et al. 2008). Early *in vitro* studies confirmed this hypothesis and showed a dose-dependent response to evolutionary distance of mtDNA within cybrid cells, in which mitochondrial dysfunction increased with increasing evolutionary distance (McKenzie et al. 2003; Trounce and Pinkert 2007).

In order to address the continuing need for animal models of mitochondrial dysfunction, a xenomitochondrial mouse model (B6NTac(129S6)-mt^{*M. terricolor*}/Cap^r; line D7) was produced. The model was created using cybrids containing mitochondria derived from an

evolutionarily divergent murine species on a laboratory mouse nuclear background (McKenzie et al. 2004; Trounce et al. 2004; Pinkert and Trounce 2007). Subsequent work resulted in production of a xenomitochondrial mouse line derived from cybrid cells and preliminary phenotypic characterization (Pinkert and Trounce 2002; Trounce et al. 2004).

To date, studies of xenomitochondrial mice have not revealed an overt phenotype (Pinkert et al. 2005). Initial studies showed that the parental background strain was a confounding factor. Xenomitochondrial mice were produced by injection of 129S6/SvEvTac (129S6) ES cells harboring *M. terricolor* mtDNA into host C57BL/6NTac blastocysts, followed by breeding chimeric xenomitochondrial females with C57BL/6NTac males (Pinkert and Trounce 2002, McKenzie et al. 2004). Fourth generation offspring were originally assessed in Barnes maze behavioral studies (unpublished data). The differences noted, however, dissipated as the xenomitochondrial mouse lineage was further backcrossed onto a C57BL/6NTac genetic background. This agreed with another report demonstrating that 129S6 mice performed poorly in these tests compared to C57BL/6NTac mice (Crawley et al. 1997). To produce a homogeneous nuclear genotype, the xenomitochondrial lineage was then backcrossed to C57BL/6NTac for 10 generations.

In this paper, experiments are outlined in which behavioral and neuromuscular phenotype were evaluated in xenomitochondrial mice. Finding no overt phenotype, experiments were designed to investigate effects of the xenomitochondrial condition on gene expression patterns in an attempt to uncover mechanisms by which experimental mice were able to compensate for mitochondrial dysfunction associated with the presence of evolutionarily divergent mtDNA. It was hypothesized that upregulation of genes related to mitochondrial function allowed xenomitochondrial mice to produce adequate energy to maintain cellular homeostasis. Further, it was expected that alteration of gene expression in genes related to metabolism of molecular intermediates of energy production aided to prevent pathogenesis. Upregulation of genes involved in reactive oxygen species scavenging was anticipated to prevent oxidative stress.

Microarray analysis measured gene expression using RNA isolated from brain tissue of three-week-old mice. Brain tissue from young mice was chosen due to high metabolic rate of the tissue and high metabolic rate of the actively growing organ in young mice. Because metabolic function is central to all cells, mitochondrial compensatory mechanisms should be common to all cells and tissues despite tissue-specific gene expression patterns.

Materials and Methods

Mice

Xenomitochondrial mice were originally produced by injection of 129S6/SvEvTac (129S6) ES cells harboring *M. terricolor* mtDNA into host C57BL/6NTac blastocysts, followed by breeding chimeric xenomitochondrial females with C57BL/6NTac males (Pinkert and Trounce 2002; McKenzie et al. 2004). To produce a homogeneous nuclear genotype, the xenomitochondrial lineage was then backcrossed to C57BL/6NTac for a minimum of 10 generations for the work done in this report. PCR analysis confirmed homoplasmy of the xenomitochondrial lineage (data not shown).

Three-week-old sex matched (three male and two female from each group) xenomitochondrial and control C57BL/6NTac mice were sacrificed for gene expression and mtDNA content analysis. Aged and young (9–14 months, 3–5 months respectively) male xenomitochondrial and C57BL/6NTac mice were subjected to a battery of neuromuscular and motor tests. Ten aged mice (five of each genotype) and seven young mice (four xenomitochondrial and three C57BL/6NTac) were used in all behavioral analyses. All mice

were maintained in an AAALAC-accredited specific pathogen-free barrier facility with *ad libitum* access to water and feed on a 14:10 light:dark cycle. All mouse procedures including euthanasia conformed to Institutional Animal Care and Use Committee (IACUC) guidelines and the Guide for the Care and Use of Laboratory Animals, under Office for Laboratory Animal Welfare (OLAW) assurance #A3152-01.

Microarray analysis

Agilent murine whole genome microarray chips were used to analyze gene expression in total brain RNA isolated from three-week-old xenomitochondrial and C57BL/6NTac control mice (n=5 per group). Whole brain was dissected from mice and placed into Trizol. A Tissue Tearor was used to homogenize the tissue and a portion of the Trizol homogenate was used to isolate RNA. RNA quantity and purity were confirmed by spectroscopy. Microarray data were analyzed using the ArrayAssist program (Stratagene, La Jolla, CA) as previously described (Mobbs et al, 2004). Briefly, data were normalized by Robust Multi-Chip Analysis (RMA), and then quality of data was tested using scatter plots. Normalized data from xenomitochondrial and C57BL/6NTac control samples were compared to calculate fold change in gene expression. Statistical inferences were assessed using Statistical Analysis of Microarrays (SAM), Bayesian statistics, and standard T statistics.

qRT-PCR

mtDNAs and RNAs from brain, heart and liver of three week old xenomitochondrial and control mice were quantified by qPCR and qRT-PCR using the $\Delta\Delta C_t$ method with the assistance of the REST program (Livak and Schmittgen 2001; Pfaffl et al. 2002).

Standard qRT-PCR was unsuitable to quantify mtDNA level and expression of mtDNA encoded transcripts. Due to sequence divergence between *M. m. domesticus* and *M. terricolor*, a carefully designed quantitative real-time PCR assay was required to avoid data artifacts resulting from sequence dissimilarities (Pogozelski et al. 2008). PCR primers were designed to amplify identical regions of *M. m. domesticus* and *M. terricolor* mtDNA and mtDNA transcripts; each primer sequence recognized given polymorphisms. If primers specific to each species amplify with comparable efficiencies, then the two primer sets can be treated as one primer set for the purposes of this analysis, allowing the quantification of expression of mt-Co2 from mtDNA. Primer sequences follow. Underlined nucleotides denote species-specific sequence differences.

- AGTCGTTCTGCCAATAGAACTTCCAATCCGT mt-Co2 *domesticus* 423F
- AGTCGTCCTACCAATGGAACTCCCAATCCGT mt-Co2 *terricolor* 423F
- TTAGATCCAAAATTTCAGAGCATTGGCCA mt-Co2 *domesticus* 608R
- TTGGAGCCGCAAATTTCGAGCATTGACCA mt-Co2 *terricolor* 608R
- GAAATCGTGCGTGACATCAAAG β -actin 619F
- TGTAGTTTCATGGATGCCACAG β -actin 834R

q-PCR was performed using SYBR green mastermix on DNA isolated from three week old xenomitochondrial and control tissues. DNA (50ng) from each sample was amplified in triplicate.

For qRT-PCR, 4 μ g of RNA were treated with DNase (Applied Biosystems Turbo DNA-free kit) to eliminate contaminating genomic and mitochondrial DNAs. After DNase inactivation, samples were split into RT positive (RT+) and RT negative (RT-) samples. Promega M-MLV reverse transcriptase was used to produce cDNAs in RT+ samples. RT- samples were treated identically to RT+ samples, but without M-MLV enzyme. qPCR of all

RT- samples confirmed absence of contaminating DNA. Samples were adequately DNA-free if Ct values from RT- samples were at least 10 cycles higher than Ct values from corresponding RT+ samples. All samples were run in triplicate using SYBR green master-mix.

Efficiency of PCR reactions was calculated as outlined elsewhere (Liu and Saint 2002). The efficiencies of mt-Co2 primer sets were 90.0% for *M. m. domesticus* primers and 93.9% for *M. terricolor* primers in qRT-PCR of mt-Co2 RNA and 95.8% for *M. m. domesticus* primers and 96.9% for *M. terricolor* primers for q-PCR of mt-Co2 DNA and were therefore calculated together for qRT-PCR and q-PCR. DNA and RNA were isolated from brain, heart and liver obtained from three week old mice and normalized to a β -actin control. Data were analyzed using the REST program, averaging replicates of each sample (Pfaffl et al. 2002).

In order to validate microarray data, RNA was isolated from brain tissue of three week old xenomitochondrial and C57BL/6NTac mice and analyzed by qRT-PCR. 4 μ g RNA samples were treated with Promega DNase for 15 minutes, inactivated, then split into RT+ and RT- samples. RT+ samples were treated with Promega M-MLV reverse transcriptase enzyme to produce cDNAs. RT- samples were treated identically, but without M-MLV enzyme. RT- samples were all amplified to confirm absence of contaminating DNA. qRT-PCR was then performed on all samples in triplicate using SYBR green mastermix. The Vector NTI program was used in design of primers. Data analyses were performed using the REST program (Pfaffl et al. 2002). Primer sequences can be found in Table 1.

Motor/neuromuscular analyses

A Rota-rod treadmill (Med Associates Inc., Georgia, Vt) was used to test motor coordination and endurance. Latency to fall from the Rota-rod was measured both at 24rpm constant rotational velocity and at an accelerating rotational velocity of 4–40rpm. Mice were removed after 240 seconds (24rpm) and 300 seconds (4–40rpm acceleration) if no fall occurred.

Balance Beam experiments tested motor coordination and balance (Carter et al. 1999). Wooden beams used were 1 meter in length and of various shapes and sizes (Square: 28mm, 12mm, 5mm; Round: 28mm 17mm 11mm). Latency of mice to traverse the beam and reach an enclosed escape box was measured.

A Pole test analyzed motor function. Mice were placed upright at the top of a 50cm gauze-wrapped pole (1cm in diameter) topped with a rubber ball. Latency to turn around and descend was measured (Sedelis et al. 2000). Mice were removed from the apparatus after four minutes if descent was not observed.

A Wire Hang test was performed to examine muscle strength. Latency of a mouse to fall 50 cm into bedding from an inverted wire cage top was measured, with the mouse returned to its cage after four minutes if no fall occurred (Paylor et al. 1998).

Gait was analyzed using footprint measurements (Klapdor et al. 1997). A corridor leading to a darkened escape box was constructed and floored with white paper. After application of nontoxic paint of differing colors to fore and hind feet, mice ran along the corridor into the escape box. Distances measured between resultant footprints included: left-front to left-front (LF-LF), left-rear to left-rear (LR-LR), right-front to right-front (RF-RF), right-rear to right-rear (RR-RR), left-front to right front (LF-RF), left-rear to right-rear (LR-RR), right-front to right rear (RF-RR), and left-front to left-rear (LF-LR).

Mitochondrial respiration

Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode as described elsewhere (Tompkins et al. 2006). Mitochondria were isolated from skeletal muscle of 5–6 month old male xenomitochondrial and control mice. A 0.25ml mitochondrial suspension, diluted to 1.0 mg/ml in respiration buffer consisting of sucrose (300mM), KCl (50mM), KH_2PO_4 (5mM), MgCl_2 (1mM), EGTA (5mM), and Tris-HCl (20mM), pH 7.35 was incubated in a sealed bottle on a magnetic stir assembly at 37°C. Complex I-linked state 4 respiration was induced by adding 2.5mM malate and 10mM glutamate followed shortly by 100 μM ADP to stimulate state 3. Respiration data were normalized to citrate synthase activity. Citrate synthase (CS) activity was measured spectrophotometrically as reported previously (Trounce et al. 1996). Respiration was expressed as nmoles of oxygen consumption per minute per unit of citrate synthase activity ($\text{nmolO}_2/\text{min}/\text{CS}$).

Statistical analyses

qPCR data were statistically analyzed using the REST program (Pfaffl et al. 2002). Hazard ratios with 95% confidence intervals were generated for motor assays that produced censored values by Cox regression analysis using PROC PHREG in SAS Version 9.1 (SAS Institute, Inc., Cary, NC) (Cox 1972). Statistical testing of Gait measurements and respiration (no censored values) was conducted with repeated measures analysis of variance using PROC MIXED in SAS. Data were expressed as least squares means with standard errors. All statistical models considered variation due to genotype.

Results

Microarray analysis

Microarray analysis of whole genome gene expression identified seven genes for which expression was altered more than two-fold (Table 2). Identified genes all belong to the immediate-early response gene superfamily; primarily representing transcription factors. Microarray data showing potential expression changes for mtDNA-encoded genes were disregarded due to sequence divergence between *M. m. domesticus* and *M. terrior*, which likely produced artifactual decreases in gene expression in xenomitochondrial mice. Microarray measurements for mtDNA-encoded transcripts from xenomitochondrial mouse tissue were near or at background levels (data not shown). Differential expression of genes associated directly with mitochondrial function was not identified. Lastly, a single xenomitochondrial sample was excluded as an outlier during data analysis due to lack of correlation with other xenomitochondrial samples. Xenomitochondrial samples 1 through 4 had intra-group correlation coefficients greater than 0.9, while sample 5 had intra-group correlation coefficients between 0.78–0.8 indicating a lack of similarity with other xenomitochondrial samples. Heat Map analysis also identified sample 5 as an outlier.

qRT-PCR analysis

No differences were observed in mtDNA content between xenomitochondrial and control brain, liver or heart tissues (data not shown). Experiments validating microarray results confirmed down-regulation of *Fos*, *Egr2* and *Egr4* in brain (Figure 1; $P=0.039$, 0.008 and 0.006 , respectively). qRT-PCR did not show significant down-regulation of *Arc*, *Dusp*, *Junb* and *Nr4a1* ($P>0.05$). Expression of mt-Co2 RNA in brain was upregulated 5.6 fold over controls (Figure 1; $P=0.003$).

Motor analyses

Tables 3 and 4 summarize data for all motor tests. Values represent mean and the 95% confidence interval. Wire hang, pole-test, Rota-rod, Accelerod, and Balance Beam assays

report data as hazard ratios using survival analysis, as time-to-completion values were truncated to a predefined maximum value if the time-to-completion surpassed that value. A hazard ratio of one represents no difference between groups. A hazard ratio less than one indicates that control mice were more likely to record an event (e.g., fall from Rota-rod, traverse Balance Beam) at any given time than xenomitochochondrial mice, while a hazard ratio greater than one indicates the opposite. Gait analysis values represent least square mean distance $\pm$ SE between footprints (R=right, L=left, F=fore, H=hind).

Young xenomitochochondrial mice were less likely to fall off a 24 rpm Rota-rod than wild-type mice ($p < 0.0001$). While older xenomitochochondrial mice and their controls did not display any difference on the Rota-rod test ($p > 0.05$), a difference remained when all mice were compared ($p = 0.025$). Similarly, young xenomitochochondrial mice performed better than C57BL/6 on accelerating Rota-rod ($p < 0.001$); while no difference was observed in their aged counterparts ($p = 0.987$).

For the most part, no differences were seen in Balance Beam analysis. The only exception occurred among the older mice on the 17mm round beam and the 5mm square beam. Aged xenomitochochondrial mice crossed the 17mm beam faster than control mice ($p = 0.04$), while wild-type mice performed better ($p < 0.0001$) on the 5mm beam. The results for the 5mm beam reflect the extreme difficulty all older mice had in crossing the 5mm beam – it was only successfully traversed three times, each time by a control mouse. Due to the limited success of mice in crossing the beam, a hazard ratio could not be calculated for this data point, which is indicated by “***” in the table.

Pole and Wire hang tests were used as additional indicators of neuromuscular function. No differences were seen at any age in Pole test analyses ($p > 0.05$). However, the Wire Hang testing of young mice indicated that xenomitochochondrial mice were less likely to fall than controls ($p = 0.004$). Surprisingly, no difference was seen between groups of aged mice ($p > 0.05$).

Analysis of footprint data revealed a difference in the space between both front feet (LFRF) in all xenomitochochondrial mice analyzed together ($p = 0.047$) but not when each age was examined separately. No other differences were observed in gait measurements.

Oxygen consumption

Complex I linked respiration of mitochondria isolated from skeletal muscle of xenomitochochondrial mice was 21.3 ± 8.1 nmolO₂/min/CS and 23.7 ± 8.1 nmolO₂/min/CS for controls. Xenomitochochondrial respiration values were not different from controls ($p > 0.05$).

Discussion

Following the cross of B6NTac(129S6)-mt^{*M. terricolor*}/Capt xenomitochochondrial mice (line D7) mice onto a C57BL/6NTac background for a minimum of 10 generations, it was surprising to find no deficiencies in behavioral or biochemical measures in xenomitochochondrial mice when compared to C57BL/6NTac controls. Observations ran contrary to expectations based on early *in vitro* cybrid studies showing a mitochondrial deficit in xenomitochochondrial cells containing *M. terricolor* mtDNA on a *M. m. domesticus* nuclear background (McKenzie and Trounce 2000; McKenzie et al. 2004). In light of the absence of measurable phenotypic deficiencies in xenomitochochondrial vs. control mice, and in consideration of data collected from *in vitro* cybrid studies, it would appear that uncharacterized compensatory mechanisms influenced *in vivo* development and mitochondrial function in xenomitochochondrial mice. Identification of this compensatory

mechanism could provide insight into pathological processes involved in mitochondrial disease and could furthermore point the way toward novel therapeutics for metabolic disorders.

We hypothesized that mitochondrial function and, in turn, neuromuscular activity might be impaired in aged xenomitochondrial mice. To test this, we examined oxygen consumption and motor function of xenomitochondrial mice. Respiration studies found no differences between xenomitochondrial and control mice. Neuromuscular analyses involved both young (3–5 months of age) and older (9–14 months of age; equivalence to “middle aged” adult humans) xenomitochondrial mice. Few differences were detected between xenomitochondrial and control mice of either age. Unexpectedly, younger mice displayed the most differences. Perhaps more surprisingly, in those few tests in which differences were identified, xenomitochondrial mice performed better than wild-type controls. Compensatory changes in mitochondrial function to the point of superior function in response to mtDNA polymorphisms might explain these results. Alternatively, the mixture of 129S6 and C57BL/6NTac nuclear backgrounds of xenomitochondrial mice may play a role in apparent superior performance, even after 10 generations of backcrossing our xenomitochondrial maternal lineage with C57BL/6NTac males.

Upon noting the lack of phenotypic changes in early experiments, our initial hypothesis was that genetic compensatory mechanisms altered expression of genes involved in mitochondrial biogenesis and function, allowing normal function of mitochondria in xenomitochondrial mice. To test this hypothesis, a full genome microarray was employed to analyze brain gene expression in three week old xenomitochondrial and control mice. Young mice were chosen and evaluated in light of their rapid growth; correlated with high rates of ATP utilization. RNA from brain was analyzed due to the high metabolic requirements of this tissue. Due to commonality of metabolic pathways between all cells and tissues, compensatory mechanisms should also be shared, with tissues utilizing the most energy showing more pronounced responses. Given the high metabolic rate of neurons it seems unlikely that genetic compensatory mechanisms would not be present in brain tissue while occurring in other tissues.

Genes identified by microarray as differentially expressed belonged to a family of immediate-early response genes; representing primarily transcription factors. qRT-PCR data validated down-regulation of Fos, Egr2 and Egr4, while expression of other genes was not significantly different than control. Considering the down regulation of these transcription factors, it was interesting that putative downstream molecular targets were unaltered in their expression profiles. Down regulation of these transcription factors in xenomitochondrial mice could represent a lower baseline ability to alter expression of specific genes when appropriate molecular signals are received. These changes in gene expression patterns could result in experimental mice less able to respond to cellular stress. Down regulation of proto-oncogenes could implicate mild mitochondrial dysfunction as a causative factor in oncogenesis if supported by other models (Gogvadze et al. 2008). In consideration of their cellular function, microarray identified changes in gene expression were not likely to function in mitochondrial compensatory mechanisms directly. The changes in gene expression were likely secondary to either mitochondrial:nuclear mismatch or compensatory mechanisms occurring in the absence of differences/perturbations in gene expression. Yet, the lack of changes in nuclear genes known to function in mitochondrial energy production or ROS scavenging was most noteworthy.

Quantification of expression of mtDNA-encoded transcripts proved much more difficult than initially expected. Microarray analysis identified massive down-regulation of mtDNA encoded genes in xenomitochondrial samples. These data turned out to be largely artifactual,

as the signal in those xenomitocondrial samples was at or near background levels for all mtDNA encoded genes. Sequence divergence between xenomitocondrial and control samples was such that control transcripts bound well to microarray probes for mtDNA genes, while xenomitocondrial transcripts bound very weakly. This sequence divergence also prohibited use of northern blot to quantify mtDNA-encoded transcripts, as differential probe affinity would lead to inaccurate data.

The PCR assay designed to measure mt-Co2 expression was suboptimal. However, given the constraints of the model system, namely the sequence divergence present between xenomitocondrial and control mice, no other approach would have given better results. While this assay is only capable of approximating expression differences between groups, the approximation should be quite good given that the efficiency of the PCR primer sets used was so similar. PCR efficiency is a primary concern in this instance because the primers had slightly differing sequence. The amplified region of the mtDNA also has polymorphisms. This can lead to differing amplification efficiencies caused by different primer binding affinity or different amplicon melting or annealing temperatures. If the primers had amplified differently, the data would have been skewed toward an artifactual upregulation of the more efficient primer set.

mtDNA contamination was problematic in qRT-PCR assays of mt-Co2 gene expression. Extensive DNase treatment was required to reduce mtDNA contamination to acceptable levels. In final analyses, Ct values from RT- samples were 10 or more cycles greater than corresponding RT+ samples. This difference indicated that mtDNA contamination levels represent less than $1/1024^{\text{th}}$ (2^{10}) the template present in RT+ samples. At this low level, any skewing of the data from mtDNA contamination was negligible.

Upregulation of mtDNA-encoded mt-Co2 is of great interest in relation to uncovering mitochondrial compensatory mechanisms. The upregulation of mt-Co2 supports the hypothesis that mitochondrial transcription is increased. It is possible that upregulation of mtDNA-encoded genes allows increased mitochondrial biogenesis and an increased mass of electron transport chain (ETC) complexes. It is particularly interesting that only mtDNA-encoded genes were upregulated. This suggests that subunits encoded by mtDNA could represent the limiting factor in assembly of ETC complexes. Treatments focusing on increased mtDNA transcription might be clinically relevant in efforts aimed at alleviation of symptoms associated with mild mitochondrial dysfunction.

While evidence of altered gene expression in xenomitocondrial mice provides insight into mitochondrial biology, identification of genes with no significant alteration in expression is also informative. Interestingly, aside from mt-Co2, no changes in genes directly associated with mitochondria were identified. Further studies involving qPCR showed that there were no mtDNA copy number alterations occurring in the xenomitocondrial mice. Clearly, when faced with polymorphic mtDNA or mild respiratory stress, cells are able to compensate very well without altering nuclear gene expression patterns. The alteration of mtDNA transcription could represent a simple way to alter mitochondrial biogenesis and function.

Aside from changes in genes encoded by mtDNA, it is also possible and likely that there are translational alterations or changes in protein or complex activation. Control of mitochondrial function without alterations in gene expression would allow rapid and precise adaptation to fluctuating energetic requirements and mitochondrial efficiency. Changes in expression of nuclear genes involved in mitochondrial function or biogenesis could occur in response to more dramatic alterations in mitochondrial function or cellular energetic requirements.

Based on minimal gene expression pattern alteration observed in xenomitochondrial mice; we propose that compensatory mechanisms at the proteomic or translational level respond to mild mitochondrial dysfunction or polymorphism to allow normal mitochondrial biogenesis and function. Compensatory mechanisms could involve altered rates of mtDNA-encoded subunit production, translation of nuclear genes involved in mitochondrial function, or activation of proteins associated with cellular respiration or mitochondrial biogenesis.

Ongoing studies will examine mice through 24 months of age, equivalent to elderly humans, in order to determine if divergent mtDNA sequence alters a characteristic senescence phenotype. Tests on mice with superimposed metabolic stressors will be conducted to determine the extent to which subclinical mitochondrial dysfunction could exacerbate effects seen in induced diabetes and neurotoxin exposure (X.Y. Kong, B.V. Bui, A.J. Vingrys, C.A. Pinkert, I.A. Trounce and J. Crowston, unpublished data). It remains possible that such stressors will expose phenotypes related to mitochondrial dysfunction not evident under normal conditions.

Conclusion

Studies using xenomitochondrial mice and related lineages shed light on mechanisms by which compensation for mild mitochondrial dysfunction occurs. That mitochondrial dysfunction, measured in an *in vitro* system, could be rescued *in vivo* entirely without alteration of nuclear genes involved in mitochondrial function is a most surprising result. Equally interesting is the noted alteration of mtDNA transcription without corresponding changes in expression of nuclear genes involved in regulation of mitochondrial transcription. This clearly indicates that it is possible to alter mtDNA transcription in response to cellular stress or energetic demands solely through activation of the involved proteins. The changes in transcription factors revealed by microarray analysis and confirmed by qRT-PCR demonstrate that diverse facets of cellular function respond to mitochondrial alterations even though mitochondrial energy production functions at normal levels.

Study of a host of animal models and clinical samples will allow examination of a graded progression of mitochondrial dysfunction and the associated progression of various cellular compensatory mechanisms (Crimi et al. 2005). After uncovering pathways by which an organism responds to different levels of mitochondrial dysfunction, efforts could focus on alternative and potentially novel therapeutic interventions for mitochondrially associated disease. At a cellular or organismal level, such inroads could result in improved treatment regimens for diseases distinguished by decreased mitochondrial capacity or dysfunction; ultimately providing a more beneficial prognosis for a host of human metabolic disorders.

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Figure 1. qRT-PCR measured fold change of mtDNA encoded mt-Co2 and genes identified by microarray as differentially expressed between xenomitochondrial and control samples. Histogram represents the fold-change of xenomitochondrial over control gene expression for identified genes. A value of one represents no change. Error designated as $\pm$ SE. Columns marked with an asterisk represent significant fold changes over control ($P < 0.05$). Fos had a fold change of 0.4, Egr2 had a fold change of 0.2, Egr4 had a fold change of 0.4 and mt-Co2 had a fold change of 5.6. Error bars are asymmetrical due to mathematical transformation of data to fold change.

Table 1

PCR primers for microarray confirmation

• CAGGGGTGAGCTGAAGCCACAAA	Arc 1936F
• CCATGTAGGCAGCTTCAGGAGAAGAGAG	Arc 2249R
• GTGTGGCCCCCTGAGGAGCAC	β-actin 364F
• AGGGACAGCACAGCCTGGAT	β-actin 505R
• GATGTTCTCGGGTTTCAACGCCGACTA	c-Fos 142F
• GCCCCTTCTGCCGATGCTCTG	c-Fos 520R
• GCGCGCTCCACTCAAGTCTTCTTTC	Dusp1 475F
• CCAGCATCCTTGATGGAGTCTATGAAGTCA	Dusp1 890R
• AAGAAGCCAACAACCTTGGTTGCTAGTTTTATTCTG	EGR-2 134F
• TTGCCCATGTAAGTGAAGGTCTGGTTTCTA	EGR-2 527R
• GCCGCGTATCCTGGAGGCGA	EGR-4 263F
• TCCGCGCAGCAAGGCATCGGG	EGR-4 558R
• AGGGAAGCGACGCCGAGAAA	Junb 86F
• GTGTAGAGACAGGCTGCCAGGG	Junb 409R
• TTGGGGGAGTGTGCTAGAAGGACTG	Nr4a1 12F
• TGAGGAGCACGGCTGGGT	Nr4a1 351R

Table 2

Microarray analysis of three-week old xenomitochondrial and C57BL/6NTac control mice.

Fold change over control	Gene name	Probe ID	Gene function	Citations
-4.42	Early growth response 2 (Egr2)	30424	Control of Schwann cell myelination, breathing pattern and early brain development	(Tourtellotte et al. 2000); (Borday et al. 2005); (Berger et al. 2006); (O'Donovan et al. 1999)
-3.69	Fos	16584	Transcription factor, part of activator protein 1 (AP-1) complex, apoptosis, control of cell cycle and differentiation	(Hess et al. 2004)
-3.69	Nuclear receptor subfamily 4, group A, member 1 (Nr4a1, Nur77)	17415	Transcription factor, dopaminergic neuron function, possible role in insulin resistance, thymocyte selection	(Fu et al. 2007); (Levesque and Rouillard 2007)
-2.96	Junb	45074	Transcription factor, part of AP-1 complex, control of cell cycle and differentiation, apoptosis, synaptic plasticity	(Raivich and Behrens 2006); (Hess et al. 2004)
-4.34	Activity-regulated cytoskeleton-associated (Arc, Arg3.1)	35280	Structural protein involved in memory and long term potentiation through a role in endocytosis of AMPA-R	(Kristensen et al. 2007); (Tzingounis and Nicoll 2006); (Guzowski 2002)
-2.65	Dual specificity phosphatase 1 (Dusp1)	5659	Innate immune control	(Maier et al. 2007)
-2.56	Early growth response 4 (Egr4)	11693	Transcription factor, male fertility, neural function undetermined	(Tourtellotte et al. 2000)

Table 3
P-values for behavioral and motor tests performed on aged and young mice: Gait Analyses.

		Gait LFLF	Gait LFLH	Gait LFRF	Gait LHLH	Gait LHRH	Gait RFRF	Gait RFRH	Gait RHRH
Old	Control	6.80 (+/- 0.30)	0.84 (+/- 0.15)	1.51 (+/- 0.09)	6.72 (+/- 0.28)	2.84 (+/- 0.13)	6.73 (+/- 0.39)	0.99 (+/- 0.14)	6.28 (+/- 0.32)
	Xeno.	7.00 (+/- 0.30)	0.86 (+/- 0.15)	1.34 (+/- 0.09)	6.85 (+/- 0.28)	2.96 (+/- 0.13)	6.93 (+/- 0.39)	0.88 (+/- 0.14)	6.81 (+/- 0.32)
p-value		0.644	0.928	0.223	0.758	0.580	0.719	0.585	0.294
Young	Control	7.90 (+/- 0.37)	0.88 (+/- 0.10)	1.63 (+/- 0.07)	7.92 (+/- 0.37)	3.26 (+/- 0.20)	8.30 (+/- 0.43)	0.96 (+/- 0.14)	8.09 (+/- 0.51)
	Xeno.	9.10 (+/- 0.33)	0.79 (+/- 0.09)	1.45 (+/- 0.06)	8.99 (+/- 0.33)	3.10 (+/- 0.18)	8.70 (+/- 0.39)	1.00 (+/- 0.12)	8.52 (+/- 0.45)
p-value		0.066	0.522	0.107	0.083	0.564	0.520	0.865	0.554
Combined	Control	7.30 (+/- 0.40)	0.86 (+/- 0.90)	1.56 (+/- 0.06)	7.23 (+/- 0.41)	3.02 (+/- 0.12)	7.40 (+/- 0.43)	0.98 (+/- 0.09)	7.06 (+/- 0.44)
	Xeno.	8.00 (+/- 0.38)	0.83 (+/- 0.80)	1.39 (+/- 0.05)	7.92 (+/- 0.39)	3.02 (+/- 0.12)	7.80 (+/- 0.40)	0.93 (+/- 0.09)	7.66 (+/- 0.42)
p-value		0.1984	0.8143	0.0466	0.2485	0.9753	0.4963	0.7028	0.3379

Table 4
P-values for behavioral and motor tests performed on aged and young mice: Pole, Rota-rod, Accelerod, Beam and Wire Hang tests.

	Pole- test	Rota- rod	Accel- erod	Beam (Sq28)	Beam (Sq12)	Beam (Sq5)	Beam (Rd28)	Beam (Rd17)	Beam (Rd11)	Wire Hang
Old	0.993 (0.683– 1.443)	1.163 (0.802– 1.685)	0.996 (0.591– 1.677)	1.404 (.523– 3.765)	1.945 (0.566– 6.683)	***	1.658 (0.648– 4.245)	2.873 (1.050– 7.866)	1.447 (0.558– 3.748)	1.120 (0.785– 1.597)
P- value	0.969	0.426	0.987	0.5	0.291	< 0.0001	0.292	0.04	0.447	0.533
Young	0.823 (0.511– 1.311)	0.384 (0.249– 0.592)	0.316 (0.164– 0.608)	2.493 (0.770– 8.076)	1.765 (0.481– 6.472)	0.907 (0.281– 2.928)	0.841 (0.265– 2.664)	0.539 (0.140– 2.081)	0.490 (0.139– 1.726)	0.484 (0.298– 0.788)
P- value	0.413	< 0.0001	0.0006	0.128	0.391	0.871	0.768	0.37	0.267	0.004
All	0.893 (0.667– 1.196)	0.717 (0.537– 0.958)	0.597 (0.393– 0.908)	1.94 (0.92– 4.091)	1.933 (0.832– 4.492)	0.523 (0.172– 1.588)	1.311 (0.648– 2.650)	1.796 (0.872– 3.699)	1.105 (0.543– 2.250)	0.730 (0.551– 0.968)
P- value	0.577	0.0246	0.0159	0.0816	0.1256	0.2528	0.4513	0.1119	0.7827	0.0287