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Development of Mitochondrial Gene Replacement Therapy

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Many "classic" mitochondrial diseases have been described that arise from single homoplasmic mutations in mitochondrial DNA (mtDNA). These diseases typically affect nonmitotic tissues (brain, retina, muscle), present with variable phenotypes, can appear sporadically, and are untreatable. Evolving evidence implicates mtDNA abnormalities in diseases such as Alzheimer's, Parkinson's, and type II diabetes, but specific causal mutations for these conditions remain to be defined. Understanding the mtDNA genotype–phenotype relationships and developing specific treatment for mtDNA-based diseases is hampered by inability to manipulate the mitochondrial genome. We present a novel protein transduction technology ("protofection") that allows insertion and expression of the human mitochondrial genome into mitochondria of living cells. With protofection, the mitochondrial genotype can be altered, or exogenous genes can be introduced to be expressed and either retained in mitochondria or be directed to other organelles. Protofection also delivers mtDNA in vivo, opening the way to rational development of mitochondrial gene replacement therapy of mtDNA-based diseases.

KEY WORDS: Mitochondria; mitochondrial DNA; protein transduction; mitochondrial genome.

Known point mutations in mitochondrial DNA (mtDNA) are relatively rare and associated with a wide variety of "mitochondrial" diseases affecting brain, retina, optic nerve, muscle, heart, endocrine organs, and liver (Graff *et al*., 2002; McFarland *et al*., 2002; Schapira, 2000; Schmiedel *et al*., 2003; Zeviani and Carelli, 2003). These conditions are notable for delayed expression of variable phenotypes, and the underlying mechanisms of cellular pathophysiology remain unclear. Because the mitochondrial genome codes for only 13 out of the ~90 electron transport proteins and the hundreds or probably >1000 of all mitochondrial proteins, it remains challenging to formulate how one or more mutations in this small genome can have such profound physiological effects. In addition, mtDNA deletions accumulate with aging and may contribute to bioenergetic failure of older muscle fibers and neurons, resulting in sarcopenia and degenerative diseases such as Alzheimer's and Parkinson's (Wallace, 2001). In all of these conditions, understanding the dynamics of mitochondrial genome replication and expression in individual cells will provide insight into disease pathophysiology. For those conditions where mitochondrial genome mutations are causal for disease expression, supplementation with normal mitochondrial genomes, or ideally replacement of defective with normal mitochondrial genomes, has great therapeutic potential. However, carrying out these critical studies has been hampered by limitations in manipulating in situ the mitochondrial genome inside mitochondria of living cells.

We have recently developed novel technologies to remove and replace the human mitochondrial genome inside mitochondria of human cells. Using lambda phage virus as a transfection vector and lambdaphage receptor targeted to mitochondria, we demonstrated that the entire human mitochondrial genome with an inserted mitochondrial-specific GFP reporter can be transfected into ρ_0 mitochondria (mitochondria without any mtDNA) of cells. In this process ("mitofection"), mtDNA replication, mitochondrial GFP expression, and restoration of bioenergetic function occur rapidly over several days (Khan *et al*., in preparation). Subsequently, the original "mitofection" technology has been significantly improved by the development of an engineered protein transduction system

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to transport mtDNA across cell membranes and target it to mitochondria. This technology ("protofection") introduces mtDNA into mitochondria within minutes, restores bioenergetic activity of ρ_0 cells within 1–2 days, and is active in vivo in animals. Protofection can be used to deliver the entire normal mitochondrial genome, or PCR-generated fragments, mutations, or deletions. Third, silencing the mtDNA polymerase (POL-γ) by RNA interference results in complete loss of detectable POL-γ activity and detectable mtDNA within 72 h with recovery of activity in 5–7 days. This allows creation of ρ_0 cells quickly without the use of mutagens or reverse transcriptase inhibitors and is applicable to nondividing cells such as neurons. The combined use of RNA interference to silence genes critical for mtDNA replication, and "protofection" to introduce healthy mitochondrial genomes into mitochondria of living cells, sets the stage for the realistic possibility for mitochondrial gene therapy of a wide variety of conditions.

A GENERAL INTRODUCTION TO MITOCHONDRIA AND THEIR GENES

Although the origins of modern mitochondria are not known with certainty, the endosymbiotic theory proposed originally by Margulis (Margulis, 2001) remains one of the most cogent. In this construct, modern mitochondria developed from bacterial precursors who inhabited early prokaryotes and provided a mechanism to detoxify oxygen. These bacterial invaders established a complex symbiotic relationship with their hosts that included improved metabolic efficiency and sharing of genomic responsibilities, with gradual transfer of mitochondrial genomic responsibilities to the host nucleus (Gray *et al*., 2001). Early in evolution, the mitochondrial genome of eukaryotes thus shrank from its large bacterial predecessor to a much-reduced size (366.9 kB in *A. thalania*; 85.8 kB in *S. cerevisiae*; 13.8 kB in *C. elegans*; 16.5 kB in *H. sapiens*). The human mitochondrial genome, similar to that of other mammals, is intron-less, circular, and codes for 13 electron transport proteins, 2 ribosomal RNAs and 22 tRNAs. The mitochondrial genetic code(m) is similar to, but not identical with, the nuclear code (n), differing in four codons (*AUA* = Ile (n), Met (m); *UGA* = Term (n), Trp(m); *AGA*, *AGG* = Arg (n), Term (m)).

The total number of mitochondrial proteins is not accurately known, but certainly numbers are in the hundreds and possibly thousands (Lescuyer *et al*., 2003; Sickman *et al*., 2003). The vast majority of mammalian mitochondrial proteins are thus coded by nuclear genes and targeted to mitochondria by N-terminal mitochondrial localization sequences (MLS). The MLS-targeted proteins are imported into mitochondria in an energy-dependent manner by membrane translocase complexes, known as the translocase of outer membrane (TOM) and translocase of inner membrane (TIM). Following importation, MLS is removed and proteins are incorporated into electron transport chain, outer or inner membrane, intermembrane space or matrix.

The myriads of critical functions performed by mitochondria, including both the historically first described role of respiration and ATP synthesis, and now including the participation in calcium signaling and buffering and control of cell death activation (Ganitkevich, 2003; Hajnoczky *et al*., 2003a, 2003b; Orrenius *et al*., 2003; Smaili *et al*., 2003; Vandecasteele *et al*., 2001), require coordinated expression and stoichiometrically regulated importation and incorporation of the hundreds (at least) of nuclear genome-encoded proteins of diverse functions and expression of 13 mitochondrial genome-encoded proteins devoted to electron transport chain function. Considering the complexity of this critical organelle, it is remarkable that it consistently is assembled in working order, repaired efficiently, and passed on regularly to progeny cells.

TRANSDUCTION DOMAINS FOR DELIVERY OF THERAPEUTIC PROTEINS

The blossoming field of genomics, through utilization of advanced transfection protocols and gene microarrays, is leading researchers to the discovery of many novel therapeutic proteins. However, delivery of these large proteins across cell membranes, into senescent cells and across the blood–brain barrier (BBB), presents a substantial hurdle to utilizing this method of therapeutic intervention. With the introduction of Protein Transduction Domain- (PTD) Protein fusions, the hurdle is diminishing and appears to be increasingly surmountable. These small regions of proteins are able to cross the cell membrane in a receptor-independent mechanism.

Although several of these PTDs have been documented, the two most commonly employed PTDs are derived from the TAT protein from HIV and Antennapedia transcription factor from Drosophila, whose PTD is known as Penetratin (Derossi *et al*., 1994).

TAT protein consists of 86 amino acids and is involved in the replication of HIV-1. The TAT PTD consists of an 11 amino acid sequence domain of the parent protein that appears to be critical for uptake (Vives *et al*., 1997). In the current literature TAT has been favored for fusion to proteins of interest for cellular import. Several modifications to TAT, including substitutions of glutamine to alanine (Q $\rightarrow$ A), have also demonstrated an increase in

cellular uptake anywhere from 90% (Wender *et al*., 2000) to up to 33-fold (Ho *et al*., 2001) in mammalian cells.

PROPERTIES OF PROTEIN TRANSDUCTION DOMAINS

Highly Efficient Uptake

Intracellular delivery of various therapeutic proteins involving TAT-PTD fusions has proven to be quite effective. This type of fusion protein was recently utilized in the delivery of biologically active antioxidant enzymes such as catalase (CAT). When exposed to H_2O_2, Hela cells demonstrated a 90% increase in cell viability as compared to controls (Jin *et al*., 2001).

Kinetic studies on the uptake of PTD have shown that an entire cell population can reach maximum uptake of PTD within as little as 30 s to 5 min of initial exposure (Ho *et al*., 2001). PTDs provide for rapid uptake of attached proteins, although these fusion proteins can vary in uptake in a tissue-specific manner and also depend on the structure and size of the protein fused.

Stability of transduced fusion proteins into cultured HeLa cells demonstrated a peak concentration at approximately 2 h of incubation with a steady decrease up to 72 h later (Jin *et al*., 2001). Tat-PTD has also been fused to Angiotensin II type I receptor (AT1R) to investigate Tat-PTD fusion's transduction efficacy and functionality in neurons. Neuronal cultures isolated from the hypothalamus and brain stem of 1-day-old Wistar–Kyoto rats (WKY) were incubated with 300 μg/mL of the recombinant protein and peak fluorescence was noted after 30 min of incubation with initial fluorescence recorded within minutes (Hammond *et al*., 2001). These are just a few of the many examples of PTD-linked proteins that demonstrate the ability of PTD to rapidly transduce cells.

PTD Fusion Proteins Allow Delivery of Large Cargo Across BBB

Viral-mediated delivery of DNA for the production of proteins is a potentially promising technology, but it is not well suited for certain conditions, as the delivery of genes via viral vector systems is time-consuming and often presents problems of immunogenicity. Protein synthesis can also be downregulated in areas of the brain which have undergone insult, such as ischemia, as well as having undergone pathophysiological change, as is seen in MELAS brain.

Another problem with the therapeutic delivery of proteins to neuronal tissues is BBB. BBB is composed of specialized endothelial cells and tight junctions, which make delivery of even low-molecular-weight proteins, such as NGF (26-kDa dimer), a very difficult and low-efficiency process.

Protein transduction domains present a new and exciting approach to the delivery of biologically active proteins across BBB. Kilic *et al*. (2003) recently demonstrated the ability of a Tat-GDNF (Glial cell line-derived neurotrophic factor) protein to cross BBB. Delivery of Tat-GDNF fusion prevented both apoptotic and necrotic injury after short- and long-term ischemia in rats. The method of application for the Tat-GDNF recombinant protein was intravenous infusion—requiring no surgical interventions.

Cao *et al*. (2002) further demonstrated the ability of PTD proteins to cross BBB, utilizing a Bcl-xL PTD fusion. The aim of this study was to introduce Bcl-XI, a known neuronal antiapoptotic factor, to provide neuroprotection during ischemia in the murine model of focal ischemia/reperfusion. Intraperitoneal injection of PTD-HA-Bcl-xL into mice demonstrated the ability of fusion proteins to cross BBB. The protein fusion was able to decrease cerebral infarction up to 40% upon initiation of cerebral ischemia (Cao *et al*., 2002).

A similar study utilized a Bcl-x mutant (FNK), with increased antiapoptotic activity, to protect SH-SY5Y neuroblastoma cells in vitro when exposed to staurosporine-induced apoptosis and glutamate-induced excitotoxicity. This PTD-FNK fusion was also injected i.p. into gerbils and prevented delayed neuronal death in the hippocampus caused by transient global ischemia (Asoh *et al*., 2002).

Cytotoxicity and Immunogenicity

A key requirement for any therapeutic intervention with a PTD fusion protein is that no untoward changes in normal cell physiology or function occur. Brain microvascular endothelial cells (BMEC) exposed to Tat demonstrate marked increased levels of cellular oxidative stress, decreased levels of intracellular glutathione, and activated DNA binding activity and transactivation of NF-κB and AP-1 (Toborek *et al*., 2003).

The protein transduction domain utilized by us is an 11 amino acid sequence that represents a poly-Arginine stretch shown to be higher in transduction efficiency that PTD of the Tat-HIV-1 protein. Although the 11 amino acid PTD sequence is similar to the small motif of the parent Tat-HIV-1 protein, the concern that it may be sufficient to elicit similar cytotoxicity when introduced into cell culture or animal models is noteworthy and has been addressed in my numerous publications since the discovery of PTDs. The literature to date indicates that the Tat-PTD can transduce proteins of interest to nearly 100%

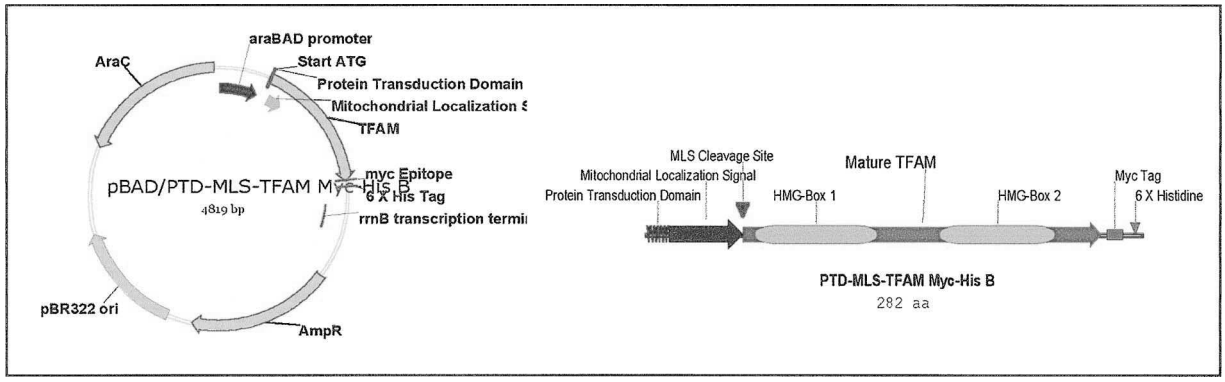

Fig. 1. Plasmid design (left) and protein structure (right) for TFAM with a PTD domain followed by an MLS.

of a cell population without exhibiting cytotoxic effects. Many groups, such as Cao *et al.* (discussed in capability of PTD to cross BBB), have also gone so far to prove the therapeutic benefits and the cell-protective capabilities that PTD-linked proteins possess. No cytotoxicity was reported upon treatment with the PTD fusion (Dolgilevich *et al.*, 2002). Jin *et al.* (2001) utilized a Tat PTD-linked SOD (super oxide dismutase) and a Tat PTD-linked hCat (human catalase) to demonstrate that the transduced fusion proteins remained enzymatically stable for 60 h. The fusion protein did not elicit any cellular toxicity and was able to increase HeLa cell viability up to 90% upon exposure to H_2O_2. Leifert *et al.* (2002) also recently reported that full-length proteins attached to the HIV-Tat protein transduction domain neither are transduced between cells nor exhibit enhanced immunogenicity. These experiments, as well as many others in the literature to date, demonstrate the potential therapeutic efficacy of PTD-linked proteins with no found toxicity or increased immunogenicity of the fusion proteins.

Mitochondrial Localization of PTD-Fusion Proteins

Del Gazio and Payne (2003) have characterized the feasibility of using protein transduction to target proteins to mitochondria. They found that a mitochondrial localization signal was necessary to enable persistence of the targeted protein inside mitochondria. Also, neither loss of the mitochondrial import machinery nor decrease in $\Delta\Psi M$ inhibited entry and retention of their fusion protein. Finally, in pregnant mice injected with their TAT-mMDH-GFP fusion protein, the protein crossed the placenta and was found in fetal and neonatal pups, indicating that the protein not only crossed multiple membrane barriers but also persisted within mitochondria.

TFAM IS A MITOCHONDRIAL HISTONE

Mitochondrial transcription factor A (TFAM) is a 246 amino acid (~25 kDa) protein first isolated and cloned as a transcription factor for mtDNA (Fisher and Clayton, 1988). It is a member of the High Mobility Group (HMG) of proteins, contains two HMG domains and a 42 amino acid mitochondrial localization sequence, binds to ~25 bp of mtDNA, and is capable of bending and unwinding mtDNA (Parisi *et al.*, 1993; Parisi and Clayton, 1991). Several important TFAM binding regions on mtDNA have been identified, and endogenous mtDNA is bound to ~1000-fold molecular excess of TFAM (Alam *et al.*, 2003). TFAM is critical for mtDNA replication (Larsson *et al.*, 1998) and is controlled by transcription factors such as NRF-1 and NRF-2 known to regulate mitochondrial biogenesis (Choi *et al.*, 2004).

Figure 1 shows the plasmid construction for creation of the recombinant PTD-MLS-TFAM and the resulting molecule. Figure 2 shows successful introduction into

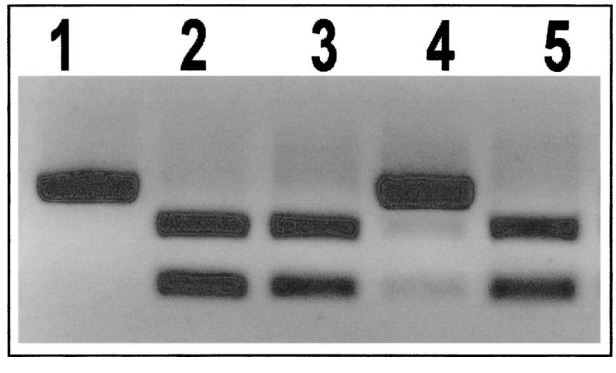

Fig. 2. Agarose gel of PCR products amplifying region around LHON 11778A mutation after SfaN1. Lane 1—LHON Cybrid; Lane 2—Sy5y; Lane 3—ρ_0; Lane 4—LHON mtDNA Protofected ρ_0; Lane 5—Protofected ρ_0 no DNA.

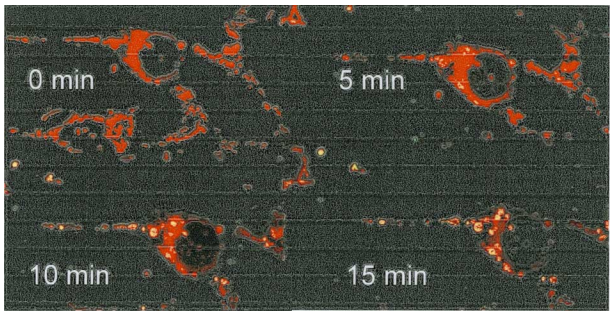

Fig. 3. Time course of Alexa 488 labeled MtDNA complexed with PTD-MLS-TFAM added to Sy5y cells. Red = Mito Tracker Red.

and replication of LHON mtDNA in ρ_0 cells. The LHON 11778A mutation causes loss of the SfaN1 site present in w.t. mtDNA. In ρ_0 cells a similar w.t.-like pseudogene is amplified and cut by SfaN1. Following protofection of LHON mtDNA into ρ_0 and passage through metabolic selection, mainly the introduced LHON mtDNA free of the SfaN1 site is found. Figure 3 shows that w.t. mtDNA that has been labeled with Alexa 488 dye and complexed with PTD-MLS-TFAM rapidly enters mitochondria of SY5Y cells and is concentrated within 15 min. Figure 4 shows

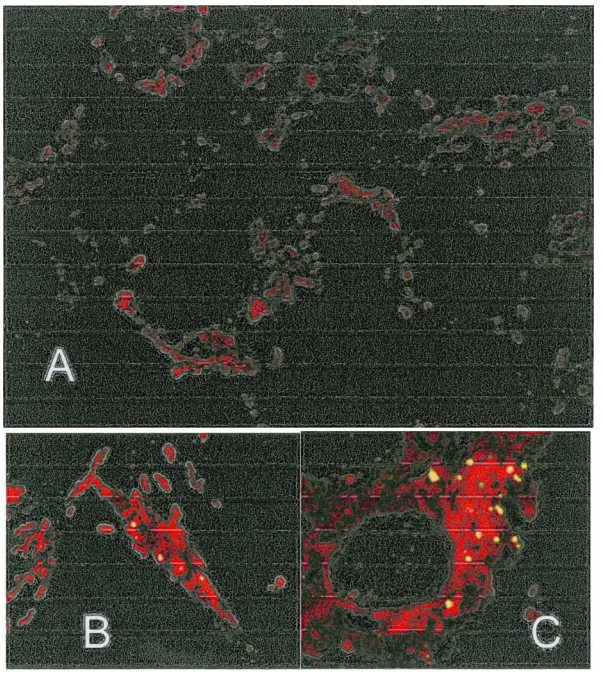

Fig. 4. MtRed and BrdU (FITC) staining of ρ_0 (A), normal SY5Y (B) and ρ_0 16 h after protofection with mtDNA complexed with PTD-MLS-TFAM (C).

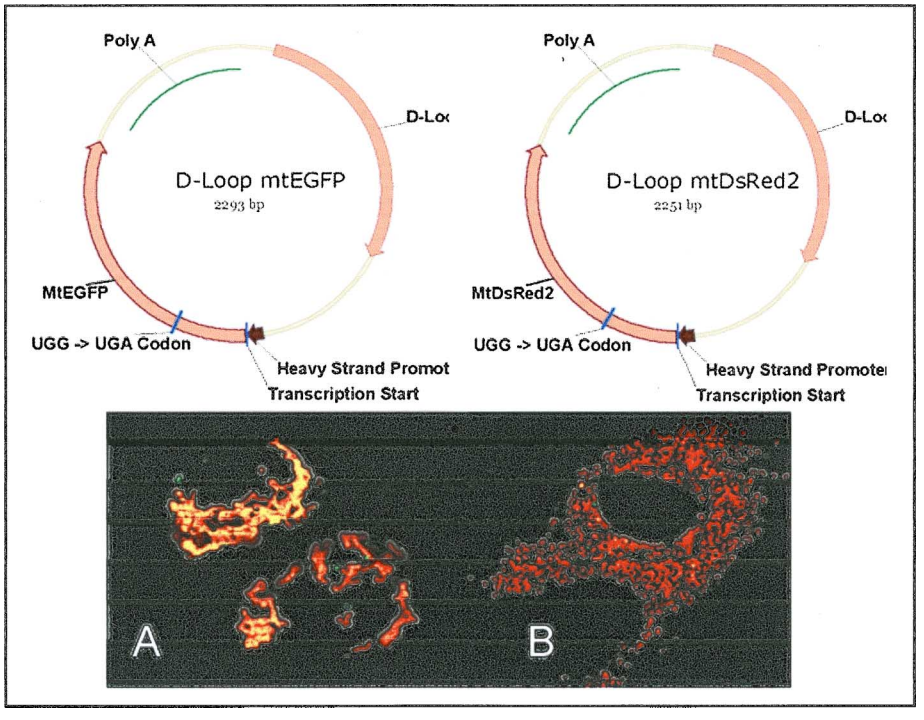

Fig. 5. (top) Constructs for generating D-loop MtEGFP (left) and D-loop MtDsRed2 (right). (bottom). Normal SY5Y cell (A) and human cortical neuron (HCN, B) 24 h after Protofection with D-loop MtEGFP construct and counterstained with Mito Tracker Red.

the rapid restoration of mtDNA replication and bioenergetic function following introduction of w.t. mtDNA by protofection. The top image (A) is of ρ_0 cells stained with Mito Tracker Red (MTRed), to localize mitochondria as a function of their $\Delta\Psi$M, and following incubation for 12 h with BrdU and immunostained for BrdU with FITC. Note the low levels of MTRed accumulation, reflecting low $\Delta\Psi$M, and absence of BrdU staining. Part (B) shows a normal SY5Y cell and part (C) shows a ρ_0 cell 16 h after protofection with PTD-MLS-TFAM complexed with w.t. mtDNA. Note the marked increase in MTRed uptake and BrdU staining.

EXPRESSION OF EXOGENOUS GENES IN MITOCHONDRIA USING PROTOFECTION

Because of our desire to be able to express individual genes inside mitochondria, in addition to the entire mitochondrial genome, we have pursued using Protofection technology to deliver small reporter genes directly to mitochondria. The premise behind the constructs is to place a gene of interest downstream of the mitochondrial D-loop and heavy strand promoter and incorporate a polyA tail. The constructs for D-loop-MtEGFP and D-loop-MtDsRed2 are shown in Fig. 5. Recall that the two reporter fluorescent proteins have been mutated so as to be specific for the mitochondrial translation apparatus. Figure 5 also shows a normal SY5Y cell (A) and a human cortical neuron (B) 24 h after Protofection with D-loop MtEGFP. There is robust mitochondrial GFP signal in mitochondria of SY5Y and several small areas of EGFP signal in the human cortical neurons.

PROTOFECTION TECHNOLOGY AND MITOCHONDRIAL GENE THERAPY

The above results show that PTD-MLS-TFAM protofection technology provides a rapid and efficient approach to providing "healthy" mitochondrial genomes to cells. Theoretically, mitochondrial diseases with homoplasmic mutations can be treated by dilution of the pathogenic mitochondrial genomes with healthy ones. Exogenous individual genes of interest can also be introduced into mitochondria, and with the appropriate codon changes, can be restricted to mitochondrial translation.

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