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Small mitochondrial-targeted RNAs modulate endogenous mitochondrial protein expression *in vivo*

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ABSTRACT

Endogenous mitochondrial genes encode critical oxidative phosphorylation components and their mutation results in a set of disorders known collectively as mitochondrial encephalomyopathies. There is intensive interest in modulating mitochondrial function as organelle dysfunction has been associated with numerous disease states. Proteins encoded by the mitochondrial genome cannot be genetically manipulated by current techniques. Here we report the development of a *mitochondrial-targeted RNA expression system* (*mtTRES*) utilizing distinct non-coding leader sequences (NCLs) and enabling *in vivo* expression of small mitochondrial-targeted RNAs. *mtTRES* expressing small chimeric antisense RNAs was used as translational inhibitors (TLIs) to target endogenous mitochondrial protein expression *in vivo*. By utilizing chimeric antisense RNA we successfully modulate expression of two mitochondrially-encoded proteins, ATP6 and COXII, and demonstrate the utility of this system *in vivo* and in human cells. This technique has important and obvious research and clinical implications.

Introduction

Mutations in the mitochondrial genome cause a set of devastating disease conditions categorized as primary respiratory chain diseases, also known as mitochondrial encephalomyopathies (MEs) (DiMauro and Schon, 2003). Mitochondrial gene therapy has been proposed as a treatment for ME, however, this approach remains controversial as there are limited preclinical data demonstrating efficacy and evidence suggesting that this approach may have significant limitations (Bokori-Brown and Holt, 2006; Manfredi et al., 2002; Perales-Clemente et al., 2011).

Endogenously encoded mitochondrial proteins function within large well-characterized respiratory complexes that perform oxidative phosphorylation (OXPHOS). The mitochondrial genome is known to harbor hundreds of pathogenic mutations, including ones affecting all of the tRNA genes and over 260 distinct coding mutations. The vast majority of protein-coding gene mutations associated with human mitochondrial disease are missense mutations, accounting for ~225 of the pathogenic mitochondrial mutations (www.mitomap.org), implying that mutant protein is usually capable of being expressed in the disease state. We previously discovered and characterized a *Drosophila* model of ME with an endogenous missense mutation in the *ATP6* gene affecting the F_1F_o-ATP synthase (complex V) (Celotto et al., 2006a; Palladino, 2010). Twenty-one distinct human missense mutations exist within the *ATP6* gene, fourteen of which have been shown to cause human MEs including Familial Bilateral Striatal Necrosis (FBSN), Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP), or Maternally Inherited Leigh's Syndrome (MILS) (D'Aurelio et al., 2010; Noji et al., 1997; Stock et al., 1999). *ATP6*^{1} mutant flies contain a missense mutation with high mutant heteroplasmy and exhibit phenotypes analogous to human symptoms including locomotor and progressive neural dysfunction, seizures, myodegeneration, and reduced longevity (Palladino, 2010).

Competition with mutant protein for incorporation into mature respiratory complexes is likely a major obstacle to a viable mitochondrial gene therapy: a fact that has largely been ignored. This competition may explain the controversial allotopic expression results and remains a formidable obstacle to the treatment of MEs resulting from any endogenous mitochondrial missense mutation. A method to specifically reduce expression of mitochondrial-encoded genes is not known.

Several RNAs are naturally imported into the mitochondria from the cytoplasm and detailed studies have provided critical insight into the import process and import substrates (Lithgow and Schneider, 2010; Schneider and Marechal-Drouard, 2000; Tarassov et al., 2007). Although the exact mechanism of RNA import into mitochondria is unknown, several pathways have been suggested to mediate mitochondrial RNA import (Mahapatra and Adhya, 1996; Schneider, 2011; Wang et al., 2010). We have identified a nuclear encoded mitochondrial *5S rRNA* isoform and engineered a novel vector to express small RNAs *in vivo*. We developed a mitochondrial-targeted translational inhibition (TLI) approach using small chimeric RNAs to regulate endogenous mitochondrial protein expression. Here we demonstrate the efficacy of mitochondrial-

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targeted TLIs by targeting two distinct loci encoding essential proteins of two different OXPHOS complexes, one *in vivo* and the other *in vitro*. The ability to selectively modulate mitochondrial protein expression in animals represents an important technological advance with obvious research and clinical applications.

Material and methods

Engineering mtTRES and mtTRES-TLI constructs

The *mtTRES* vector was created using the available *pUAST-attB* vector as a backbone (Bischof et al., 2007). A StuI site was added by site directed mutagenesis 5′ to the *attP* integration site using Quick Change Lightning (Invitrogen, USA). The *5S rRNA RNAPIII* promoter (AE013599.4) and termination (AE013599.4) sequences were PCR amplified from wild type *Drosophila* genomic DNA and directionally inserted using standard cloning methods and the HindIII–EcoRI and StuI–KpnI cloning sites, respectively. For the mammalian *mtTRES* vector the human *U6* promoter (NT_010194.17) was PCR amplified from p*Silencer* 2.1 (Invitrogen, USA), purified and inserted in place of the fly *RNAP III* promoter. The EcoRI–EagI cloning sites were used to insert NCLs. The *5S rRNA*^{mt} variant was identified as the most abundant mitochondrial isoform by clonal analyses (88%) from three independent cloning events and sequence analysis of 135 clones. The *5S rRNA*^{mt} was the major mitochondrial isoform in all three independent clonal populations (Supplementary material: Fig. S1 and GenBank: CR33451). The *5S rRNA*^{mt} sequence was synthesized with flanking EcoRI–EagI cloning sites (GeneWiz, South Plainfield NJ, USA). The *MRP* and *RNAseP* (*RNP*) oligonucleotides were annealed and directionally cloned into EcoRI–EagI cloning sites using published sequences (Wang et al., 2012). TLI complementary sequences were synthesized as oligonucleotides, annealed and directionally cloned into EagI–KpnI cloning sites. *TLI-5S*^{mt}*::ATP6(a)* is 25 bases long, whereas *TLI-5S*^{mt}*::ATP6(b)* is 26 nucleotides in length and the complementary region is shifted 3 nucleotides 5′. All oligonucleotides were commercially synthesized by IDT (Coralville IA, USA). The final constructs were sequence verified (GeneWiz, South Plainfield NJ, USA).

Drosophila transgenesis, longevity and locomotor assays

mtTRES vectors allow site-directed PhiC31-mediated *attP/B* transgenesis. We used the *VK00027 attP* insertion site and flies bearing the *VK27 attP* chromosome are the control for all transgenic experiments. DNA injections were performed by Genetic Services (Cambridge MA, USA) and successful transgenesis events were identified using *white*$^{mc+}$. Homozygous transgenic strains were tested. Previously established methods were used to test longevity (Palladino et al., 2003) and locomotor assays (Fergestad et al., 2006; Fergestad et al., 2008).

Western blotting and antisera production

Standard methods were used for western blot analyses (Celotto et al., 2012). Briefly, flies were carbon dioxide anesthetized and snap frozen in liquid nitrogen. Thoraces from 8 flies were dissected and homogenized in sample buffer (125 μl), heated at 95 °C for 5 min, and loaded into the wells of an SDS-PAGE gel. Antisera was generated to fly ATP6 protein using purified HKEFKTLLGPSGHNGS peptide (hc17), immunized New Zealand rabbits and antigen affinity purification (NeoBioSci, Cambridge MA, USA). Anti-ATP6 antibody recognition specificity of hc17 peptide was confirmed by Southern Blot and ELISA (by NeoBioSci, Cambridge MA, USA). Western blotting identifies a single ~25 kDa protein that enriches with mitochondria. Competitive ELISA (kit by Cell Biolabs Inc., USA) using fly lysates and increasing concentrations of hc17 peptide was used to further validate the specificity of the anti-ATP6 antibody (Supplementary material, Fig. S2). ATP6 antisera are used at 1:2000. Anti-COXII antibodies (Proteintech, Chicago IL, USA) and anti-SOD2 antibodies (LSBio, Seattle WA, USA) were used at 1:2500 and 1:2000, respectively. Anti-ATP-alpha (a5-c antibody, Developmental Studies Hybridoma Bank, University of Iowa, USA) was used as a loading control. ATPalpha is a nuclear encoded plasma membrane protein (the catalytic subunit of the Na^+/K^+ ATPase). For HeLa cells, 1×10^6 cells were electroporated and harvested after ~48–72 h for western blot. GAPDH (1:3000) (Abcam, USA) was used as a loading control. Secondary detection was performed using anti-rabbit (1:4000) (Biorad, USA) and anti-mouse (1:10000) (Biorad, USA) HRP conjugated antibodies. For all Western blots sub-saturation images have been quantified. In some cases, darker exposures of the quantified images are used in the figures.

RNA isolation and quantitative RT-PCR

RNA was extracted from 12 whole flies, using 250 μl Trizol (Invitrogen, San Diego, USA) and the RNeasy mini kit (Qiagen, Valencia, USA). RNA was eluted in 100 μl dH_2O and quantified. 5 μg RNA was used to perform a reverse transcription reaction (Superscript RT, Invitrogen). Quantitative Real-Time PCR [Mx3000P QPCR System, Stratagene] was performed using standard techniques with normalization to *RP49* expression (Celotto et al., 2006b). Only DNA-free cDNA samples were used. In a total reaction of 25 μl, 12.5 μl 2X-SYBR Green Supermix (Qiagen, Valencia, USA), 2 μl of cDNA and 400 nM each of forward and reverse primers (*ATP6*, and *COXII*) were used. Fold change (FC) was determined using the equation, $FC = 2^{-\Delta(\Delta Ct)}$. All QPCR experiments were performed with four biological replicates and the data were normalized to mRNA expression levels of *RP49*.

Isolation of mitochondria from HeLa cells

Mitochondria were isolated using a standard differential centrifugation procedure. In short, 24 million cells were trypsinized and homogenized by a Dounce homogenizer. Nuclear fraction was pelleted at 1000 *g* for 15 min. The supernatant was then centrifuged at 10,000 *g* for 15 min. The pellet contained the enriched mitochondria.

In vitro transcription and radiolabeling

Primers were designed to amplify *5S*^{mt} and *TLI-5S*^{mt}*::COXII* sequences from previously engineered *mtTRES-5S*^{mt} and *mtTRES-5S*^{mt}*-TLI::COXII* plasmids. *T7 + 5s_For* (TAATACGACTCACTATAGGGGCCAACGACCATAC CACGCTGAATAC) and *5s_Rev* (AGGCCAACAACACGCGGT GTTC) primers were used for 5S DNA amplification. For *TLI-5S*^{mt}*::COXII*, *T7 + 5s_For* and *COX2_Rev* (TCCAAAAAATCTTAATGGCACATGCAGC) primers were used.

Using a Thermo Scientific TranscriptAid T7 High Yield Transcription Kit and [α-32P] adenosine 5′-triphosphate (MP Biomedicals) *in vitro* transcription was performed, as per the manufacturer's instructions. Unincorporated [α-32P] ATP was removed using NucAway™ Spin Columns (Ambion Inc. Austin, Texas). Specific activities of radiolabeled RNA products were quantified by a LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter) and equal amounts were used in the mitochondrial import assay.

Mitochondrial RNA import assay

Mitochondrial RNA import assay was modified from Bhattacharyya et al. (2002); Magalhaes et al. (1998); and Wang et al. (2012). In short, mitochondrial pellets were suspended with RNA probes in the import buffer (200 μl final volume) containing 0.25 M sucrose, 2 mM KH_2PO_4, 50 mM KCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 5 mM L-methionine, 1 mg/ml BSA, 5 mM ATP, 2 mM DTT, 20 mM succinate, and 50 mM HEPES, [pH 7.1]. The mixture was incubated for 20–30 min at room temperature. Mitochondria were spun at 11,000 *g* for 5 min and washed once with wash buffer (0.6 M sorbitol, 20 mM Tris, [pH 8.0]). To remove