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Recombinant Mitochondrial Transcription Factor A with N-terminal Mitochondrial Transduction Domain Increases Respiration and Mitochondrial Gene Expression

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Abstract

We developed a scalable procedure to produce human mitochondrial transcription factor A (TFAM) modified with an N-terminal protein transduction domain (PTD) and mitochondrial localization signal (MLS) that allow it to cross membranes and enter mitochondria through its “mitochondrial transduction domain” (MTD=PTD+MLS). Alexa488-labeled MTD-TFAM rapidly entered the mitochondrial compartment of cybrid cells carrying the G11778A LHON mutation. MTD-TFAM reversibly increased respiration and levels of respiratory proteins. *In vivo* treatment of mice with MTD-TFAM increased motor endurance and complex I-driven respiration in mitochondria from brain and skeletal muscle. MTD-TFAM increases mitochondrial bioenergetics and holds promise for treatment of mitochondrial diseases involving deficiencies of energy production.

Keywords

TFAM; respiration; mitochondrial gene expression; respiratory proteins

1. Introduction

Mammalian mitochondrial DNA (mtDNA) is a ~16.6 kilobase circular genome that consists of a regulatory control region (“D-loop”), 13 genes for essential catalytic proteins of the ~87 proteins in the electron transport chain (ETC), 22 tRNA's and two ribosomal RNA's that facilitate translation of the mtDNA-encoded ETC proteins in the mitochondrial matrix (Dimauro and Schon 2008). The remainder of the ETC proteins and ~1200–1500 of the other mitochondrial catalytic and structural proteins are imported using multi-protein translocase complexes of the outer (TOM) and inner (TIM) mitochondrial membranes that direct protein

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Disclosures

FRP is an officer in Gencia Corporation and has a personal financial interest in the development of the MTD-TFAM technology described in this paper. None of the other authors has a personal financial interest in Gencia Corp. or the technology described.

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precursors formed outside mitochondria to their appropriate location by means of specific N-terminal mitochondrial localization sequences (Kutik, Guiard et al. 2007). After reaching their final destinations, the localization sequences are cleaved by mitochondrial proteases (Kutik, Guiard et al. 2007). Because most catalytic ETC proteins coded by mtDNA are hydrophobic, special mitochondrial chaperones are believed to assist in proper folding and insertion into their respective ETC macrocomplexes (Leidhold and Voos 2007; Szabadkai and Rizzuto 2007).

Although some of the basics of mtDNA replication and transcription are known, much is either controversial or remains to be discovered (Fernandez-Silva, Enriquez et al. 2003; Brown, Cecconi et al. 2005; Scarpulla 2008; Shadel 2008). Abnormalities of mtDNA replication and transcription (such as production of deleted species) or translation (due to mutations in tRNA or coding ETC genes) are responsible for illnesses present in childhood or early adulthood involving high energy, post-mitotic tissues such as brain, retina, heart and skeletal muscle (Wallace 2005; Dimauro and Schon 2008). These “mitochondrial” diseases can display variable and overlapping phenotypes, and understanding their genotype-phenotype relationships remains a great challenge (Wallace 2005; Dimauro and Schon 2008).

Further insights into understanding mitochondrial genome replication and expression, in addition to development of novel therapies for mitochondrial diseases, would benefit from technology that allows external manipulation of the mitochondrial genome. Mitochondrial transcription factor A (TFAM) is a member of the high-mobility group (HMG) of DNA-binding proteins that participate in mtDNA replication and transcription (Garstka, Schmitt et al. 2003; Ekstrand, Falkenberg et al. 2004; Maniura-Weber, Goffart et al. 2004; Pohjoismaki, Wanrooij et al. 2006; Cotney, Wang et al. 2007; Kang, Kim et al. 2007; Scarpulla 2008). Genetic deletion of TFAM is embryonic lethal (Wallace 2002), demonstrating its essential role in mitochondrial function.

Earlier (Khan and Bennett 2004), we proposed an approach to delivery of mtDNA cargo to the mitochondrial matrix based on the use of recombinant TFAM engineered with an N-terminal protein transduction domain (PTD), followed by a matrix mitochondrial localization sequence (MLS). We refer to the combination of PTD and MLS as “mitochondrial transduction domain” (MTD). We now report the development of a scaled-up technology to produce a recombinant MTD-TFAM possessing similar overall properties but a different primary structure (Figure 1A). We then asked whether this MTD-TFAM protein by itself could affect mitochondrial function *in vitro* and *in vivo* and describe effects of this recombinant MTD-TFAM on respiratory physiology in SH-SY5Y cybrid cells carrying the G11778A LHON mutation and in normal adult mice.

2. Materials and Methods

Expression of MTD-TFAM

We included a HA epitope after the 11-Arginine PTD based on reports that the HA epitope facilitates cytosolic escape of transduced protein after macropinocytosis (Wadia, Stan et al. 2004). The nucleotide sequence corresponding to PTD-HA-MLS-TFAM (see below) was subcloned into PE-Sumo3 (Life Sensors). The construct was transformed into Tuner (DE3) pLysS cells (Novagen). Recombinant protein was expressed by the transformed bacteria cultured in Overnight Express TB medium (Novagen), an auto-induction media, supplemented with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol in a Bioflo 310 Fermentor/Bioreactor (New Brunswick). Growth and expression of the bacteria culture were performed at 37° C, dissolved oxygen 30%, with variable agitation and airflow. When culture achieved an optical density of 25 the bacteria were harvested and pelleted by centrifugation at 3500 g and stored at -80 degrees.

[6X-His]-[SUMO3]-[11Arg PTD] –[HA]-[SODMLS]-[MatureTFAM]

[MGHHHHHHGG]-

[MSEKPKKEGVKTENDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSMR
QIRFRF DGQPINETDTPAQLEMEDEDTIDVFQQQTGG]-[RRRRRRRRRRR]-
[GEGDIMGEWGNEIFGAI AGFLGGE]-[MLSRAVCGTSRQLPPVLGYLGSRQ]-
[SSVLASCPKKPVSSYLRSKEQLPIFK AQNPDAKTTELIRRIAQRWR
ELPDSKKKIYQDAYRAEWQVYKEEISRFKE QLTPSQIM SLEKEIMD KHLKRKAM
TKKKELTLLGKPKRPRSAYN VYVAERFQEA KGDSPQEKLK TVKENWKNLS
DSEKELIYQH AKEDETRYHN EMKSWEEQ MIEVGRKD LLRRTIKKQR KYGAEEC)
KGDSPQEKLK TVKENWKNLS DSEKELIYQH AKEDETRYHN EMKSWEEQ
MIEVGRKD LLRRTIKKQR KYGAEEC]

Protein Purification

Cell pellets were resuspended in modified Bugbuster (Novagen) lysis buffer (1 M TBS, 1M Urea, 250mM Sucrose, 15% glycerol, 80mM imidazole). Benzonase (Novagen) was then added to the lysate (250 U/ml) and allowed to incubate under agitation at room temperature for six hours. The lysate was then clarified by centrifugation at 35,000 g for 35 minutes and then 0.2 μ filtered to remove micro-aggregates. Isolation of the vector protein was performed on a GE AKTA purifier utilizing Histrap HP 5mL columns. Imidazole was removed from the eluted protein utilizing Slide-a-lyzer (Pierce) dialysis cassette (20 K MWCO) in dialysis buffer (1M TBS, 1M Urea, 250 mM sucrose, 250 mM NaCl, 20% glycerol.) Sumo protease was then added to the elution to allow for cleavage of the sumo-fusion fragment from the target protein vector. The solution was then applied to a 1mL Histrap (GE) column and the sumo fusion, and other nickel binding contaminants were removed from the solution. The flow-through was collected, dialyzed to remove NaCl (.5M TBS, 1M Urea, 250 mM sucrose) and applied to a Histrap heparin column to ensure proper conformation of the target vector protein and remove remaining contaminants from the preparation. The elution was then screened via SDS page analysis for proper size, complete removal of sumo fusion, and purity. Western blot analysis was also performed utilizing TFAM antibodies (Santa Cruz) to verify the success of target protein purification. Each lot of protein vector was tested for DNA binding using EMSA (Electrophoretic Mobility Shift Assay) as a measure of biological activity. Protein was stored in 50% glycerol at –20 degrees.

DS9 LHON cell culture

DS9 cybrid cells containing the G11778A LHON mutation in high abundance were a kind gift of Dr. Russell Swerdlow and were created in a SH-SY5Y rho0 cell line by fusion with platelets from a 42 year-old male with LHON. Cells were grown in DMEM containing 10% FCS and passed with trypsin.

Exposure to MTD-TFAM

DS-9 cells were grown to ~50% confluency in T25 flasks. MTD-TFAM sufficient to bind ~10 ug of mtDNA, based on EMSA (typically 100 uL of protein in 50% glycerol solution; 45 ug protein) was mixed with 50 uL of Roche Expand Long Template PCR buffer #3, water added to 500 uL and incubated for 30 min at 37 degrees. The Roche PCR buffer #3 is a high Mg^{+2} buffer we used to facilitate protein binding to DNA. The resulting solution was mixed with 4.5 ml of DMEM and added to DS9 cells in T25 flasks that had regular media removed and had been rinsed with DMEM. The cells were incubated with MTD-TFAM (or buffer control) for 5 hours at 37 degrees, then the MTD-TFAM solution was removed and replaced with regular media. Exposure to MTD-TFAM occurred only once, and the same batch of MTD-TFAM was used for all experiments.

Whole cell respiration

MTD-TFAM and buffer control cells were passed in parallel, expanded in culture and harvested with trypsin. 4.5 million Trypan-blue excluding cells/ml of DMEM with glucose were added to each chamber of an Oroboros Oxygraph 2 respirometer and studied intact with “high resolution respirometry” (Hutter, Unterluggauer et al. 2006). Briefly, after assay of basal respiration rates, ATP synthase was inhibited with oligomycin and mitochondria were incrementally uncoupled with FCCP injection to estimate maximal uncoupled respiration that was sequentially inhibited with rotenone to inhibit complex I followed by antimycin A/ myxothiazole to inhibit complex III.

Real Time-quantitative PCR (RT-qPCR) analysis of mitochondrial gene expression

Total genomic DNA and total RNA were isolated from cell pellets using All-Prep kits from Qiagen and amounts assayed with Quant-IT DNA and RNA assays (Invitrogen). 1 µg of total RNA was reverse transcribed into cDNA (complimentary DNA) using iScript (BioRad). Levels of 18S rRNA or mtDNA D-loop were assayed using SybrGreen detection (BioRad) with Roche human genomic DNA or a full-length mtDNA PCR product, respectively, as standards. Copy numbers of 12S rRNA, ND2, CO3 and ND4 mitochondrial genes were assayed in a multiplex qPCR assay (BioRad Powermix) using the full-length PCR product of human mtDNA as standard. All RT-qPCR was carried out in an iQ5 thermocycler (BioRad) using primers and probes designed with Beacon Designer software. Levels of mitochondrial genes were normalized to 18S rRNA in either DNA or RNA (cDNA) samples.

SfaN1 restriction analysis

A PCR product in the ND4 gene spanning the *Sfa*N1 site removed by the G11778A mutation was amplified from genomic DNA and cDNA of the LHON cybrid cells at different passages over time, and digested with *Sfa*N1. Digestion products were analyzed using automated electrophoresis (Experion, BioRad).

Western blot analysis of ETC proteins

100 µg of total cell protein were loaded onto 4–12% Bis-Tris Criterion precast gels (BioRad) and separated. The proteins were then transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). Complex I subunits were detected by immunoblotting using the following antibodies purchased from Mitosciences: MS111 against subunit NDUFA9 at 1.125 µg/mL, MS110 against subunit NDUF33 at 0.5 µg/mL, MS109 against an 8kDa subunit at 1 µg/mL, MS107 against subunit NDUF44 at 0.5 µg/mL, and MS105 against subunit NDUF48 followed by an IRDye® 800 goat anti-mouse secondary at 1:15,000 (Li-cor). Subunits from complexes I–V were detected by immunoblotting using the Mitoprofile® human total OXPHOS complexes detection kit at 1:575 (Mitosciences) followed by an IRDye® 800 goat anti-mouse secondary at 1:25,000 (Li-cor). Mitofilin was assayed as an estimate of mitochondrial mass in each sample and was detected by immunoblotting using the MSM02 antibody at 2 µg/mL purchased from Mitosciences followed by an IRDye® 800 goat anti-mouse secondary (Li-cor). Beta-actin was used as a loading control and was detected by immunoblotting using a polyclonal beta-actin antibody purchased from Abcam followed by an IRDye® 680 goat anti-rabbit secondary at 1:15,000 (Li-cor). The membranes were visualized and bands quantitated using the Odyssey infrared imaging system (Li-cor).

Immunohistochemical analysis of ETC macrocomplex assembly

Cells were grown to subconfluence (70–80%) in polylysine coated Mattek dishes, fixed with 4% paraformaldehyde in 0.1M PBS for 20min., rinsed in PBS and stained following the protocol recommended by Mitosciences (www.mitosciences.com) Briefly, fixed cells were treated with antigen retrieval buffer (5% urea in 100mM Tris, pH 9.5) at 95°C for 20 min, followed by 3

washes with PBS, 0.2% Triton-X-100 in PBS for 15 min and 3 more washes with PBS. Cells were blocked in 10% goat serum for one hour at room temp.; primary antibodies (Mitosciences) were added to each dish in 10% goat serum: anti-complex 1 (MS602-1 NDUFB4) 50µg/ml; anti-complex 4, subunit 1 (MS602-IV) 6.25µg/ml; anti-complex 5α control (MS602-CVα) 1.25µg/ml and incubated at 4°C overnight. Cells were washed in 1% goat serum; secondary antibodies were added to each as recommended by the protocol and incubated at room temperature for 2 hours. Following 3 washes in 1% goat serum, dishes were rinsed twice in PBS, then 45nM TOTO-3 (Invitrogen, USA) was added for 10 min. at room temp. to stain nuclei. Cells were washed twice in PBS and mounted with Vectashield (Vector Labs, Burlingame, CA). Single plane confocal images were made using 60x objective on an Olympus IX70 microscope with Olympus Confocal Laser Scanning System and argon ion and helium/neon ion lasers.

MTD-TFAM injections, motor endurance testing and mitochondrial fraction respiration

MTD-TFAM capable of binding ~100ug of DNA (450 ug purified protein in 1 ml of 50% glycerol solution) was dialyzed against 5% glycerol in PBS then concentrated to ~0.5 ml using Amicon centrifuge filters. Testing of the Amicon flow-through revealed no DNA binding capacity. Adult male C57BL/6 mice were injected IV through tail veins with the dialyzed, concentrated MTD-TFAM (4 mice) or buffer vehicle control (4 mice). Mice were treated in pairs each day (MTD-TFAM or CTL); each mouse received one IV injection every week for a total of four injections.

Before starting injections, each mouse was trained for endurance on a rotarod (Columbus Instruments). The training session consisted of two sessions of 120 sec at 5 rpm, two sessions of 120 sec at 10 rpm, two sessions of 120 sec at 15 rpm; there was a 10 minute rest period between each session. Each testing period consisted of three consecutive sessions at 15 rpm, followed by three consecutive sessions at 20 rpm for weeks 0–4. Because of increasing endurance, during weeks 3–4, three consecutive 30 rpm sessions were added after the 20 rpm sessions. There was a 10-minute rest period between each session; each session lasted no longer than 2000 sec.

One week after the fourth injection, each pair of mice (MTD-TFAM treated or CTL) was sacrificed. In parallel each brain, heart, and equal weights of skeletal muscle were removed, weighed and homogenized in buffered mannitol-sucrose (200 mM mannitol, 50 mM sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.4) in the same Teflon-glass homogenizer. P2 pellets were kept on ice until resuspension in “MiRO5” mitochondrial respiration media (EGTA 0.5 mM; MgCl₂·6H₂O 3 mM; K⁺-lactobionate 60 mM; taurine 20 mM; KH₂PO₄ 10 mM; HEPES 20 mM; sucrose 110 mM; BSA, fatty acid free, fraction V1 g/l; <http://www.oro-boros.at/index.php?id=524#857>). One fourth of each tissue's P2 pellet from MTD-TFAM-treated or buffer control-treated mouse was added to the respirometer chamber, followed by sequential additions of substrates to assay State 3 respiration (+ 5 mM ADP) through complex I (glutamate-10mM/malate-2mM), complex II/III (rotenone-0.5uM + succinate-5mM) and complex IV (antimycinA-2.5uM/myxothiazole-0.5uM+ascorbate-/TMPD-+ cytochrome C-) followed by sodium azide.

Statistical analyses

All statistical analyses were carried out with InStat for Macintosh (GraphPad, v3.0b). Parametric or non-parametric tests were used based on whether the data being analyzed were distributed normally or not. Statistical significance was considered for P values <0.05.

3. Results

MTD-TFAM (Figure 1A) was produced initially as a N-terminal 6XHis-SUMO derivative to increase its intracellular solubility and with a rapid induction approach to minimize toxicity. The initial protein extract was treated with benzonase to remove contaminating DNA; 6XHis-SUMO-MTD-TFAM was isolated on a nickel column, eluted and treated with SUMO protease. Subsequent passage through a nickel column isolated the 6XHis-SUMO, and the eluted MTD-TFAM was purified further and shown to bind and retard electrophoresis of mtDNA by EMSA (Figure 1B).

We then labeled and purified MTD-TFAM with Alexa488 dye according to manufacturer's instructions (Alexa Fluor®488 Protein Labeling Kit, Invitrogen) and incubated the Alexa488-MTD-TFAM with SH-SY5Y neuroblastoma cybrid cells carrying a G11778A mtDNA mutation in the ND4 gene from a patient afflicted with Leber's Hereditary Optic Neuropathy (LHON), a cause of retinal ganglion cell degeneration and blindness in young adults (Wallace, Singh et al. 1988; Yen, Wang et al. 2006). Incubation with Alexa488-labeled MTD-TFAM revealed rapid entry of MTD-TFAM into the mitochondrial compartment (Figure 1C).

We next investigated if MTD-TFAM could alter the mitochondrial physiology in these LHON cybrid cells. Three consecutive independent experiments were carried out over several months in which LHON cybrid cells at the same initial passage numbers were treated with MTD-TFAM or buffer control (CTL). The two groups of cells in each of the three independent experiments were passed in parallel to generate adequate cell densities to carry out multiple, simultaneous "high-resolution" oximetry-respiration experiments using intact cells metabolizing glucose (Hutter, Unterluggauer et al. 2006). In this approach, basal, ETC complex-dependent and incrementally uncoupled respiration rates were assayed in real time where all metabolic control systems were otherwise intact. Nine million living cells from individual experiments and their CTL were added to each 2 ml. respiration chamber. We expanded CTL and MTD-TFAM treated cells in culture in each experiment and studied cells at equivalent passage numbers (P2, P3, P4) after initial treatment. The basal respiration values were depicted as a function of the same number of live cells expressed at each of the same three passages after MTD-TFAM exposure. We observed that exposure to MTD-TFAM caused a time-dependent, reversible increase in basal respiration rates that reached a maximal ~2.5-fold increase over control samples at the second passage around 2 weeks (Figure 2). The resulting effect of MTD-TFAM on respiration across passage number was highly significant ($p=0.044$) by ANOVA (Figure 2). Table 1 shows that MTD-TFAM treatment did not alter basic respiratory parameters related to respiratory chain coupling, indicating that basic respiratory chain physiology was not altered by MTD-TFAM exposure.

Because TFAM is a recognized essential factor for mitochondrial genome replication and transcription, one possible explanation for this result is that MTD-TFAM exposure was increasing mitochondrial gene replication, transcription and translation into respiratory proteins. We used primers for the D-loop with SyberGreen detection and multiplex qPCR for several mitochondrial genes (12S rRNA, ND2, ND4, CO3) to monitor alterations in mitochondrial gene copy numbers in genomic DNA samples or mitochondrial gene expression in cDNA samples (primer and probe sequences are in Supplemental Table 1). Figure 3 shows that mtDNA copy number from the averages of D-loop, ND2, ND4 and CO3 qPCR for each sample did not change over the time course examined (one-way ANOVA $p=0.56$). Comparison between data for days 8–9 compared to days 11–12 approached significance ($p=0.092$). Mitochondrial gene expression in RNA samples, normalized to 12S rRNA expression in each sample, were highest at the earliest time points examined and declined subsequently (Figure 4). Changes in ND2 and ND4 expression across time approached significance (one-way ANOVA $p=0.061$, 0.095 , respectively). Restriction analysis using *SfaNI* treatment of an ND4

PCR product followed by automated electrophoresis revealed that MTD-TFAM treatment did not alter the near-homoplasmic distribution (>97%) of the G11778A mutation in the LHON cybrid cells (not shown).

In the third experimental series of LHON cybrid cell samples exposed to MTD-TFAM, we examined the levels of multiple individual ETC proteins with Western blots. We also studied assembly of ETC macrocomplexes with immunohistochemistry using antibodies directed against mtDNA-encoded catalytic subunits of complexes I and IV, compared to that of an antibody against a nuclear genome-encoded component of complex V (ATP synthase) as a marker for general mitochondrial distribution. Our Western blot analyses (Figure 5) revealed that multiple complex I proteins, all encoded by nuclear genes, increased many fold at the earliest time point examined and then declined to near control values afterwards. The relative mitochondrial mass in cells, expressed as a ratio of the outer mitochondrial membrane protein mitofilin to that of cytosolic beta actin, ~doubled (1.9-fold) in MTD-TFAM treated cells at the earliest time point examined (9 days) and was slightly below control cells by the last time point (20 days). The levels of a mtDNA-encoded (CIV, subunit 2) and multiple nuclear genome-encoded ETC proteins from several complexes also increased substantially and reversibly in the MTD-TFAM treated cells (Figure 5). Confocal microscopy did not reveal any effects of exposure of the LHON cybrid cells to MTD-TFAM on the proportions of cells (97–100%) with intact ETC complex I or complex IV macroassemblies (Figure 6).

Because our results with MTD-TFAM treatment of LHON cybrid cells showed increases in several aspects of mitochondrial physiology, we next wished to determine if similar changes could be observed *in vivo*. We treated normal adult male mice with tail vein I.V. injections of MTD-TFAM or buffer control and assayed motor endurance and respiration in mitochondrial preparations from brain, heart and muscle. Mice were injected once a week for four weeks with MTD-TFAM sufficient to bind ~100 ug of DNA in each injection. Motor endurance was quantitated by the time each mouse could remain on a rotarod that rotated at a constant velocity, which was slowly increased in 10 rpm increments up to 30 rpm. We observed in both treatment groups a substantial effect of conditioning alone on motor endurance, with a 3–4 fold increase in time spent at 20 rpm over the four week testing period (not shown). After 3 weeks of treatment, mice receiving MTD-TFAM injections showed a ~3-fold increase in 30 rpm rotarod endurance that was statistically significant (Figure 7, top). After 4 weeks of treatment, MTD-TFAM treated mice showed a non-significant ~2-fold increase in 30 rpm rotarod endurance.

The mice were sacrificed 7 days after the last MTD-TFAM injection and rotarod test, organs were harvested, equal weights homogenized, centrifuged and studied with respirometry in parallel from the buffer CTL and MTD-TFAM injected mice. Crude mitochondrial P2 pellets from each organ pair (MTD-TFAM and CTL) were resuspended in mitochondrial respiration buffer and sequentially exposed to substrates that provide electrons to complex I (glutamate/malate), complex II (rotenone/succinate) and complex IV (antimycinA-myoxothiazole/ascorbate/TMPD/cytochrome C) with ADP present to estimate State 3 respiration.

We observed significant increases in complex I-driven respiration in brain and skeletal muscle mitochondria isolated from MTD-TFAM treated mice (Figure 7, bottom). We observed variable and non-significant increases in respiration among the mitochondrial preparations from different organs and other ETC complex substrates in the MTD-TFAM treated mice (Figure 7, bottom).

4. Discussion

In this study, we have shown that the naturally occurring TFAM protein which is essential for mtDNA expression and replication can be engineered with a protein transduction domain and

mitochondrial localization signal (“MTD-TFAM”) so as to be able to enter rapidly into the mitochondrial compartment of cells. After developing a scalable production procedure for this recombinant protein, we found that incubation for only a few hours with MTD-TFAM, followed by return of cells to regular culture medium, substantially and reversibly increased cell respiration after an interval of ~2 weeks. We observed no significant or consistent changes in mtDNA levels based on qPCR analysis for several mtDNA sequences but found increases in mitochondrial gene expression (cDNA) levels that were greatest at the early time points after MTD-TFAM treatments. Multiple nuclear genome-encoded and one mitochondrial genome-encoded ETC proteins also increased reversibly after MTD-TFAM exposure of cells. MTD-TFAM injections into mice increased motor endurance and complex I-mediated respiration in brain and skeletal muscle, post-mitotic tissues commonly involved in mitochondrial diseases.

Our *in vivo* findings with weekly single MTD-TFAM injections into mice contrast with a study of human TFAM expression in mice where mtDNA copy number increased without change in respiratory capacity (Ekstrand, Falkenberg et al. 2004). In contrast, human TFAM expression in mice prevented loss of mitochondrial respiratory capacity in experimental myocardial infarction (Ikeuchi, Matsusaka et al. 2005). While our preliminary findings are encouraging for the therapeutic potential of systemically delivered MTD-TFAM to increase mitochondrial function *in vivo*, much remains to be explored in terms of whether human TFAM can reliably stimulate mouse mtDNA transcription, and the dose-response, timing and reversibility of the increased mitochondrial respiration we observed.

Most interestingly, we also found evidence in the cell culture experiments for a stimulation of mitochondrial biogenesis based on the increase in mitofilin/beta-actin ratios and increases in multiple ETC proteins coded for by the nuclear genome. These findings suggest that MTD-TFAM exposure is capable of triggering in cells a more substantial transcriptional response beyond that associated only with mtDNA gene expression. If true, the mechanisms for increasing mitochondrial biogenesis are not yet known.

These encouraging initial results show that the human mitochondrial genome can now be manipulated from outside the cell to change expression so as to increase mitochondrial respiration, arguably the most essential physiological role of mitochondria. However, many important questions remain to be answered about this technology and its mechanisms and potential therapeutic applications.

First, it is not known if exogenous MTD-TFAM that has migrated to the mitochondrial compartment has the same intra-mitochondrial localization as endogenous TFAM, which is believed to complex with mtDNA and multiple other proteins in mtDNA-protein complexes known as nucleoids (Wang and Bogenhagen 2006; Holt, He et al. 2007; Kang, Kim et al. 2007; Kucej and Butow 2007; Bogenhagen, Rousseau et al. 2008). Our observations of increased mitochondrial gene expression after exposure to MTD-TFAM suggest that exogenous MTD-TFAM, which would be cleaved to native TFAM after mitochondrial importation, is capable of entering the matrix and nucleoids and positively regulating mitochondrial transcription and replication. Whether this occurs remains to be determined.

Second, since MTD-TFAM enters the mitochondrial compartment within minutes, based on colocalization observations through confocal microscopy, it is not obvious why there is a 1–2 week interval between MTD-TFAM exposure and increases in mitochondrial gene expression and respiration. Studies to determine more precisely the time courses of MTD-TFAM stimulation of mtDNA replication and gene expression are underway to address this question.

Third, our findings of an apparent global increase in mitochondrial ETC protein expression were unexpected. We have also observed the same phenomenon in cybrid cells made from mtDNA of patients with Parkinson’s disease that were treated with MTD-TFAM and examined

10–11 weeks later (Keeney, et al, unpublished data). Further investigation is required on how exposure to a single mtDNA transcription factor can potentially activate a complex mitochondrial biogenesis program.

These initial observations demonstrate that MTD-TFAM can be produced in a scalable manner in quantities sufficient for *in vivo* studies, and that MTD-TFAM alters mitochondrial respiratory physiology *in vitro* and *in vivo*. Much remains to be characterized about the mechanisms underlying our observations, and the therapeutic potential of MTD-TFAM for treating diseases associated with bioenergetic deficiency is worthy of further investigation. More importantly, our findings show that the mitochondrial genome is no longer an isolated site and can be manipulated from outside the cell with targeted protein transduction technology.

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Figure 1.

(a) schematic of MTD-TFAM. PTD=11 arginine protein transduction domain; HA=hemagglutinin epitope; SOD2=mitochondrial localization sequence for matrix enzyme SOD2; HMG=high mobility group domain. (b) (left) Coumassie stained gel showing purification of recombinant MTD-TFAM. Lanes 1=SUMO-TFAM; 2= after removal of SUMO; 3, 4= dialysis; 5, 6=further column purification. (right) agarose gel EMSA of circular human mtDNA incubated with increasing amounts of purified MTD-TFAM. (c) MTD-TFAM was labeled with Alexa488 and incubated with LHON cybrid cells where mitochondria were stained with MitoTracker Red. Shown are images obtained 39 minutes after exposing the cells to Alexa488-MTD-TFAM.

Figure 2.

Effects of MTD-TFAM on respiration of intact cells metabolizing glucose. In three independent, consecutive experiments G11778A LHON cells were incubated with 32 ng of MTD-TFAM in 4 ml of DMEM media (or DMEM media with buffer salts) for 4 hours, then rinsed and passed in parallel in regular DMEM growth media. At varying passages after MTD-TFAM incubation, aliquots of cells were harvested with trypsin and utilized for intact cell respiration at densities of 4.5 million live cells/ml. Shown are mean $\pm$ SEM basal respiration rates in glucose for CTL or MTD-TFAM exposed cells. P1=combined CTL respirations (n=8). P2, P3, P4= passage numbers after initial treatment with MTD-TFAM (n=3 each) or buffer CTL (n=3 for P2, P4; n=2 for P3). One-way parametric ANOVA across passage number for MTD-TFAM treatment $p=0.0044$.

Figure 3. Bennett MTD-TFAM and mitochondrial respiration

Time course of effects of MTD-TFAM exposure on mitochondrial DNA copy number. Aliquots of cells used for respiration experiments were extracted for genomic DNA and analyzed for mtDNA copy number using qPCR and primers that amplified portions of the D-loop and 12S rRNA, ND2, ND4, and CO3 genes. Shown are averages of all determinations for each sample. Data are presented as mean $\pm$ SEM of CTL values at each time point. One-way ANOVA was not significant ($P=0.56$).

Figure 4. Bennett MTD-TFAM and Mitochondrial Respiration

Effects of MTD-TFAM exposure on mitochondrial gene expression. Aliquots of cells used for respiration experiments were extracted for RNA; 1 ug RNA was reverse transcribed to cDNA and assayed using qPCR and multiplex TaqMan probes for 12S rRNA, ND2, ND4 and CO3 genes with a linear complete mtDNA PCR product serving as external standard. Shown are 12S rRNA normalized mean $\pm$ SEM copy numbers (ND2, ND4, CO3) for MTD-TFAM expressed as percentages of CTL at each time point. One-way ANOVA's showed $P=0.061$ for ND2 and $P=0.095$ for ND4.

Figure 5. Bennett MTD-TFAM and Mitochondrial Respiration
Effects of MTD-TFAM exposure on electron transport chain protein levels. Aliquots of cells from the third MTD-TFAM experiment were analyzed for ETC proteins using a panel of mouse monoclonal antibodies from MitoSciences. Beta actin served as loading control. Shown on the left are blot images for the complex I antibodies (top) and complexes II-V (bottom). Shown on the right are quantitations of Western blot data from for the cells exposed to MTD-TFAM and expressed as percentage of buffer CTL.

Figure 6.

Exposure to MTD-TFAM does not alter ETC macrocomplex assembly. Shown are confocal images of LHON cells without and with MTD-TFAM exposure that were immunostained with MitoSciences antibodies against complex I and complex V subunits. There were no differences in the very high percentages of cells showing positive staining for both complex subunits.

Figure 7.

Effects of MTD-TFAM injected *in vivo* on motor endurance and mitochondrial respiration in mouse organs. MTD-TFAM was dialyzed against 5% glycerol in PBS, concentrated to ~0.5 ml using an Amicon filter and injected IV once a week for 4 weeks. Mice underwent motor endurance training and testing as described in Methods. One week after the last (fourth) injection mice were sacrificed and organs from MTD-TFAM or buffer CTL injected mice were processed in equal weights in parallel to make P2 pellets, which were studied for substrate-specific State 3 (+ADP) respiration. Equal weights of tissue were studied in each respiration chamber. (a) Shown are mean $\pm$ SEM times on the rotarod at 30 rpm for CTL and MTD-TFAM treated mice. (* $P < 0.05$ by t-test compared to CTL). (b) Shown are mean $\pm$ SEM respiration rates for MTD-TFAM and CTL injected mice for brain, heart and skeletal muscle

mitochondria expressed as pmol O₂/ml-sec. CI=complex I (rotenone-sensitive malate/glutamate); CII=complex II (antimycinA/myxothiazole-sensitive succinate); CIV=complex IV (azide-sensitive ascorbate/TMPD/cyt C). (*P<0.05 by t-test compared to CTL).

Table 1

Effects of MTD-TFAM Treatment on Respiratory Parameters of G11778A LHON Cybrid Cells

	Buffer CTL	MTD-TFAM treated
Respiratory control ratio (RCtIR)	3.84 $\pm$ 0.46 (14)	3.51 $\pm$ 0.33 (11)
Respiratory coupling ratio (RCplR)	2.75 $\pm$ 0.24 (14)	2.70 $\pm$ 0.22 (12)
Uncoupling ratio (UnCplR)	1.36 $\pm$ 0.06 (14)	1.35 $\pm$ 0.03 (11)

Shown are mean $\pm$ SEM values (number of determinations) for LHON cybrid cells exposed to buffer salts or MTD-TFAM and studied at different passages. RCtIR= FCCP/oligomycin; RCplR= basal/oligomycin; UnCplR= FCCP/basal.