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EXPERT REVIEW

Transduction of Human Recombinant Proteins into Mitochondria as a Protein Therapeutic Approach for Mitochondrial Disorders

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ABSTRACT Protein therapy is considered an alternative approach to gene therapy for treatment of genetic-metabolic disorders. Human protein therapeutics (PTs), developed via recombinant DNA technology and used for the treatment of these illnesses, act upon membrane-bound receptors to achieve their pharmacological response. On the contrary, proteins that normally act inside the cells cannot be developed as PTs in the conventional way, since they are not able to "cross" the plasma membrane. Furthermore, in mitochondrial disorders, attributed either to depleted or malfunctioned mitochondrial proteins, PTs should also have to reach the subcellular mitochondria to exert their therapeutic potential. Nowadays, there is no effective therapy for mitochondrial disorders. The development of PTs, however, via the Protein Transduction Domain (PTD) technology offered new opportunities for the deliberate delivery of human recombinant proteins inside eukaryotic subcellular organelles. To this end, mitochondrial disorders could be clinically encountered with the delivery of human mitochondrial proteins (engineered via recombinant DNA and PTD technologies) at specific intra-mitochondrial sites to exert their function. Overall, PTD-mediated Protein Replacement Therapy emerges as a suitable model system for the therapeutic approach for mitochondrial disorders.

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ABBREVIATIONS

aa	amino acids
CDS	coding sequence
CNS	central nervous system
COX	Cytochrome *c* Oxidase
E. coli	*Escherichia coli*
ERT	Enzyme Replacement Therapy
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
GMOs	genetically modified organisms
IBs	inclusion bodies
IMS	intermembrane space
IPTG	isopropyl-beta-D-thiogalactopyranoside
L	N-terminal Leader Peptide
LAD	Lipoamide Dehydrogenase
mab	monoclonal antibody
MPP	Mitochondrial Processing Peptidase
mtDNA	mitochondrial DNA
MTS	Mitochondrial Targeting Signal Peptide
NABs	Neutralizing Antibodies
nDNA	muclear DNA
OXPHOS	Oxidative Phosphorylation System
PDHC	Pyruvate Dehydrogenase Complex
PEG	poly(ethylene glycol)
PRT	Protein Replacement Therapy
PTD	Protein Transduction Domain
PTs	Protein Therapeutics
ROS	Reactive Oxygen Species
TAT	a Protein Transduction Domain

TCA Tricarboxylic acid cycle
TFAM Mitochondrial Transcription Factor A

INTRODUCTION

It has been known over the years that the majority of metabolic, genetic, and degenerative diseases represent disorders of tissue homeostasis and cell regulation. In these cases, homeostasis has been broken down either because physiological regulators are not produced at all or produced inadequately. Alternatively, these regulators are mutated and become unable to carry out their intended function (e.g., cell-cell communication, cell replication, signaling, and gene transcription). The establishment of functional genomic technologies in therapeutics has addressed questions concerning the tissue specificity of the protein of interest as well as the level of protein(s) under normal and diseased states (1).

One of the first and best examples of metabolic diseases efficiently treated with a protein therapeutic (PT) was the juvenille diabetes mellitus in 1922 (2). Recombinant DNA technology permitted the expression of human insulin gene in *Escherichia coli (E. coli)* bacteria cells (3). The produced biosynthetic insulin was approved by the FDA in 1982 for the therapy of diabetic patients. The use of insulin to treat human diabetes mellitus suggested that similar pathophysiological disorders could be treated by Protein Replacement Therapy (PRT). Human PTs are either isolated from animal tissues or developed via recombinant DNA technology. Until now, a large number of biopharmaceuticals (recombinant proteins of therapeutic and/or diagnostic value) have been developed via DNA biotechnology (4,5). According to Golan and his colleaques (4), more than 130 PTs are used in clinical practice and classified into four groups: Group I—PTs with enzymatic or regulatory activity; Group II—PTs with special targeting activity; Group III—Protein Vaccines; Group IV—Protein Diagnostics (Table I).

The majority of human PTs act upon membrane-bound receptors to initiate a cell-signaling pathway for exerting their pharmacological response. In contrast, proteins that normally act inside the cells (enzymes, signaling intermediates, or transcription factors) or even inside subcellular organelles cannot be developed to PTs in the conventional way, since they are unable to "cross" the plasma membrane and reach their specific target sites. To this point, the Protein Transduction Domain (PTD) technology, complemented with recombinant DNA technology, has been able to produce PTs for intracellular delivery.

Table I Classification of PTs (4)

Group	Function	PTs (examples)	Clinical application (examples)
I	Enzymatic or regulatory activity	Insulin	Diabetes Mellitus
		Growth Hormone	Growth Hormone Deficiency
		Factor VIII	Haemophilia A
		Erythropoietin (Epo)	Anaemias
		β-Glucocerebrosidase	Gaucher's disease
		α-galactosidase A	Fabry disease
		PEGinterferon-α2a	Chronic hepatitis c (HCV)
		Interferon-β1a	Multiple Sclerosis
		Tissue Plasminogen Activator (tPA)	Myocardial infraction, Acute ischemic stroke
		L-Asparaginase	Acute lymphocytic leukemia
		Lepirudin	Heparin-induced thrombocytopenia (HIT)
II	Special targeting activity	Trastuzumab	Breast cancer
		Infliximab	Rheumatoid arthritis,
		Etanercept	Psoriatic arthritis
		Muromonab-CD3	Renal or hepatic allograft rejection
		Enfuvirtide	Advanced HIV infection
III	Protein vaccines	Hepatitis B surface antigen (HBsAg)	Hepatitis B vaccination
		Human Pappiloma virus (HPV) major capsid Proteins	HPV vaccination
IV	Protein Diagnostics	Recombinant Purified Protein Derivative (DPPD)	Tuberculosis exposure
		Thyroid Stimulating hormone (TSH)	Thyroid diseases
		HIV, HBV, HCV Antigens	HIV, HBV, HCV Infection

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The scope of this article is to review a) the mitochondrial genetic-metabolic disorders and their current therapeutic medical practice; b) the principles of design and production of human recombinant PTs, bearing PTDs; and c) the ability of such PTs to enter mammalian cells and then translocate inside subcellular organelles, like mitochondria. Emphasis is given on the potential application of PTD-mediated PRT as a therapeutic approach for the mitochondrial disorders.

MITOCHONDRIAL GENETIC / METABOLIC DISORDERS

Mitochondria: Basic Characteristics

Mitochondria are unique, semiautonomous, subcellular organelles with distinct morphology. They have an outer (OMM) and an inner (IMM) membrane, which create the two aqueous spaces, the intermembrane space (IMS) and the innermost mitochondrial matrix space. Across the inner membrane, which is folded into the cristae, the five complexes of the oxidative phosphorylation system (OXPHOS) are embedded, while in matrix space the tricarboxylic acid cycle (TCA), the β-oxidation of fatty acids, and the urea cycle take place. Mitochondria, beyond their vital role in bioenergetics of cell, are also involved in the regulation of apoptosis (programmed cell death), redox potential, antiviral signaling as well as calcium homeostasis (6–10).

Mitochondria have been originally derived by the endosymbiosis of aerobic proteobacteria within anaerobic primordial eukaryotic cells. Based on the similarities between bacteria and mitochondria, it was proposed that at some point during the evolution bacterial genes moved into the eukaryote's genome (11,12).

Each mammalian cell has a large number (>100) of mitochondria, and each mitochondrion carries various copies (~2 to 10) of mtDNA molecules. Human mitochondria have their own DNA (mtDNA), a closed, intronless, circular, double-stranded molecule of 16,569 bps, being replicated, transcribed, and translated in the matrix space, with mitochondrial genetic code (see http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi?mode=c#SG2) differing from the universal one in four codons. The two strands (heavy and light) of mtDNA contain 37 genes, encoding 13 structural subunits of OXPHOS complexes plus the 22 tRNAs and 2 rRNAs (needed for the protein biosynthesis in mitochondria). More than 80 structural subunits of OXPHOS complexes plus the assembly proteins and the enzymes required for mitochondrial biogenesis are encoded by nuclear DNA (nDNA). Thus, the OXPHOS metabolic pathway is under the dual control of mtDNA and nDNA (11,13–15). Due to the complexity of their biogenesis, mitochondria are subjected to quality control through molecular chaperones and proteases, proteins involved in nucleoside/nucleotide metabolism (like the thymidine phosphorylase) (16), fusion and fission of mitochondrial organelles as well as through mitophagy (mitochondrial autophagy to remove abnormal mitochondria) (17).

mtDNA is maternally inherited (18). Mature oocytes contain a high mtDNA copy number (~100,000), of which less than 0.01% is inherited, due to the mitochondrial genetic bottleneck during postnatal folliculogenesis (19–21). Paternal mtDNA was detected in muscle tissue only in one case (22).

mtDNA is highly polymorphic among individuals (~50–60 neutral polymorphisms between two individuals). Due to the close proximity of mtDNA to inner membrane and the lack of histones and significant DNA repair system, mtDNA is highly susceptible to reactive oxygen and nitrogen species (ROS and RNS), the byproducts of OXPHOS that contribute to high frequency mutation rate (23,24).

More than 200 pathogenic mutations in mtDNA contribute to the heteroplasmy, the co-existence of wild-type and mutant mtDNAs within mitochondria of cells. Due to the random distribution of mtDNA molecules during cellular division, a genetic drift usually occurs in favor of wild-type or mutant mtDNA. This could finally lead to homoplasmy towards the one or the other type of mtDNA (15,21). When mutant mtDNAs outreach a threshold, cells in tissues, like brain, heart and muscle, become unable to meet their high energy demands and pathologic symptoms prevail.

Oxidative Phosphorylation System

Oxidative Phosphorylation System (OXPHOS) converts substrates from glycolysis, fatty acid oxidation and TCA to ATP through five complexes (I–V) (http://www.prism.gatech.edu/~gh19/b1510/glytca.htm). Electron transport chain reactions are carried out by the first four (I–IV) complexes of OXPHOS. First, electrons are transferred from NADH and $FADH_2$ into complex I (NADH: ubiquinone oxidoreductase) or II (succinate dehydrogenase), respectively, and then to coenzyme Q or ubiquinone, a soluble electron carrier. Reduced ubiquinol transfers electrons to complex III (ubiquinol: cytochrome *c* oxidoreductase or bc_1 complex) and, more specifically, to cytochrome c_1. Then, cytochrome *c*, the second soluble electron carrier, transfers the electrons to complex IV (cytochrome *c* oxidase or COX), the terminal complex of electron transport chain, in order to reduce the molecular oxygen to water at the final stage (13,25,26). Electron transfer is a redox reaction producing energy, part of which is used for proton translocation from mitochondrial matrix space to IMS through complexes I, III, and IV, thus creating an electrochemical gradient. The latter is required for the

synthesis of ATP by translocating protons back to mitochondrial matrix through the complex V (ATPase synthase). ATP is then transferred to cytoplasm in exchange of ADP through the ATP translocator.

Protein Import Machinery of Mitochondria

Beyond the 13 structural subunits of OXPHOS encoded by mtDNA, ~1,500 human mitochondrial proteins, encoded by nDNA (spread in almost all chromosomes) are synthesized in the cytosol as preproteins (precursors) and then imported inside the mitochondria using the mitochondrial protein import machinery. According to Pfanner's group (27), protein import follows at least four pathways: a) the β-barrel pathway towards the outer membrane; b) the redox-regulated import pathway to the IMS; c) the carrier protein pathway to the inner membrane; and d) the presequence pathway to the inner membrane or matrix space. As a first step, TOM (translocase of outer membrane) complex is used by mitochondrial preproteins to be imported in the mitochondria. Depending on the final destination of mitochondrial proteins, distinct pathways should be followed, like SAM (sorting and assembly machinery), MIA (mitochondrial IMS assembly), TIM (TIM22 or TIM23: carrier or presequence translocase of inner membrane) and OXA (insertase/export machinery of the inner membrane). A large number of mitochondrial preproteins carry an N-terminal leader peptide (L) or Mitochondrial Targeting Signal peptide (MTS) (predicted by the Mitoprot logismic program at http://ihg.gsf.de/ihg/mitoprot.html). MTS (15–50 aa) is usually cleaved off by the Mitochondrial Processing Peptidase (MPP) in the matrix space, thus giving rise to the corresponding mature protein. MTS can also be located on the C-terminus of the preprotein, like in the case of the mitochondrial DNA helicase, Hmi1 (28). There are also mitochondrial preproteins with non-cleavable, internal (dispersed inside the primary protein structure) targeting signals, which regulate the pathway to be followed inside the mitochondria (7,27).

Mitochondrial Disorders of mtDNA or nDNA Origin

Mitochondrial dysfunction can lead to a variety of clinical phenotypes (Table II), ranging from the "primary" mitochondrial disorders to other neurodegenerative disorders, like Friedrich ataxia, Parkinson disease, Alzheimer disease, metabolic disorders, diabetes, obesity, cancer, and aging (14,17,23,29–31).

"Primary" mitochondrial disorders are associated with defects of respiratory chain or OXPHOS. They represent a heterogeneous group of rare genetic diseases (~1/5,000), affecting mainly organs with high energy (ATP) demands, like brain, muscle, and heart (25). These disorders are classified as either systemic or organ-specific, and their

Table II Examples of Genes of Mitochondrial or Nuclear Origin, Encoding Mitochondrial Proteins Involved in Mitochondrial Disorders

	Gene	OXPHOS complex	Function	Clinical phenotype	OMIM
Disorders of mtDNA origin (structural genes)	*MTND1*	I		Dystonia	
	MTCYB	III		Mitochondrial Myopathy	
	MTCO2	IV		Encephalopathy	
	MTCO3	IV		Encephalomyopathy, MELAS	
	MTATP6	V		Leigh Syndrome	
Disorders of nDNA origin (structural genes)	*NDUFS1*	I	iron-sulfur protein fraction	Leigh Syndrome	157655
	SDH-A	II	flavoprotein fraction	Leigh Syndrome	600857
	UQCRB	III	Electron transfer	Hypoglycemia, lactic acidosis	191330
Disorders of nDNA origin (non-structural genes)	*B17.2L*	I	Complex assembly	Early onset progressive encephalopathy	609653
	BCS1L	III	Complex assembly	Encephalopathy, hepatic failure and tubulopathy	603647
	SURF1	IV	Complex assembly	Leigh Syndrome	185620
	SCO2	IV	Complex assembly / Copper transport	Neonatal cardioencephalomyopathy due to COX deficiency	604272
	ECGF1		Thymidine phosphorylase/ mtDNA stability	MNGIE, multiple mtDNA deletions	131222
	DDP		Mitochondrial protein import	Deafness-dystonia	300356
	FRDA		Frataxin Trinucleotide Repeat	Friedreich ataxia	606829
	MFN2		Mitofusin-Mitochondrial fusion	Charcot-Marie-Tooth disease-2A2	608507
	PDHA1		Pyruvate dehydrogenase E1-α subunit	X-linked Leigh Syndrome	300502

Based on WebHome < MITOMAP < MITOWEB (http://www.mitomap.org/bin/view.pl/MITOMAP/ClinicalPhenotypesPolypeptide; http://www.mitomap.org/bin/view.pl/MITOMAP/NuclearGenesStructural; http://www.mitomap.org/bin/view.pl/MITOMAP/NuclearGenesNonStructural).

onset varies from infancy to late adulthood. Since OXPHOS disorders can be due to mutations occuring in both nuclear or mitochondrial genes, they are classified as mitochondrial disorders of mtDNA or nDNA origin and can follow all modes of inheritance, maternal and mendelian (autosomal recessive or dominant as well as X-linked) (32).

Mitochondrial disorders of mtDNA origin are due to point mutations, rearrangements or large-scale deletions in mtDNA (implicated either in protein coding or in protein synthesis), with symptoms being apparent when mutated mtDNA exceeds a threshold (60–90%) level.

Mitochondrial disorders of nDNA origin are due to mutations occuring in a) genes encoding respiratory chain subunits, respiratory chain assembly proteins and electron carrier CoQ_{10}, and b) genes affecting mtDNA integrity, mitochondrial protein import, lipid composition of the inner mitochondrial membrane, intergenomic signaling and mitochondrial dynamics (like motility, fusion and fission) (14,17,21,26).

Identification of the causative gene has been found only in a small percentage (~20%) of patients with "primary" mitochondrial disorders (32). Among them, ~80% are attributed to nDNA defects.

Deficiency in complex IV (COX) represents one of the most frequent causes of respiratory chain defects, often associated with severe or fatal clinical phenotypes (32,33). COX is a 13-subunit holoenzyme embedded in the inner mitochondrial membrane, where it catalyses the electron transfer from reduced cytochrome *c* to molecular oxygen (34). The three mtDNA-encoded COX subunits form the catalytic core of the holoenzyme, while the ten nDNA-encoded COX subunits are important for the overall stability and assembly of the holoenzyme (25,33,35). The biogenesis of COX is a multi-step process that occurs via short-term discrete intermediates. More than 20 nDNA-encoded ancillary proteins, so-called COX assembly proteins, are involved in the biogenesis of COX holoenzyme by contributing to the functional assembly, the mitochondrial copper transfer pathway or the biosynthesis of heme prosthetic groups (34).

Mutations in mtDNA genes encoding COX subunits have been implicated in myopathies and multisystemic diseases, while rearrangements or point mutations in tRNA genes of mtDNA have impaired COX function (21,36). Despite the pathogenic mutation found in the nuclear *COX6B1* gene (37), no other pathogenic mutations have been found in the nuclear genes encoding COX subunits (25), probably due to their lethal role, early in embryonic life. However, a number of cases with encephalomyopathy and COX deficiency opened the door for the identification of nuclear genes, encoding the COX assembly proteins. Briefly, mutations in eight human nuclear COX assembly genes *(SURF1, SCO2, SCO1, COX10, COX15, LRPPRC, TACO1* and *FASTKD2*) have been found to be associated with mitochondrial dysfunction and COX deficiency (25,38–46).

Mitochondrial Medicine

Nowadays, there is no effective therapy for mitochondrial disorders. However, a number of therapeutic approaches have been already applied or are still under development (15,26,47–51). Some of these approaches are indicated below.

Symptomatic therapy is applied to confront a variety of clinical manifestations of mitochondrial disorders, such as seizures, diabetes, hearing loss, ptosis of eyelids obscuring vision, hypertrophic cardiomyopathy, endocrine dysfunction and liver failure. Symptomatic drug therapy (with antiepileptics and antipsychotics), hemodialysis and surgery (cochlear implants and heart transplantation) can improve the quality of life of people suffering from such disorders.

Exercise training seems to alter the balance of mutated towards wild-type mtDNA. Aerobic exercise has been reported to induce OXPHOS and facilitate mitochondrial functions.

Ketogenic diet (>90% fats, enriched in proteins, vitamins, minerals, but low in carbohydrates) replaces glucose by ketone precursors, thus "shifting" metabolism from glycolysis to β-oxidation of fatty acids in mitochondria. Ketones have also been found to reduce the mitochondrial ROS production and enhance the mitochondrial biogenesis.

Metabolic and pharmacological therapy is focused on i) removal of toxic metabolites, like the neurotoxic lactic acid (leading to lactic acidosis and imbalance of cellular pH) with either sodium bicarbonate or dichloroacetate (DCA); ii) administration of electron acceptors (menadiol, vitamin C), metabolites and cofactors (CoQ10, vitamins B1 and B2, L-carnitine, creatine, folic acid, copper); iii) administration of ROS scavengers (vitamin E, CoQ10, idebenone, dihydrolipoate); and iv) administration of mitochondrial calcium channel blockers to rebalance calcium homeostasis.

Enhancement of mitochondrial biogenesis is based on i) sirtuin analogs, a group of NAD-dependent deacetylases; ii) agonists (bezafibrate and pyrroloquinoline quinine) of nuclear transcriptional coactivators, like the PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1alpha); iii) upregulation of mitochondrial soluble adenylyl cyclase, which activates mitochondrial protein kinase A and improves COX activity in cases, where the residual COX activity is not too low (52–55).

Enhancement of mitochondrial fusion occurs with the quinazolinone mdivi-1 (56).

Gene therapy of mitochondrial disorders of nDNA origin encounters the same obstacles as other gene therapy trials for the treatment of monogenetic disorders. For mitochondrial disorders of mtDNA origin, application of

gene therapy (replacement or repair) becomes even more complicated due to i) the heteroplasmy effect; ii) the necessity for mitochondrial targeting; iii) the translocation of gene products inside mitochondria; and iv) the mitochondrial genetic code. Promising genetic approaches can be i) "gene shifting" by reducing the mutant in favor of wild-type mtDNA; ii) "allotopic" or "xenotopic" expression of protein of interest; iii) bypassing the defective OXPHOS complexes with parallel expression of alternatives enzymes (like NDi1 and AOX) (57); iv) transduction into mitochondria of specific restriction endonucleases (like SmaI), that are able to recognize and cut out only the mutant mtDNA (58); and v) inhibition of mutant mtDNA replication by peptide nucleic acids (PNAs) (59).

Somatic stem cell therapy was carried out via the allogeneic hematopoietic stem cell (HSCs) transplantation in patients with mitochondrial neurogastrointestinal encephalomyopathy syndrome (MNGIE) in order to reduce thymidine and deoxyuridine levels in the blood (60).

According to germline therapy nuclear transfer technology aims to remove mitochondria carrying mutant mtDNA from maternal oocytes and replace them with normal mitochondria from a healthy donor's ooplasm (ooplasmic transfer) (61,62). Such innovative technologies may be useful in the near future to prevent transmission of mtDNA disorders at the preconception or conception stage.

Genetic counselling for families with a history of mitochondrial disorders to prevent transmission to offsprings is based on prenatal (analysis of mutations in amniocytes or chorionic villi) or preimplantation diagnosis.

It is obvious, however, that due to a lot of drawbacks, complexities, and adverse reactions associated with many of these approaches presented, the current strategies have to be improved and combined with new ones. The focus of this review is an alternative therapeutic approach of mitochondrial disorders: the PTD-mediated PRT. The potential applications of the selective transduction of exogenously administered recombinant PTs in mitochondria can be fruitful overall.

Targeting Mitochondria

Systemic administration or tissue-targeted delivery of PTs followed by "intracellular trafficking" and entrance into mitochondrial sites would facilitate the treatment of mitochondrial disorders (51,63). A number of examples of transduction of proteins or other molecules into mitochondria have been reported. Restriction endonucleases, able to restrict specifically mutant mtDNA haplotypes in cases of heteroplasmy, were fused to an MTS to selectively target the mitochondrial matrix space and deplete the mutant mtDNA (58,64). Vitamin E, conjugated to TPP (a lipophilic triphenylphosphonium cation) was also delivered selectively to mitochondria (65). "Mitochondriotropics," a term proposed by Weissig and Torchilin to describe the ability of molecules to diffuse into the intramitochondrial sites, has been an interesting concept (66,67). According to this, the properties of each one of the proposed therapeutics for mitochondrial disorders can influence its "trafficking" towards specific mitochondrial sites and in a state to "deliver" its intended therapeutic action. Liposomes or nano-based carrier systems have been developed to encapsulate the pharmaceutical molecules in order to target specific tissues. Subsequent internalization, usually occurring by endocytosis, is followed by release of the bioactive molecules into the cytoplasm in order to be translocated into the mitochondria. Therefore, "intramitochondrial trafficking" of the molecules should be assessed based on the desired therapeutic effect. Potential therapeutic molecules, required for the therapy of "primary" mitochondrial disorders of mtDNA origin or mitochondrial metabolic disorders affecting the TCA or the β-oxidation of fatty acids, should be directed to mitochondrial matrix space (31,51,58,64,68). In other cases of "primary" mitochondrial disorders, therapeutics should be targeted to the IMS or inner membrane, whereas the respiratory complexes are embedded (69). In cases of cancer or ischemia (cardiac or brain), where apoptosis can be manipulated, PTs should target mitochondria in order to influence the mitochondrial outer membrane permeabilization and thus the release of anti-apoptotic proteins (cytochrome *c*, apoptosis-inducing factor, endonuclease G) from the mitochondrial IMS to the cytoplasm (51,70,71).

Therapeutic molecules conjugated to MTS, delocalized lipophilic cations (easily "crossing" mitochondrial membranes), vehicle-based carriers (like MITO-Porter or DQA-somes), Mitochondria Penetrating Peptides (MPPs), PTDs and other appropriate agents could be used to target mitochondria (70,72–77). It is crucial, however, at this stage to underline the wide diversity in structure, properties, and physicochemical behavior of candidate mitochondrial therapeutics. Therefore, parameters that apply to conventional pharmaceuticals may not necessarily apply to macromolecules, like mitochondrial PTs.

Delivery of human recombinant proteins as putative mitochondrial PTs at intramitochondrial compartments may not only be affected by the intramitochondrial processing system of peptides, but also by protein-protein interactions, interactions with protein carriers, or even other accessory proteins at the targeted sites of delivery. No doubt, the complicated questions need extensive investigation. Prerequisites for the succeful outcome of mitochondrial PTs are their transduction through the plasma membrane and their delivery at specific sites within the mitochondria via intramitochondrial trafficking. Finally, the transduced PTs must be quite stable at the intramitochondrial site to exert their therapeutic function.

PRODUCTION AND INTRACELLULAR DELIVERY OF PTs–PROTEIN TRANSDUCTION DOMAIN (PTD) TECHNOLOGY

Design and Production of Human Recombinant PTs

The production of recombinant PTs starts with the isolation of the gene of interest that is cloned and subsequently expressed in a host system. Genetic manipulations of bacterial *E. coli* host strains (see Fig. 1) make these cells a powerful system to produce large quantities of therapeutic, non-glycosylated, recombinant proteins with proper protein folding at reasonable cost (78). Generation of inactive species of the heterologous proteins of interest in bacteria frequently result in the formation of inclusion bodies (IBs) or proteolysis, due to improper folding and aggregation. Several methods have been developed to recover the recombinant proteins produced in bacteria in soluble form, like optimization of growth conditions, refolding of recombinant proteins with the use of chaotropic agents or cloning of the coding sequence (CDS) in affinity tag fusion vectors. Alternatively, L-Arginine solution has been used for the recovery and refolding of IBs' enriched recombinant proteins (69,79).

In addition, several recombinant host expression systems (including yeast, filamentous fungi, insect baculovirus SF9 cells, transgenic plants, animal cell cultures and even transgenic animals), all known as genetically modified organisms (GMOs), have been developed for the production of PTs (80,81). GMOs differ in their capacity to produce glycosylated or non-glycosylated heterologous proteins with proper protein folding and solubility as well as their ability to secrete them into the growth medium. Purification of the expressed protein is based on the expression system used, the size, the pI and the affinity of the recombinant protein to a specific ligand. Such systems are expected to facilitate production at high yield and increase world supply of recombinant proteins (Biopharmaceuticals) of second generation (recently termed Biosimilars) with proper folding, conformation, and stability (82,83).

Many eukaryotic proteins are subjected to post-translational modifications (~200 different types), which affect solubility, stability in serum (plasma), binding to specific receptors as well as degradation via ubiquitination. Some structural modifications (like glycosylation and thioester formation) concern proteins mainly acting extracellularly, while others (phosphorylation and ADP ribosylation) concern intracellular proteins (84). While bacteria do not glycosylate their proteins, yeast cells and plant-based systems do, but in a different manner than mammalian cells. Genetically modified yeast cells could acquire a glycosylation pattern quite similar to that achieved by human cells (85).

Delivery of PTs: Adverse Reactions—Immunogenicity

Since PTs are quite susceptible to proteolytic degradation and thus administered parenterally, they must be stable enough to survive their journey up to the targeted tissue. PTs administered intraperitoneally have to bypass the reticuloendothelial system and/or evade the renal clearance. This can be achieved either by chemical modification or encapsulation into the micro- / nano-particulate carriers, like liposomes, micelles, nanocarriers, or in cases of tumors, by taking advantage of the EPR (Enhanced Permeability and Retention) effect (6,86). The most popular chemical modification of PTs is PEGylation, which is conjugation of proteins with poly(ethylene glycol) (PEG) residues (87). PEGylation increases their solubility, enhances stability and structural integrity (serum half life), yet minimizes kidney elimination. PEGylation also reduces immunogenicity of PTs, due to the steric hindrance provided (88). PEGylated proteins, however, being administered on a chronic base, can lead to accumulation of PEG inside cells, formation of vacuoles in kidney epithelium as well as anti-PEG antibodies (89). PTs, unlike conventional pharmaceuticals, are not metabolized, but rather degraded into peptides of smaller size inside the body.

High dose schedule or repeated doses of PTs can induce adverse reactions or provoke immunogenicity in several instances (90), although these phenomena are not so common (5). Impurities (bacterial remnants or degradation of formulation components) could act like the adjuvants in vaccines to induce immunological response (desired, however, in that situation). Process- and product-related factors, leading to modification of protein structure (aggregates, pattern of glycosylation), may expose neo-epitopes that are recognized as foreign (non-self) antigens by the immune system (91,92). Changes in the formulation (replacement of human serum albumin by polysorbate and glycine) of biosimilar erythropoietin resulted in a very severe disorder, known as antibody-mediated pure red cell aplasia (PRCA) (93). Adverse reactions provoked by PTs could be due to either pharmacodynamic effects, like cardiotoxicity provoked by Trastuzumab (Herceptin®), flu-like symptoms, or even hypersensitivity reactions (5). Since in most cases there is a minimal requirement (1-5%) for the exogenously added protein-enzyme to replace the endogenous malfunctioned protein-enzyme and complement the deficiency, biodistribution and pharmacodynamic issues could be managed appropriately in a dose-based manner (94).

Theoretically, almost all biological medicinal agents can provoke immunological response and formation of antibodies of low (IgM) or higher (IgG) affinity (95,96). In cases of immune response, whereas no neutralizing IgG antibodies (NABs) are formed, prescription of antihistamines and antipyretics can be administered. Alternatively, the infusion

rate of the administered PT can be modified. Development of NABs will reduce the overall efficacy of the corresponding PTs or influence their pharmacokinetic profile (97,98). The ability to predict putative immunogenicity of a given PT, like prediction of existence of T- or B-cell epitopes on protein molecule, could permit either removal or modification of the most efficient of them in order to produce more safe biopharmaceuticals. Immunoinformatics using computer algorithms, like Immune Epitope Database, T Cell Epitope Database (TCED) and EpiMatrix system, can screen for 9mers (or even 25mers) as putatives T-helper-cell epitopes on amino acid protein sequence. Substitution of key amino acids could reduce the potential of the respective T-cell epitope to induce immunogenicity (de-immunization) (96,99).

Intracellular Delivery of PTs

Protein therapy as the direct intracellular delivery of the protein (the gene translational product itself) is considered an alternative approach to gene therapy of genetic-metabolic disorders. For monogenetic diseases, where the gene of interest normally encodes an extracellular acting protein (hormone, growth factor, signaling molecule), protein therapy could offer an effective therapeutic approach. For those diseases, however, where the gene of interest normally encodes an intracellular protein, like in many metabolic-genetic disorders (e.g. mitochondrial disorders), the putative PT has to "cross" the plasma membrane of cells and even target a specific organelle (e.g. mitochondria) to exert its therapeutic effect (100).

Early last century, patients with phenylketonuria (a metabolic disorder characterized by dysfunction or deficiency of liver-specific phenylalanine hydroxylase enzyme that leads to accumulation of phenylalanine in plasma) were restricted to food low in phenylalanine and rich in tyrosine (101). Unfortunately, other metabolic (lysosomal) disorders did not respond to such a diet restriction approach.

It was in early 1960s when Christian Deduve proposed the Enzyme Replacement Therapy (ERT) to treat lysosomal disorders (102), developed by accumulation of metabolites-intermediates (103). The first ERT was applied to treat Gaucher's disease, a chronic disease of lipid metabolism, in which the β-glucocerebrosidase enzyme deficiency does not permit the intracellular lipids to be processed. The exogenously administered mannose-terminal β-glucocerebrosidase (prepared by exoglycosidase digestion of human placental glucocerebrosidase) could be recognized by mannose lectin on the plasma membrane of macrophages, where lipids are stored (4,104). More specifically, site-directed mutagenesis of 495Arginine (R) to 495Histidine (H) permitted the addition of mannose residues on the produced recombinant human β-glucocerebrosidase that facilitated the entrance in macrophage / monocyte cells and cleavage of the intacellular lipids (4,105). The ability to intervene in the post-translational glycosylation of human recombinant lysosomal proteins, by adding and/or exposing mannose residues as well as by rising the levels of mannose-6-phosphate for recognition by lysosomal mannose-6-phosphate receptors, could lead to potential therapeutic approaches of untreated as yet lysosomal disorders (6,105).

Intracellular delivery of PTs (varying in size, structure, polarity and hydrophobicity (106)), depends on the existence of either an active carrier-mediated process, absorption pathway, or receptor-mediated endocytosis. A number of ligands use receptor-mediated endocytosis for internalization purposes, including proteins, like transferrin; lipoproteins, like LDL; vitamins, like riboflavin (vitamin B2); and mabs (107–109). Internalization pathways are activated to facilitate uptake of macromolecules (including proteins) from the surrounding environment (107). Lack of an appropriate transport mechanism makes intracellular delivery of PTs difficult. More stringent restrictions occur in the case of transport through the blood-brain barrier (BBB) (110,111). The ability of antibodies to interact with their cell surface receptors and provoke internalization led to a new class of antibody-based PTs. Such PTs, derived as conjugation products of small molecular size drugs or toxins to tissue-specific mabs, are already in the market (112) to target and then eliminate specific cell types (113,114). Since ICAM-1 (Intercellular Adhesion Molecule-1) is expressed primarily by endothelial cells (115), anti-ICAM carriers developed as PTs for ERT of lysosomal storage disorders (116). The ability of tetanus toxin to cross the BBB led to the use of the carboxyl tetanus toxin fragment (CTTF) as a brain-targeting moiety (117). In conclusion, one can design and produce PTs fused to targeting ligands in order to deliver them selectively into a given tissue.

Despite the fact that few exogenously administered PTs enter the cells by interacting with their cell surface receptors, in most cases PTs fail to enter the cells. Therefore, the intracellular transduction of recombinant PTs and subsequent targeting at specific malfunctioned subcellular organelles, involved in inherited metabolic disorders, remains a challenge.

PTD Technology

PTD technology refers to the technology that uses small peptides of less than 30 amino acids able to penetrate almost all biological membranes. These small peptides, termed Protein Transduction Domains (PTDs) or Cell Penetrating Peptides (CPPs), carry intracellularly a variety of cargos ranging from small molecules (e.g. cyclosporine,

doxorubicin) to siRNAs and macromolecules (proteins, plasmids and liposomes) (118–121). PTDs can be either covalently attached to the cargos or could form non-covalent complexes with them (122–124) (Table III).

The first demonstration of protein transduction via PTDs in an animal model was reported from Dowdy's group (125). Intraperitoneally injected beta-galactosidase protein, fused to TAT, in mice was found in all tissues examined, including brain. TAT, the most popular and widely used PTD (Table III), is an 11 aa sequence, derived from the TAT protein of the human immunodeficiency virus-1 (HIV-1) (119,125). Historically, TAT was identified as PTD, based on the observation that HIV-1 TAT protein (a transactivator factor of transcription; 86 aa) was able to penetrate the plasma membrane (126) and transduce heterologous proteins intracellularly (127). In parallel, Penetratin or Antp, a Drosophila homeotic transcription factor derived peptide (Table III), was also developed as PTD by Prochiantz's group (128). Both TAT and Penetratin are enriched in cationic amino acids, like Arginine (R) and Lysine (K). Since the mid-1990s, a large number of peptides have been used as PTDs with many applications both *in vitro* and *in vivo* (119,120,129). These PTDs are either derived from proteins with inherent property to penetrate membranes or are produced via chemical synthesis (Table III). Moreover, other technologies have been used, like the Phage Display technology, to detect novel, efficient PTDs (130). Phage Display technology can potentially identify peptides able to bind to specific molecules on the surface of targeted cells and may overcome the lack of tissue specificity observed in therapeutic applications of PTD technology (131).

There is still an open question of how PTDs are taken up by the cells. A number of models have been proposed, including the inverted micelle driven delivery model (model 1), the direct penetration-driven delivery model (model 2), and the endocytosis-driven delivery model (model 3) (132). The latter seems to be the most widely accepted, although other models have also been proposed, due to the resemblance of PTDs to many antimicrobial peptides (133). The precise mechanism of internalization depends on the nature of the PTD used, the nature (sequence length, conformation) and concentration of cargo, the targeted cell type, the density and distribution of heparan sulphate proteoglycans on cell surface, the specific phase of cell cycle, the incubation time, the fate of transduced protein, and the peptide-to-cell ratio employed (134–136).

At the very first step of PTD-mediated transduction process, positive charged PTDs interact electrostatically with the heparan sulphate proteoglycans. There are PTDs, like the Pep-1 (Table III), however, that interact with membrane negatively charged phospholipids, thus minimizing their association with cell surface proteoglycans and their uptake through endocytosis (137). This way they bypass the loss of cargo being trapped in the endosome compartment. Transmembrane potential, membrane fluidity, and salt environment appear to contribute also to the transduction of PTDs across the membranes (138,139).

Pharmacokinetic studies in animals have shown that systemically administered PTDs are rapidly cleared from the circulation, with the kidney, liver, and spleen being the organs with the higher uptake rate. PTDs were even transduced into the brain tissue via the BBB (140). Data show that TAT can cross the BBB at a rate comparable to that of the cytokines (interleucin-1 and tumor necrosis factor-α) and accumulates in the central nervous system (CNS) (141). PTs conjugated with PTDs (like TAT-FNK and TAT-Bcl-x_L) was delivered in the brain, especially in diseased state with BBB disruption (142,143). Neuroglobin was conjugated with TAT and efficiently transduced into mouse neurons to protect them from ischemic lesions (144).

PEGylated PTs, produced via PTD technology, complemented by liposomal and nanoparticle delivery systems, can improve the pharmacokinetic profile and overcome a number of obstacles. Thus, penetration of all biological barriers, including BBB or blood-spinal cord barrier (BSCB) (145) could be achieved. Moreover, selective transduction of PTs via PTD in targeted tissues will reduce possible systemic toxicity. To this direction, novel approaches like the heparin-protamine regulation (on/off) system will enhance specific cargo delivery (ATTEMPTS: Antibody Targeted, Triggered, Electrically Modified Prodrug-Type Strategy) (146).

Construction of Proteins for Intracellular Delivery

In early days of protein transduction (127), the TAT peptides (1–72 aa or 37–72 aa of HIV-1 TAT full length protein) were chemically cross-linked to proteins like β-galactosidase (~120 kDa). β-galactosidase was dissolved in PBS, treated with iodoacetamide, esterified with 4-(maleimidomethyl)-cyclohexanecarboxylic acid N-hydroxysuccinimide ester and cross-linked with the TAT peptide. Conjugation chemistry gives either stable linkage or creates thioester bridges to be cleaved under reducing conditions, thus releasing the desired cargo (124,147).

Later on, Dowdy's group (124,125,148) engineered in frame TAT-X fusion proteins by cloning the DNA (*X*) sequence (corresponding to the open reading frame (ORF) or CDS of the protein X) into the pTAT/TAT-HA expression vector and transforming appropriately competent bacterial cells (see Fig. 1).

In cases when post-translational modification (e.g. glycosylation) of the produced PT is necessary for its biological activity, suitable eukaryotic expression systems

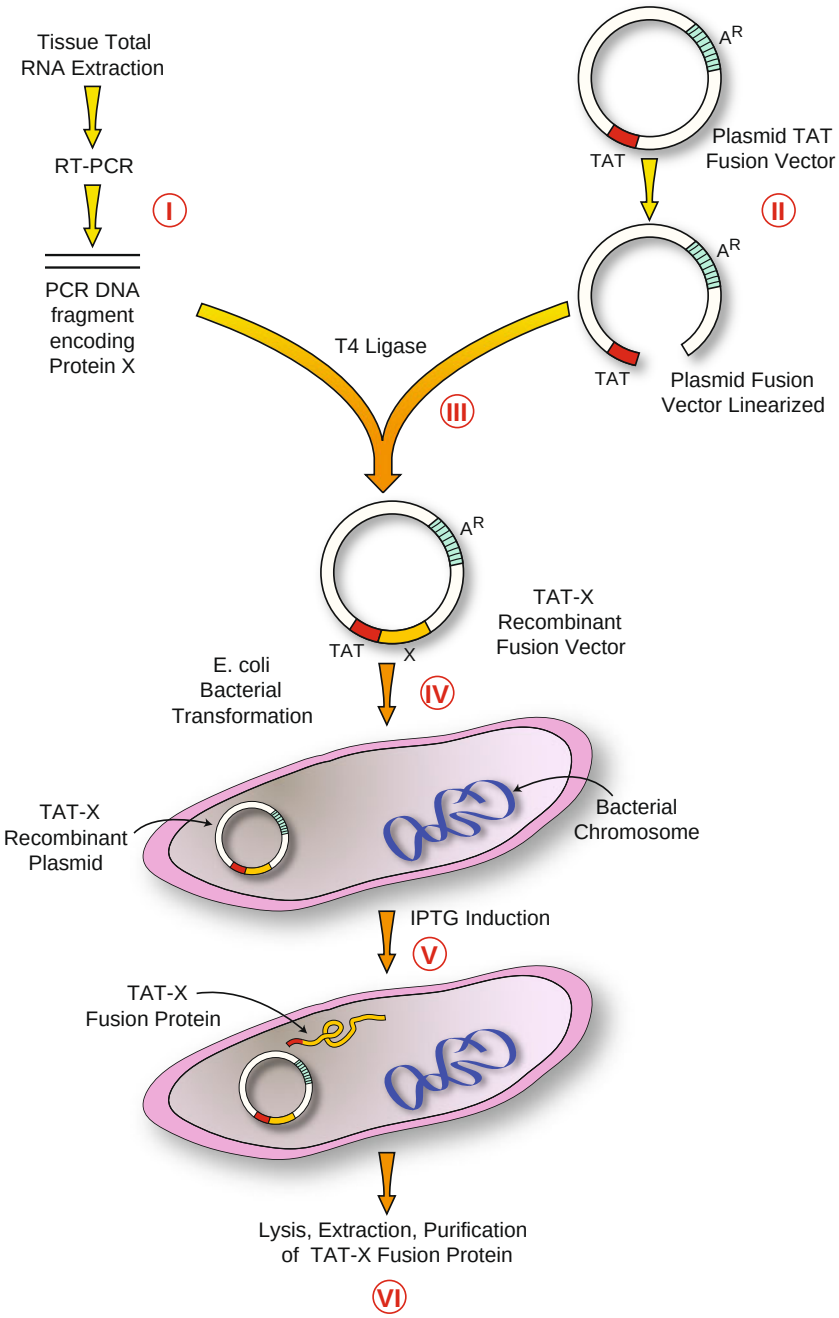

Fig. 1 Stages of bacterial production of recombinant TAT-X fusion protein intended for protein transduction. Total RNA extracted from a given tissue served as template for RT-PCR to amplify CDS of protein X (I). PCR fragment is subsequently cloned either in pTAT/pTAT-HA bacterial expression vector (148) in frame with the sequence for TAT domain or in another appropriately modified bacterial expression vector carrying TAT sequence (69) (II, III). Following transformation of competent bacterial cells (IV) and IPTG induction of protein expression (V), bacterial cells are collected, harvested, and lysed, and finally the fusion protein (TAT-X) is purified (VI). The solubilised recombinant fusion TAT-X protein would then be transduced into cultured mammalian cells (A^R: Antibiotic Resistance gene).

can be used to facilitate secretion of the produced glycosylated TAT-X fusion protein (149).

Protein transduction can be also approached in a non-covalent manner. Non-covalent protein transduction (NCPT) strategy can be based on premixing the selected PTD with the cargo or formulating the PTs in liposomes or nanoparticles (150). Amphipathic peptides used as PTDs, Pep-1 and Pep-2, carrying an acetyl and a cysteamide group at their N- and C-terminus respectively, form stable complexes with the cargos in solution through hydrophobic interactions (123,137,151). Of critical importance for non-covalent intracellular delivery is the molecular ratio of PTD molecule to the cargo (122,150).

Organelle Targeting

In certain pathological cases, internalization and intracellular trafficking are key elements for successful delivery of PTs in certain tissues (108). The "signal hypothesis" proposed by Gunter Blobel (152), which led to the Nobel price for Physiology or Medicine in 1999, opened a whole new horizon in therapeutic targeting of subcellular compart-

Table III Most Representative PTDs

PTD	Origin	Amino acid sequence / physicochemical nature	Amino acids (aa)	Refs
TAT	HIV-1 transactivator factor of transcription	YGRKKRRQRRR Cationic peptide	11	(125,126)
Penetratin or Antp	*Drosophila* homeotic transcription factor encoded by *antennapedia* gene	RQIKIWFQNRRMKWKK Amphipathic peptide	16	(128)
VP22	Herpes simplex virus VP22 transcription factor	DAATATRGRSAASRPTERPRAPARSASRPRRPVD Amphipathic peptide	35	(184)
Poly- Arginines	Chemically synthesized	R9 / R8 Cationic peptide	9 / 8	(185)
Transportan	Galanin-mastoparan	GWTLNSAGYLLGKINLKALAALAKKIL Chimeric -Amphipathic peptide	27	(186)
Pep-1	Trp-rich motif-SV40 NLS	KETWWETWWTEWSQPKKKRKV Amphipathic peptide	21	(137)

ments (6). Subcellular organelle targeting can be exploited through organelle-specific targeted sequenses and is emerging as a powerful approach in the pharmaceutical field (6,63,153).

Since PTDs like TAT "open the door to cell" (154) for a variety of cargos, PTDs also "open the door to subcellular organelle targeting" by incorporating organelle-specific targeted sequences in the designed structure of the PT.

TAT, due to its inherent ability to target nucleus (155), has already been used for the transduction of plasmid DNA or biological molecules, like p53 and p16 transcriptional suppressors (156–158). TAT has also been used for lysosomal delivery of PTs under the frame of ERT in some lysosomal disorders (159). Ohta's group (143) ligated the TAT domain to the super-antiapoptotic factor FNK to produce the PTD-FNK fusion protein. PTD-FNK fusion protein was transduced efficiently into cells, localized into mitochondria, and protected both neuronal and cardiac cells from apoptosis induced by ischemia *in culture* as well as in animal models (71,143,160). Similarly, TAT-Bcl-2 fusion protein protected neurons from apoptotic cell death *in culture* (161).

Toxicity Studies

PTs are characterized by high specificity, although physical instability, proteolysis, aggregation, hemolytic activity (as an indicator of cell membrane disruption), cytotoxicity, and even immunogenicity can be observed upon their use (90). Although the toxicity studies carried out with PTDs are quite limited, variable response to various PTDs used has been recorded. These differences may be attributed to the nature and structural characteristics (cationic, amphipathic) of each PTD used, the nature of its linkage with the cargo, the molecular mass, and/or the pI of the attached cargo (162–164). In addition, internalization process of PTDs may influence the homeostasis of targeted cells. Metabolomic analysis of cells treated with various PTDs has shown different pattern of behaviour, with TAT weakly influencing the expression status of three genes (165,166).

Clinical Studies and Therapeutic Applications

Animal models of various diseases have been testified for the functionality of transduced bioactive cargos through PTD technology (164). Fusion of recombinant phenylalanine hydroxylase with TAT peptide lowered the phenylalanine levels in plasma, thus providing an alternative therapeutic approach for phenylketonuria (167). PTDs have been used for ERT approaches, for the therapeutic approach of disorders involving CNS, and for the differentiation of induced pluripotent stem cells (90,168,169). There is a number of obstacles that have to be solved (adverse effects, immunogenicity, stability, avoidance of proteases-peptidases, circulation half time). However, preclinical studies and clinical trials (concerning systemic or topical application of PTDs-conjugated PTs / small molecules) are under evaluation, with first results being quite encouraging (118,129,170,171). Most of these studies include neuroprotection after stroke incidence, cardioprotection following ischemia injury, enhancement of apoptosis towards cancer therapy, and enhanced induction of intestinal insulin uptake. Other pathological conditions, proposed to be treated via the PTD technology, are angiogenesis, age-related macular degeneration leading to loss of vision, pathological fibrosis and scarring through keloid formation, inflammatory disorders associated with various mediators like cytokines, and lysosomal disorders (164). According to Van den Berg and Dowdy, data reviewed from Phase I and Phase II clinical trials on more than 2,000 patients, treated with PTs developed via PTD technology, indicated no substantial adverse effects till now (124).

PTD-MEDIATED PROTEIN REPLACEMENT THERAPY (PRT) AS A THERAPEUTIC APPROACH FOR MITOCHONDRIAL DISORDERS

The ability of PTDs (like TAT) to penetrate not only the plasma membrane but also the mitochondrial membranes (154,172,173) "opened the door" for the application of PTD technology as a putative protein therapeutic approach of mitochondrial disorders. In general, TAT could mediate transduction of the PT either from its N-terminal (TAT-X fusion protein) or its C-terminal (X-TAT fusion protein). Subcellular targeting to mitochondria can be exploited by the insertion of an MTS in the recombinant fusion protein produced, like TAT-MTS-X (Fig. 2). In this case, cleavage of TAT-MTS peptide by MPP in mitochondrial matrix space inhibits the reverse movement of the protein from the mitochondria out to cytosol or even into the extracellular space, through the TAT. Thus, it enhances the retention of the mature X protein (the desired PT) inside the mitochondria (Fig. 2).

Payne's group (172,174) successfully targeted GFP (Green Fluorescent Protein) into the mitochondria of cells *in culture* and *in vivo*. GFP was expressed as a fusion protein carrying both TAT and an MTS, derived from the mitochondrial protein malate dehydrogenase. Designed TAT-MTS-GFP fusion protein was targeted to mitochondria of cultured cells, and the TAT-MTS peptide was then recognized and cleaved by the MPP in the matrix space of mitochondria. In this case, the released GFP was trapped inside the matrix space of mitochondria. TAT-MTS-GFP fusion protein, injected *i.p.* into pregnant mice, was detected in mitochondria of both maternal and fetal organs, like heart and brain. A novel mechanism for TAT transduction of mitochondrial membranes through the mitochondrial sodium channels has been proposed (173), different from the endocytotic mechanisms proposed for TAT transduction of plasma membranes.

With respect to TAT-mediated PRT in mitochondria, a number of studies *in culture* and in animal models have been reported thus far. Khan and Bennett proposed PTD technology as an efficient approach to transduce mtDNA, in complex with the recombinant fusion PTD-MLS-TFAM protein, into the ρ^o (depleted of mtDNA) mitochondria (175). TFAM, a mitochondrial transcription factor A, is a high mobility group box (HMGB) protein that binds to mtDNA, playing crucial role in mtDNA packaging and nucleoid formation and thus in mitochondrial biogenesis (176). Moreover, a modified recombinant MTD-TFAM (MTD is a mitochondrial transduction domain, derived from a combination of a PTD and a MLS, a matrix

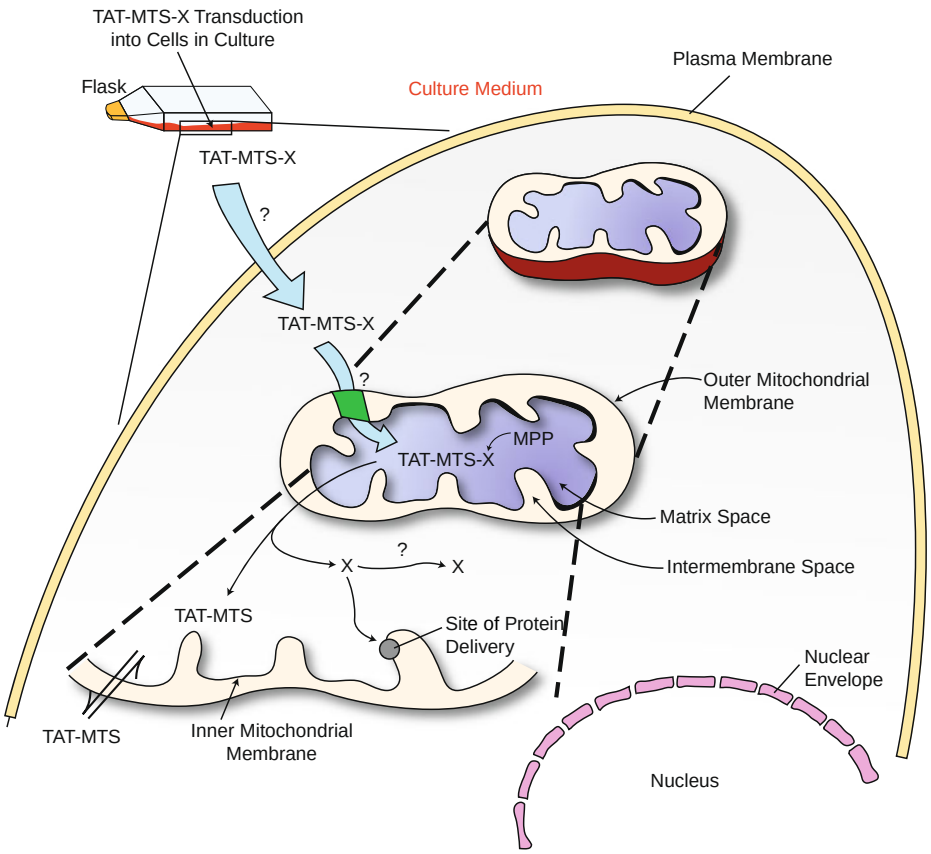

Fig. 2 Diagrammatic presentation of intramitochondrial transduction of recombinant fusion TAT-MTS-X protein into cultured mammalian cells. Recombinant TAT-MTS-X fusion protein (prepared from bacteria (as shown in Fig. 1) in order to carry also an appropriate MTS (68,69,154)) is administered into cultured cells. First, TAT-MTS-X fusion protein penetrates the plasma membrane. Subsequently, TAT-MTS-X fusion protein is translocated through MTS from cytosol to mitochondria and reaches the mitochondrial matrix space. The fusion protein is then processed by MPP, and the released mitochondrial mature X protein either remains inside the matrix (68) or is embedded in the inner mitochondrial membrane (69).

mitochondrial localization sequence) fusion protein transduced cells, translocated into mitochondria and increased cell respiration by increasing expression of genes involved in respiratory chain both *in culture* and in animal models (177). Furthermore, MTD-TFAM fusion protein improved motor function in mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which is able to induce experimental parkinsonism (178).

Wilson's group (49,179) used an MTS (from the human MnSOD mitochondrial protein) to target the DNA repair enzyme Exonuclease III (ExoIII) into the mitochondria of breast cancer cells via the PTD technology. The goal of this strategy was to sensitize these cells to oxidative stress in the general frame of increasing the mtDNA's repair capacity. The produced recombinant MTS-ExoIII-TAT fusion protein was successfully targeted into the mitochondria of cultured cells, playing crucial role on the survival of these cells.

TAT-mediated PRT concerning a mitochondrial metabolic disorder, named Lipoamide Dehydrogenase (LAD) Deficiency, was reported by the Lorberboum-Galski's group (68,154). LAD refers to the E3, a catalytic subunit of a multicomponent enzymatic complex, the α-ketoacid dehydrogenase complex (including the pyruvate dehydrogenase complex (PDHC)), which is localized in the mitochondrial matrix space and plays a very important role in the metabolism of carbohydrates and lipids. LAD deficiency results in lactic acidosis, Krebs cycle dysfunction and impaired branched-chain amino acid degradation. Exogenously added recombinant TAT-LAD fusion protein in fibroblasts, derived from patients with LAD deficiency, led to transduction of this TAT-LAD fusion protein inside the cytosol of fibroblasts, translocation to mitochondria, penetration of mitochondrial membranes, processing of the MTS sequence of fusion protein by the MPP in the matrix space, and, more importantly, restoration of the enzymatic activities of both LAD and PDHC. The latter indicates that transduced TAT-LAD fusion protein efficiently complemented the mutant LAD subunit in patients' fibroblasts (68). In additional *in vivo* studies, exogenously single *i.v.* administration of TAT-LAD fusion protein in E3-deficient mice (an animal model for LAD) improved the enzymatic activities of both the LAD and PDHC in mouse liver, heart, and brain (180). This work strongly suggested TAT-mediated PRT in mitochondria as an efficient approach to treat human mitochondrial metabolic disorders due to a deficient or mutated mitochondrial protein. Moreover, this approach maybe also manifest neurological symptoms, since TAT-LAD was found able to cross the BBB and be delivered in the brain tissue, as it was earlier proposed (125).

TAT-mediated PRT in a tissue culture model was reported also by our group in a case of a mitochondrial respiratory chain disorder, the fatal infantile cardioencephalomyopathy and COX deficiency due to mutations of the *SCO2* gene (42,69). Human *SCO2* is a nuclear gene on chromosome 22 (22q13), encoding the full length L-Sco2 protein (266 aa), a COX assembly protein. L-Sco2 protein is synthesized into the cytosol and targeted mitochondria through its N-terminal leader peptide (L: first 41 aa of L-Sco2, its own MTS). After being processed by the MPP in the matrix space, the released mature Sco2 protein is embedded in the inner mitochondrial membrane. Human mature Sco2 has been found to be involved not only in the mitochondrial copper pathway (42,46), but also in mitochondrial redox signaling (181) and the p53 regulatory pathway in mitochondria (182).

Our group applied the PTD technology as a therapeutic approach for fatal infantile cardioencephalomyopathy and COX deficiency due to mutations of the *SCO2* gene. By using recombinant DNA technology (rapid gene cloning and expression in *E. coli* bacteria), we produced the recombinant TAT-L-Sco2 fusion protein in bacteria. Purified bacterial IBs, enriched in fusion TAT-L-Sco2 protein, were solubilised in L-Arginine solution. Human recombinant TAT-L-Sco2 fusion protein was successfully transduced into mammalian cells (U-87 MG and T24 cell lines) and translocated into the mitochondria in a concentration- and time-dependent manner (69). In addition, (^{35}S)Methionine-labeled fusion TAT-L-Sco2 protein, produced in a cell-free *in vitro* transcription / translation system, was efficiently processed by the MPP to the corresponding mature Sco2 protein after incubation with isolated intact mitochondria. To demonstrate whether this transduced recombinant fusion TAT-L-Sco2 protein was also able to restore the enzymatic activity of COX, two model systems were used: Imatinib-treated human CML K-562 erythroleukemia cells, with low steady state levels of *SCO2* mRNA and COX deficiency (69); and primary fibroblasts from *SCO2* / COX deficient patients (42). Exogenously administered recombinant fusion TAT-L-Sco2 protein resulted in enzymatic restoration of COX activity in both models (69). Complementation of COX enzymatic activity by TAT-L-Sco2 protein in early (T9, T13) but not late (T20) passages of patients' fibroblasts maybe means that senescent COX-deficient cells cannot support membrane transduction or the OXPHOS dysfunction is far away to be reversed. Thus, protein therapy should gain better results in the early stages of such mitochondrial disorders. Eventually, the successful transduction of TAT-L-Sco2 protein in cultured cells should be applied to mice, specifically to the compound heterozygous KI/KO (harboring an *SCO2* knock-out (KO) and *SCO2* knock-in (KI) allele), a relevant mouse model for fatal infantile cardioencephalomyopathy and COX deficiency (183).

CONCLUSIONS AND PERSPECTIVES

The use of diverse PTDs as cargo systems opens new horizons in the transduction of recombinant PTs inside mammalian cells, an event that was hardly approachable a few years ago. Examples of successful intracellular delivery of PTs further support the value of PTD technology as a powerful protein therapeutic approach with obvious applications in certain serious genetic diseases.

A number of studies reported so far are directly related to targeted delivery of proteins into mitochondria. The cases of "intramitochondrial trafficking" of both human recombinant TAT-L-Sco2 and TAT-LAD fusion proteins indicate that TAT-mediated transduction of recombinant PTs can lead to TAT-mediated PRT for mitochondrial disorders. This approach, however, needs to be more thoroughly investigated in order to provide clear cut answers to certain as yet unsolved questions: a) to what extent PTs transduced inside the mitochondria exhibit the correct protein conformation; b) how stable the transduced PTs are; and c) how efficiently do they express their intended biological activity. Therefore, one has to follow the fate of the transduced PTs via their trafficking from the intracellular space into mitochondria, and get a sense of whether the transduced PTs have reached the desired intramitochondrial site of delivery, remained structurally intact and biologically active, or been attached to other proteins in such a way to fail "delivering" their intended therapeutic function—that is, to examine how the delivered PT could interact with the "gatekeeper proteins" of the mitochondrial membranes into such a way to "slip" inside the mitochondria and then "fit" with other proteins in the targeted site (e.g. OXPHOS complexes, Table II). Answers to these questions are critical in order to assess the short- or long-term therapeutic efficacy of transduced PTs in dysfunctional mitochondria as well as the well being of recipient cells and even the whole organism upon treatment *in vivo*. Another critical issue is the selective biodistribution of the transduced PTs into the target site *in vivo*. TAT-mediated PRT must be also evaluated *in vivo* for their potential to provoke immunogenicity and/or other adverse reactions.

Considering that a large number of rare diseases are primarily attributed to abnormal or depleted mitochondrial proteins (Table II), the TAT-mediated PRT approach looks very promising (Fig. 2). This approach might work well in cases where the transduced PT (like TAT-MTS-X fusion protein) is stable enough and delivered in sufficient quantities in the right intracellular-intramitochondrial target, thus achieving complementation of the deficient native protein. Futrhermore, in cases of mitochondrial disorders of mtDNA origin, all the parameters involved in the gene therapy approaches (the heteroplasmy effect, the mitochondrial targeting, the mitochondrial genetic code) should be taken into serious consideration.

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