# Validation of the use of an artificial mitochondrial reporter DNA vector containing a Cytomegalovirus promoter for mitochondrial transgene expression

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ABSTRACT

Mitochondria have their own gene expression system that is independent of the nuclear system, and control cellular functions in cooperation with the nucleus. While a number of useful technologies for achieving nuclear transgene expression have been reported, only a few have focused on mitochondria. In this study, we validated the utility of an artificial mitochondrial DNA vector with a virus promoter on mitochondrial transgene expression. We designed and constructed pCMV-mtLuc (CGG) that contains a CMV promotor derived from Cytomegalovirus and an artificial mitochondrial genome with a NanoLuc (Nluc) luciferase gene that records adjustments to the mitochondrial codon system. Nluc luciferase activity measurements showed that the pCMV-mtLuc (CGG) efficiently produced the Nluc luciferase protein in human HeLa cells. Moreover, we optimized the mitochondrial transfection of pCMV-mtLuc (CGG) using a MITO-Porter system, a liposome-based carrier for mitochondrial delivery *via* membrane fusion. As a result, we found that transfection of pCMV-mtLuc (CGG) by MITO-Porter modified with the KALA peptide (cationic amphipathic cell-penetrating peptide) showed a high mitochondrial transgene expression. The developed mitochondrial transgene expression system represents a potentially useful tool for the fields of nanoscience and nanotechnology for controlling the intracellular microenvironment *via* the regulation of mitochondrial function and promises to open additional innovative research fields of study.

## INTRODUCTION

Controlling the function of an intracellular microenvironment such as an organelle could potentially create innovative research opportunities and discoveries in nanoscience and nanotechnology. Mitochondria, a prime target organelle, carry out various essential cellular functions including ATP production, regulating apoptosis and mitochondrial biosynthesis. Mitochondria possess their own genome, mitochondrial DNA (mtDNA), with a gene expression system that is independent of the nuclear system, and regulate cellular functions in cooperation with the nucleus [1]. To date, many useful technologies regarding nuclear transgene expression have been reported [2-5], and a wide variety of plasmid DNA (pDNA) vectors have been developed and are now used as convenient and useful tools for transgene expression by many researchers and clinicians world-wide [5]. While, there are a few reports regarding the development of pDNA vectors for mitochondrial transgene expression, and a convenient and established method for achieving mitochondrial transgene would be highly desirable. The acceleration of mitochondrial DNA vector development would open innovative fields including the life sciences, drug discovery and gene therapy.

It is well known that mitochondria possess their own transcription/translation system and a unique codon usage that is different from universal codon usage. Because of this, a mitochondrial DNA vector needs to be designed to meet essential components for mitochondrial transgene expression, including an optimal promoter for mitochondrial transcription and mitochondrial codon usage. Several studies regarding artificial mitochondrial DNA vectors have been reported, most of which involved the design of DNA vectors containing a gene optimized for the mitochondrial codon system and a mitochondrial endogenous promotor such as a heavy strand mtDNA promoter (HSP) [6-9]. Among the previous excellent reports, we focused on the use of a

virus vector for achieving mitochondrial transgene expression that was originally reported by Yu et al [9]. In this study, the authors constructed the Adenoassociated virus (AAV) with mitochondrial targeting activity, in which the human NADH ubiquinone oxidoreductase subunit 4 (*ND4*) gene linked with HSP (pTR-UF11-*ND4FLAG*) was packaged, and showed that the expression of WT *ND4* in cells with a point mutation in the *ND4* gene, which is implicated in Leber's hereditary optic neuropathy (LHON) restored a defect in ATP synthesis [9]. This report prompted us to hypothesize that a pDNA containing HSP and the endogenous gene (ND4) could achieve mitochondrial transgene expression even without the need for a virus vector, if the successful mitochondrial delivery of pDNA could be achieved.

We recently constructed a pHSP-mtLuc (CGG) that possesses the HSP, *Nd 4* gene derived from mouse mtDNA and an artificial mitochondrial genome with the reporter *NanoLuc (Nluc) luciferase* gene that records adjustments to the mitochondrial codon system [10]. The basic structure of pHSP-mtLuc (CGG) was designed based on pTR-UF11-*ND4FLAG* [9], and an additional sequence was further linked to the 3' terminus of the artificial mitochondrial gene to improve mitochondrial protein translation. In this study, we examined the *in vivo* mitochondrial delivery of pHSP-mtLuc (CGG) to the liver and skeletal muscle of mice *via* hydrodynamic injection, and confirmed that mitochondrial transgene expression had occurred. Hydrodynamic injection, in which a large volume of naked pDNA is rapidly injected, is an efficient method for the *in vivo* nuclear delivery of naked pDNA and has been used in a wide variety of basic and translational studies [11-13]. When naked pDNA was delivered to the livers of mice by hydrodynamic tail vein (HTV) injection, 2 mL of saline containing naked pDNA was injected into the tail vein within a period of 5 sec [14]. A sufficient volume of saline was used to facilitate the extravasation of the pDNA from the vasculature and into the liver tissue through multiple physical

barriers. It has been suggested that hydrodynamic force could induce the transient opening of the cellular membrane, permitting pDNA to be internalized into cells. This may account for the subsequent localization of pDNA in the nucleus. We also succeeded in *in vivo* mitochondrial gene delivery *via* hydrodynamic injection [14]. Moreover, we showed that the hydrodynamic injection of pHSP-mtLuc (CGG) resulted in the expression of the mitochondrial Nluc luciferase protein in liver and skeletal muscle [10].

We next investigated the mitochondrial transgene expression of pHSP-mtLuc (CGG) using cultured cells, because it can be assumed that a mitochondrial transgene expression system for living cells would accelerate mitochondrial gene therapy and further studies directed at mitochondrial molecular biology. However, the transfection of pHSP-mtLuc (CGG) into cultured HeLa cells by lipofection using Lipofectamine 2000 resulted in no luciferase activity [10] (Figure S1). The efficiency of the transfection of pHSP-mtLuc (CGG) using Lipofectamine 2000 therefore appears to be lower than that for hydrodynamic injection, and, as a result, lipofection failed to result in successful mitochondrial transgene expression. Based on these results, our research has focused on improving mitochondrial transgene expression efficiency, to develop a new artificial DNA construct, which can achieve mitochondrial transgene expression in living cells, even when a non-viral vector is used.

In this study, we focused on the Cytomegalovirus (CMV) promoter to improve mitochondrial transgene expression efficiency. The CMV promoter has been used frequently to achieve effective nuclear transgene expression in mammalian cells [15, 16]. However, a virus promotor that is capable of functioning in mitochondrial transgene expression has not been reported. In this study, we validated the utility of an artificial mitochondrial DNA vector with a

virus promoter on mitochondrial transgene expression. For this validation, we designed pCMV-mtLuc (CGG) that contains a CMV promotor and an Nluc luciferase gene optimized for a mitochondrial codon system based on pHSP-mtLuc (CGG) [10] (Figure 1A).We first investigated whether pCMV-mtLuc (CGG) could lead to transgene expression to produce a reporter Nluc luciferase protein in *in vivo* and *in vitro* experiments based on Nluc luciferase activity measurements. Hydrodynamic injection and lipofection were used for *in vivo* and *in vitro* transfection, respectively. Moreover, *in vitro* transcription and translation assays in a nucleo-cytoplasmic translation system were performed to validate the mitochondrial transgene expression of pCMV-mtLuc (CGG) in detail. Finally, we attempted to optimize the mitochondrial transfection of pCMV-mtLuc (CGG) using MITO-Porter system, which liposome-based carrier for mitochondrial delivery *via* membrane fusion [17, 18].

## EXPERIMENTAL SECTION

**Materials.** Cholesteryl hemisuccinate (CHEMS) were purchased from Sigma (St. Louis, MO, USA). 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE) and sphingomyelin (SM) were purchased from Avanti Polar lipids (Alabaster, AL, USA). Stearylated octaarginine (STR-R8) [19] and stearylated KALA (STR-KALA) [20] was obtained from KURABO Industries Ltd (Osaka, Japan). Protamine was purchased from CALBIO CHEM (Darmstadt, Germany). pNL1.1 CMV [Nluc/CMV] Vector was purchased from Promega (Madison, WI, USA). For the visualization of pDNA, Cy5-labeled pDNA was prepared by labeling the molecule with a Mirus Label IT® Cy™5 Labeling Kit (Mirus Corp., Madison, WI, USA), according to the manufacturer's recommended protocol. HeLa human cervix carcinoma cells were obtained from the RIKEN Cell

Bank (Tsukuba, Japan). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Lipofectamine 2000 (LFN 2000) were purchased from Life technologies Corporation (Carlsbad, CA, USA). MitoFluor Red 589 were purchased from Thermo Fisher Scientific Life Sciences (Waltham, MA, USA). Oligonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified form. All other chemicals used were commercially available reagent-grade products.

**Construction and preparation of pDNA containing an artificially designed gene.** The gene fragments for pCMV-mtLuc (CGG), pmtLuc (CGG), pCMV-mtLuc (TAG) and pCMV-mtLuc (CGG)/3xTGA were synthesized by GENEWIZ (South Plainfield, NJ, USA), and the synthesized genes were inserted into the pBluescript II SK(-) (Stratagene, La Jolla, CA, USA) that had been pretreated with restriction enzymes (*EcoR* I and *Sma* I sites). As shown in Figure 1 and Table 1, the pCMV-mtLuc (CGG) contains the CMV promoter, mtLuc (CGG) gene that codes mitochondrial Nluc luciferase containing a TGA codon. If the Nluc luciferase were to be translated in the cytosol, translational arrest would be expected at the TGA codon that is a stop codon as a nuclear genetic codon. The full length version of mitochondrial Nluc luciferase was produced in mitochondrion, because the TGA codon encodes for Typ in mitochondria. We also designed pmtLuc (CGG) without the CMV promoter, pCMV-mtLuc (TAG) that contains a mitochondrial/universal stop codon (TAG) in the mitochondrial Nluc luciferase gene and pCMV-mtLuc (CGG)/3xTGA that contains three TGA codons in the mitochondrial Nluc luciferase gene. Sequence information regarding these plasmids is summarized in the Supporting Information

(Sequences S1-S4). pDNA used in this experiment was amplified in E. coli strain DH5α, purified using an Endfree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany).

**Experimental animals.** Male C57BL/6 mice (6 weeks old) were purchased from Sankyo Labo Service (Sapporo, Japan). All animal protocols were approved by the institutional animal care and research advisory committee at the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan (date: 22 March 2013, registration No. 13-0062).

***In vivo* delivery of pDNA using hydrodynamic injection.** HTV injection was used to deliver pDNA to mice livers, as described in a previous report [14, 21]. The naked pDNA in Saline (2 mL) was injected into the tail vein within a period of 5 sec.

**Cell cultures.** HeLa cells were maintained in complete medium, which is DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were cultured under an atmosphere of 5% $CO_2$/air at 37°C.

**Nluc luciferase assay of liver tissue after hydrodynamic injection of pCMV-mtLuc (CGG) and HeLa cells transfected with the pDNA.** To evaluate the extent of the transgene expression of mitochondrial Nluc luciferase in liver tissue, a 100 μg of pCMV-mtLuc (CGG) that codes for mitochondrial Nluc luciferase was administered to the tail vein of mice *via* HTV injection. In this experiment, we also evaluated the transgene expression of pmtLuc (CGG), pCMV-mtLuc (TAG)

and pCMV-mtLuc (CGG)/3xTGA. At 6 hr post injection, the livers were harvested and minced with scissors. Approximately 200 mg of the minced liver tissue was then homogenized using a PreCellys in 1 mL of Reporter lysis buffer (Promega). After centrifugation at 15,000*g* for 10 min at 4ºC, the supernatant was used in an Nluc luciferase assay using the Nano-Glo Luciferase Assay System (Promega). Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Nluc luciferase activities are expressed as relative light units (RLU) per mg of protein.

To evaluate the transgene expression of mitochondrial Nluc luciferase in HeLa cells, the HeLa cells ($4 \times 10^4$ cells/well) were seeded on a 24 well plate (Corning, NY, USA) one day before the transfection of pDNA by LFN 2000. The cells were incubated with carriers containing 0.4-μg pDNA suspended in 0.25 mL of serum-free DMEM were added to the cells, followed by incubation under 5% $CO_2$ at 37ºC for 3 hr. The medium was then replaced to 1 mL of fresh DMEM containing 10% serum, followed by incubation for 21 hr. LFN 2000 was used according to the manufacturer's recommended protocol. The cells were then washed with phosphate-buffer saline (PBS (−)) and solubilized with reporter lysis buffer (Promega), and the resulting cell lysate was used to estimate Nluc luciferase activity, as described above.

To estimate the extent of inhibition of nuclear transcription, the cells were treated with α-amanitin (final concentration 1, 10 ng/mL), a specific inhibitor of RNA polymerase II [22], during the transfection. In this experiment, we applied 0.4 μg of pCMV-mtLuc (CGG) or 0.4 fg of pNL1.1 CMV [Nluc/CMV] Vector, to obtain similar Nluc activity in the absence of α-amanitin. Before transfection with LFN 2000, the pNL1.1 CMV [Nluc/CMV] Vector (0.4 fg) was mixed with 'carrier DNA', the pBluescript II SK(-), to give a total amount of 0.4 μg.

**Detection exogenous mRNA in mice liver using RT-PCR.** A 100 μg portion of pCMV-mtLuc (CGG), pCMV-mtLuc (TAG), pCMV-mtLuc (CGG)/3xTGA or pmtLuc(CGG) were administered *via* the tail vein of mice *via* HTV injection. At 6 hr after the injection, the livers were harvested and minced with scissors. Total RNA was then purified with the TRIzol reagent (Life technologies Corporation) according to the manufacturer's recommended protocols, combined with DNase I digestion for the degradation of DNA in total RNA samples using recombinant DNase I (20U, Takara, Kusatsu, Japan) and recombinant RNase Inhibitor (Takara). The resulting RNA suspension (1 μg) was reverse transcribed using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). PCR was performed on cDNA using GoTaqGreen Master Mix kit (Promega, Madison, WI, USA) to detect exogenous mRNA coding *Nluc luciferase*, using primers (Nluc (+): CCTTGAACAGGGAGGTGTGT and Nluc (-): TTGATGGTTACTCGGAACAGC). Each PCR products (5 μL) were subjected to electrophoresis in a 1.5% agarose gel in TAE (40 mM TrisHCl, 40 mM acetic acid, 1 mM EDTA, pH 8.0) at 100 V for 30 min. The DNA bands were visualized by UV after staining with GelRed (Biotium, Hayward, CA, USA).

***In vitro* transcription and translation.** To demonstrate that pCMV-mtLuc (CGG) produces no Nluc luciferase in the nucleo-cytoplasmic translation system, we performed *in vitro* translation assays in the nucleo-cytoplasmic translation system. We first prepared mRNAs coding mtLuc (CGG), mtLuc (TAG) and wild type Nluc luciferase (Structural information of mRNAs is shown in Figure S2) using template DNAs containing the CMV promoter and using a HeLaScribe Nuclear Extract *in vitro* Transcription System (Promega), according to the manufacture's recommended protocol and the transcribed RNA was purified by ethanol precipitation. The RNA pellet was hydrated with sterilized water containing recombinant DNase I (20U, Takara). We used 10 ng of

pCMV-mtLuc (CGG), pCMV-mtLuc (TAG) or pNL1.1 [CMV/nLuc] Vector as template DNAs for mtLuc (CGG), mtLuc (TAG) and wild type Nluc luciferase. The resulting mRNA suspension (5 ng) was added to the Rabbit Reticulocyte Lysate (Promega), followed by in vitro nucleo-cytoplasmic translation according to the manufacture's recommended protocol. The translational products (Nluc luciferase) were used to estimate the Nluc luciferase activity, as described above.

**Packaging of pCMV-mtLuc (CGG) in MITO-Porter.** Condensed particles of pCMV-mtLuc (CGG) (final DNA concentration, 35 μg/mL) were prepared as follows; a solution of pDNA was added to the protamine solution in 10 mM HEPES buffer (pH7.4) under vortexing at a nitrogen/phosphate ratio of 2.3. A lipid film was prepared by the evaporation of a chloroform solution of lipid (DOPE/SM/CHEMS = 9:2:1, molar ratio) on the bottom of a glass tube, follow by hydration with 250 μL of the condensed particles of pCMV-mtLuc (CGG) suspensions for 15 min at room temperature. The glass tube was sonicated for 30 sec in a bath-type sonicator (AU-25C; Aiwa Co., Tokyo, Japan) to package the condensed particles of pCMV-mtLuc (CGG) in lipid envelope. The resulting suspension was incubated with STR-R8 or STR-KALA (10 mol% of total lipids) for 30 min at room temperature to produce R8-MITO-Porter or KALA-MITO-Porter. The diameters and ζ-potentials were measured using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

**Evaluation of mitochondrial transfection activity of MITO-Porter using HeLa cells.** HeLa cells ($8 \times 10^4$ cells/well) were seeded on a 24 well plate (Corning) one day before the transfection of pCMV-mtLuc (CGG). The cells were incubated with carriers containing 0.4-μg pDNA

suspended in 0.25 mL of serum-free DMEM, followed by measuring Nluc luciferase activity after 24 hr incubation, as described above.

**Evaluation of the cellular uptake of pCMV-mtLuc (CGG) by flow cytometry.** HeLa cells ($1x10^5$ cells/well) were seeded on a six-well plate (Corning), and incubated with DMEM, containing 10% FBS under an atmosphere of 5% $CO_2$/air at 37°C for 24 hr. Cells were washed with PBS (−) before incubation with the carriers. The cells were incubated with carriers containing 1-μg Cy-5 labeled pCMV-mtLuc (CGG) suspended in 1 mL of serum-free medium, followed by incubation under 5% $CO_2$ at 37ºC for 1 hr. After removing the medium, the cells were washed once with ice-cold PBS (−) and then twice with ice-cold PBS (−) containing heparin (20 U/mL). The cells were trypsinized and then suspended in DMEM with serum. After centrifugation (700 g, 4 °C, 3 min), the supernatant was removed and cells were resuspended in 0.5 mL of PBS (−) containing 0.5% bovine serum albumin (BSA) and 0.1% $NaN_3$. The cell suspension was filtered through a nylon mesh followed by analysis by flow cytometry (Gallious; Beckman Coulter Inc., Pasadena, CA, USA). Cy5 was excited at 638nm light and fluorescence detection channel was set to FL6 filter for Cy5.

**Intracellular observation of pCMV-mtLuc (CGG) transfected by MITO-Porter.** HeLa cells ($1 \times 10^5$ cells/dish) were seeded on a 3.5 cm glass-base dish (Iwaki; ASAHI GLASS Company, Ltd., Tokyo, Japan) at one day before transfection and incubated in DMEM, which contained 10% FBS, under an atmosphere of 5% $CO_2$/air at 37°C. The cells were incubated with carriers containing 1-μg Cy-5 labeled pCMV-mtLuc (CGG) suspended in 1 mL of phenol red-free medium without FBS, followed by incubation under 5% $CO_2$ at 37ºC. After 1-hr incubation, the medium

was replaced with fresh phenol red-free medium containing FBS, and the cells were incubated for 2 hr. The medium was replaced with fresh medium containing MitoFluor Red 589 (final concentration, 100 nM) 20 min before the acquisition of the fluorescence images, and the cells were incubated in this solution. After this incubation, the cells were washed with the phenol red-free medium containing FBS, and then observed by CLSM (FV10i-LIV; Olympus Corporation, Tokyo, Japan). The cells were excited with a 559 nm light for detecting MitoFluor Red 589 and a 635 nm light for detecting Cy5 using a LD laser. Images were obtained using a FV10i-LIV equipped with a water immersion objective lens (UPlanSApo 60x/NA = 1.2) and a diachronic mirror (DM 405/473/559/635). The two fluorescence detections Chs were set to the following filters: Ch1: BP 570-620 (red color) for MitoFluor Red 589 and Ch2: BP 660-710 (green pseudo color) for Cy5.

**Statistical analysis.** Data are expressed as the mean ± S.D. for the indicated number of experiments. For multiple comparisons in Figures 2A, 2B, 3, 4 and 6A, one way ANOVA followed by bonfferoni test was performed. In Figure 5, we performed two-way ANOVA analysis to compare the effect of two factors that are "pDNA type" and "applied dose of α-amanitin". $p < 0.05$ was considered to be statistically significant.

## RESULTS

### Design of pCMV-mtLuc (CGG) with a CMV promotor to express mitochondrial Nluc Luciferase.

We recently constructed pHSP-mtLuc (CGG) that contained HSP as a promoter, and showed that the hydrodynamic injection of pHSP-mtLuc (CGG) resulted in the expression of the mitochondrial Nluc luciferase protein in the liver and skeletal muscle [10]. However, the transfection of pHSP-mtLuc (CGG) into cultured HeLa cells resulted in no luciferase activity [10] (Figure S1). Thus, we focused on the use of a CMV promoter to improve mitochondrial transgene expression efficiency, and designed a new candidate mitochondrial reporter DNA vector (pCMV-mtLuc (CGG)).

As shown in Figure 1A, the pCMV-mtLuc (CGG) contains the CMV promoter, *mtLuc (CGG)* gene that codes mitochondrial Nluc luciferase, and $tRNA^{Asp}$ with the 5'UTR. The conventional *Nluc luciferase* gene can be translated on cytoplasmic ribosomes, but not in mitochondria, because the AGG codon at amino acid 45 (bases 133-135) encodes for Arg in the nuclear genetic code, while the AGG codon leads to the arrest of mitochondrial translation (Table 1). While, in the case of the *mtLuc (CGG)* gene contained in pCMV-mtLuc (CGG), the AGG codon at amino acid 45 (bases 133-135) was changed into a CGG codon which encodes for Arg in the both the nuclear genetic and the mitochondrial code. Moreover, the *mtLuc (CGG)* gene contains a TGA codon at amino acid 12 (bases 34-36), which encodes for mitochondrial Trp and the nuclear genetic stop codon (Table 1), therefore mitochondrial Nluc luciferase cannot be produced outside mitochondria. Thus, we expected that luciferase activity would be detected as the result of the transfection of pCMV-mtLuc (CGG) resulting from mitochondrial transgene expression *via* mitochondrial translation.

To date, there are no reports that indicate that a CMV promoter contributes to mitochondrial transgene expression, although the CMV promoter is an effective and well-known

promoter for nuclear transgene expression [15, 16]. In this study, we designed pmtLuc (CGG) without a CMV promoter (Figure 1B) for comparison with pCMV-mtLuc (CGG) to validate the utility of the CMV promoter for mitochondrial transgene expression. We also designed a pCMV-mtLuc (TAG) containing the *mtLuc (TAG)* gene, in which the TAG codon at amino acid 45 (bases 133-135), a mitochondrial/universal stop codon, as a dual stop codon variant (Figure 1C). Moreover, we designed pCMV-mtLuc (CGG)/3xTGA that contains the *mtLuc (CGG)/3xTGA* gene which possesses three TGA codons in the mitochondrial Nluc luciferase gene (Figure 1D). The positions of point mutations in the conventional *Nluc luciferase* gene in the design of new constructs are summarized in Table 1. Sequence information regarding these plasmids is shown in Supplementary Vector sequences (Sequences S1-S4).

**Investigation of the effect of a CMV promoter contained by pCMV-mtLuc (CGG) on mitochondrial transgene expression.**

To evaluate the transgene expression of pCMV-mtLuc (CGG), we measured Nluc luciferase activity produced by mice liver mitochondria, after the HTV injection of pCMV-mtLuc (CGG). We also evaluated the transgene expression of pmtLuc (CGG) without the CMV promoter as a negative control. The findings showed that the Nluc luciferase activity in liver tissue administered pCMV-mtLuc (CGG) ($10^4$ - $10^5$ RLU/mg protein) was significantly higher than that produced using pmtLuc (Figure 2A). While, previous studies reported that the value of Nluc luciferase activity with pHSP-mtLuc was low ($10^3$ – $10^4$ RLU/mg protein) [10], and that no activity was detected in cultured cells (Figure S1). We expected that pCMV-mtLuc (CGG) would achieve mitochondrial transgene expression, even in cultured cells, because the efficiency of the

mitochondria transgene expression of pCMV-mtLuc (CGG) was much higher than that of pHSP-mtLuc (CGG) in the case of the HTV injection.

Nluc luciferase activities were measured after a 24 hr period of transfection with pCMV-mtLuc (CGG) or pmtLuc(CGG) in HeLa cells using LFN 2000, which is a well-known cationic lipid-based transfection reagent for achieving nuclear transgene. We expected that lipofection using LFN 2000 would deliver pDNA to mitochondria as well as the nucleus, because it has been reported that certain types of cationic liposomes have a high mitochondrial affinity [17, 23-25]. As shown in Figure 2B, Nluc luciferase activity with pCMV-mtLuc (CGG) was detected in HeLa cells, and the value was significantly higher than that with pmtLuc (CGG). These results suggest that pCMV-mtLuc (CGG) has a higher mitochondrial transgene expression efficiency than pHSP-mtLuc (CGG), indicating that the combination of a CMV promoter and a gene optimized for use in a mitochondrial codon system can be useful for mitochondria transgene expression.

**Validation of transgene expression of mitochondrial Nluc luciferase in liver tissue following HTV injection of pCMV-mtLuc (CGG).**

To validate whether the luciferase activities detected in Figure 2 resulted from the transgene expression of the mitochondrial Nluc luciferase protein, we evaluated the transgene expression of pCMV-mtLuc (TAG), which contains the mitochondrial/universal stop codon (TAG), pCMV-mtLuc (CGG)/3xTGA that contains three TGA codons, which encode for mitochondrial Trp and a nuclear genetic stop codon and pmtLuc (CGG) without a CMV promoter as controls. The pCMV-mtLuc (CGG) or other sample pDNA molecules were administered to the tail vein of mice *via* HTV injection, and the livers were harvested at 6 hr postinjection.

Before measuring Nluc luciferase activity, we investigated the mRNA levels of *Nluc luciferase* following HTV injection (Figure 3A). PCR involved the use of specific primers to detect the cDNA derived from exogenous mRNA coding the *Nluc luciferase* after reverse transcription. As a result, the mRNAs coding for the *Nluc luciferase* (405 bp) derived from pCMV-mtLuc (CGG), pCMV-mtLuc (TAG) and pCMV-mtLuc (CGG)/3xTGA were detected, while the corresponding mRNA was not detected in the case of pmtLuc (CGG). We also confirmed the absence of contaminating DNA when this RT–PCR assay was peformed. Gel images of the RT–PCR detection showed that the target DNA bands appeared in the case of reverse transcription (RT(+)) (upper panels in Figure 3A) but were not observed in the absence of reverse transcription (RT(-)) (lower panels in Figure 3A). These results indicate that the DNase I treatment procedure during the RT-PCR assay was properly performed.

As shown in Figure 3B, the Nluc luciferase activity in liver tissue that had been administered pCMV-mtLuc (CGG) was significantly higher than that with pCMV-mtLuc (TAG). This result indicates that the TGA codon for mitochondrial Trp and the nuclear genetic stop codon in pCMV-mtLuc can achieve mitochondrial translation successfully, while the TAG codon for the mitochondrial/universal stop codon arrests mitochondrial translation as well as cytosolic translation. Moreover, it was confirmed that the Nluc luciferase activity achieved with pCMV-mtLuc (CGG)/3xTGA that contains three TGA codons was high, comparable to that with pCMV-mtLuc (CGG) which contains one TGA codon (Figure 3B), strongly suggesting that these detected luciferase activities were derived from the Nluc luciferase protein produced in mitochondria and not in the nucleus.

***In vitro* translation assay of the nucleo-cytoplasmic translation system.**

The results described above indicate that pCMV-mtLuc (CGG) can be transcribed into mtLuc (CGG)-mRNA in both the nucleus and mitochondria. Thus, we were concerned about the possibility that mtLuc (CGG)-mRNA might be translated to a protein *via* a cytosolic translation system. To demonstrate that mtLuc (CGG)-mRNA produces no Nluc luciferase in the cytoplasmic translation system, we performed *in vitro* translation assays in the nucleo-cytoplasmic translation system using a Rabbit Reticulocyte Lysate system. It was expected that only the nuclear genetic codon would function in this system, because the Rabbit Reticulocyte Lysate system is not optimal for the mitochondrial translation system.

For this validation, we used mRNAs coding *mtLuc (CGG)* and *mtLuc (TAG)*, which contain a universal stop codon "TGA", and mRNA coding *wild type Nluc luciferase* (positive control). These mRNAs were added to a Rabbit Reticulocyte Lysate for nucleo-cytoplasmic translation, and Nluc luciferase activities were measured. Structural information regarding the mRNAs used in this experiment is summarized in Figure S2. As a result, no Nluc luciferase activity was detected when mRNAs coding *mtLuc (CGG)* and *mtLuc (TAG)* were used, while strong Nluc luciferase activity was observed in the case of mRNA coding for *wild type Nluc luciferase* (Figure 4). The result suggests that the "TGA" codon substitution prevented the correct nucleo-cytoplasmic translation. These results support the conclusion that the mRNA coding mtLuc (CGG) is, in fact, translated in mitochondria.

**Investigation of transcription of pCMV-mtLuc (CGG) on transgene expression.**

The findings shown in Figure 3A suggest that pCMV-mtLuc (CGG) was transcribed inside cells, however we were unable to determine whether pCMV-mtLuc (CGG) could be

transcribed in mitochondria as well as nuclei. Although the results in Figure 4 indicate that the mRNA coding mtLuc (CGG) could be translated in mitochondria, we were unable to clarify whether the mRNA was produced *via* mitochondrial transcription. To validate this point, we investigated the transcription of pCMV-mtLuc (CGG) on transgene expression.

pCMV-mtLuc (CGG) was transfected to HeLa cells using LFN 2000, after treatment with α-amanitin. We also used a pNL1.1 CMV [Nluc/CMV] Vector that codes Nluc luciferase optimized for the nuclear genetic codon system, and produces the Nluc luciferase protein *via* nuclear transcription/cytosolic translation. The α-amanitin molecule inhibits the nuclear transcription process, thus the amounts of the mRNA transcribed in nuclei would be expected to be decreased as a function of the concentration of α-amanitin, resulting in a decrease in Nluc luciferase activity derived from nuclear transcripts. While, it is expected that we would be able to detect Nluc luciferase activity derived from the mRNA transcribed in mitochondria in presence/absence of α-amanitin.

In this experiment, we applied 0.4 μg of the pCMV-mtLuc (CGG) or 0.4 fg of the pNL1.1 CMV [Nluc/CMV] Vector, to produce a similar Nluc activity in absence of α-amanitin (left two bars in Figure 5). We performed two-way ANOVA analysis, and found significant differences between pCMV-mtLuc (CGG) and pNL1.1 CMV [Nluc/CMV] Vector in the presence of α-amanitin. In the case of the transfection with pCMV-mtLuc (CGG), Nluc luciferase activities were observed in absence/presence of α-amanitin (closed bars in Figure 5). This result suggests that pCMV-mtLuc (CGG) can produce the protein *via* mitochondrial transcription/translation. On the other hand, the Nluc luciferase activities were drastically decreased with increasing concentrations of α-amanitin, when the pNL1.1 CMV [Nluc/CMV] Vector was transfected (open bars in Figure

5), indicating that α-amanitin decreases the amounts of the mRNA transcribed in nuclei, resulting in a decrease in Nluc luciferase activity.

**Mitochondrial transfection of pCMV-mtLuc (CGG) using MITO-Porter system.**

We attempted to optimize the mitochondrial transfection of pCMV-mtLuc (CGG) using a MITO-Porter system, which is a liposome-based carrier for mitochondrial delivery *via* membrane fusion [17] [18]. It was expected that a strategy involving membrane fusion using a MITO-Porter system would permit the cargo to be delivered to mitochondria, independent of the size and physical properties of the cargoes. To date, we have shown that the mitochondrial delivery of antisense oligo RNA by the MITO-Porter results in the knockdown of mitochondrial RNA and has a functional impact on mitochondria [26]. Based on our previous report, we conclude that the MITO-Porter has the ability to deliver pCMV-mtLuc (CGG) to the mitochondrial matrix and achieve mitochondrial gene expression.

We packaged pCMV-mtLuc (CGG) in MITO-Porter, and R8 or KALA-peptides were modified on the surface of the carrier to produce R8-MITO-Porter and KAKA-MITO-Porter. R8 [19], which mimics Trans Activator Transcription (TAT) peptide, has been used for cellular uptake and mitochondrial targeting of MITO-Porter [17]. KAKA peptide is cationic amphipathic cell-penetrating peptide [27], and the repeat sequence in this peptide (Lys-Lue-Ala) is expected to function as mitochondrial targeting sequence [28, 29]. The diameter and the ζ potential of the carriers are listed in Table 2. Based on these results, positively charged particles were prepared in both the case of the R8-MITO-Porter and KALA-MITO-Porter encapsulating pCMV-mtLuc (CGG).

After a 24 hr period of transfection of pCMV-mtLuc (CGG) to HeLa cells by R8-MITO-Porter, KALA-MITO-Porter and LFN 2000, Nluc luciferase activity was measured in order to evaluate the mitochondrial transfection activity. As shown in Figure 6A, the transfection by KALA-MITO-Porter showed high Nluc luciferase activity (more than $10^4$ RLU/mg protein), and the value was significantly higher than that by R8-MITO-Porter and LFN 2000. To verify that efficient transgene expression by KALA-MITO-Porter was the result of the efficient delivery of pCMV-mtLuc (CGG) to mitochondria, we analyzed the intracellular distribution of the pDNA-mtLuc (CGG). In this experiment, the Cy-5 labeled pCMV-mtLuc (CGG) was used, and cellular uptake was evaluated using flow cytometry (Figure 6B) and the intracellular observation was performed using CLSM (Figure 6C).

Flow cytometry analyses showed that pCMV-mtLuc (CGG) was efficiently taken up by HeLa cells in the case of KALA-MITO-Porter and LFN 2000, the MFI values was considerable higher than that in the case of R8-MITO-Porter (Figure 6B). Images of intracellular observations were shown in Figure 6C. In the case of the KALA-MITO-Porter, numerous green and some yellow dots were observed in cells (Figure 6C), indicating that the Cy5-labeled pCMV-mtLuc (CGG) (pseudo green color) were mainly localized in the cytosol and some pDNAs were localized in red-stained mitochondria. When R8-MITO-Porter was used, some yellow signals were observed (Figure 6C). While, the yellow signals were hardly observed in the case of LFN 2000 (Figure 6C). Collectively, the KALA-MITO-Porter was efficiently internalized by the cells, and delivered large amounts of the pCMV-mtLuc (CGG) to mitochondria, thus contributing to the high mitochondrial transgene expression.

## DISCUSSION

To achieve nuclear transgene expression, a number of useful technologies, including pDNA vectors have been reported to date [2-5]. Based on such a current trend, many researchers have focused on the mitochondrial targeting of exogenous protein *via* allotropic expression, where an engineered gene coding a protein fused with a mitochondrial targeting signal peptide (MTS) is transcribed/translated *via* nuclear transcription/cytosolic translation, and the gene product is then delivered to mitochondria *via* the MTS import machinery. The current state of research concerning mitochondrial delivery by allotropic expression have recently been summarized in an excellent review [30].

The majority of mitochondrial proteins are synthesized on cytosolic ribosomes, and the these proteins are imported into mitochondria *via* MTS import machinery [31]. Thus, allotropic expression using MTS would be expected to be a useful method for the mitochondrial targeting of exogenous protein, however this method faces severe problems as a tool for certain of mitochondrial proteins encoded in mtDNA. One is that the gene sequence of the mtDNA encoded proteins needs to be adjusted to achieve cytosolic translation. Even if the mitochondrial endogenous proteins fused with MTS were produced in cytosol, it would not be possible to effectively deliver them to mitochondria, because of the highly hydrophobic nature of mtDNA-encoded proteins [32].

Corral-Debrinski and colleagues recently reported on the development of a AAV vector harboring a gene in which the mtDNA sequences were combined with the MTS in 5′ and the 3′UTR of the nuclear *COX10* gene, to optimize allotropic expression aimed at a gene therapy for *ND4* [33]. As explained by the authors, after nuclear transcription, the hybrid mRNA is associated with the *cis*-acting elements of the *COX10* mRNA and becomes localized on the mitochondrial surface, where cytosolic translation and mitochondrial transport *via* the MTS import machinery are tightly

coupled. This finding promises to accelerate research for mitochondrial gene therapy *via* allotropic expression, although there remains a concern regarding the utility of this strategy for mtDNA-encoded proteins other than ND4.

On the other hand, mitochondrial gene therapy could be also achieved by other strategies including the import of therapeutic agents including wild-type mtDNA into mitochondria (mitochondrial transfection). To achieve such an innovative strategy, several promising studies focusing on the combination of using a mitochondrial codon and a mitochondrial endogenous promotor have been reported to date [6-9]. Based on previous interesting reports, we are in the process of continuously developing more efficient methods of mitochondrial gene delivery *via* hydrodynamic injection [14]. The hydrodynamic injection originally reported by Liu *et al.* [21] and Zhang *et al.* [34] is frequently as an efficient *in vivo* nuclear gene transfer method of naked pDNA [11-13]. We also reported that hydrodynamic injection could achieve *in vivo* mitochondrial gene delivery, and that this technique had no significant effect on mitochondrial function [14]. Moreover, we showed that the hydrodynamic injection of pHSP-mtLuc (CGG) resulted in mitochondrial transgene expression in both the liver and skeletal muscle [10].

While, the transfection of pHSP-mtLuc (CGG) into cultured cells by lipofection failed to result in mitochondrial transgene expression [10] (Figure S1). We concluded that the establishment of a simple and convenient mitochondrial transgene method for living cells would be very valuable, because such a method could largely contribute to the research efforts focusing on mitochondrial gene therapy and mitochondrial molecular biology. Thus, in this study, we validated that the use of pCMV-mtLuc (CGG) with a CMV promoter, which is a well-known strong promoter of nuclear transcription, resulted in mitochondrial transgene expression in HeLa cells even by lipofection using LFN 2000. As shown in Figure 2B, the transfection of pCMV-mtLuc (CGG) using LFN

2000 resulted in successful mitochondrial transgene expression in HeLa cells.

This result suggests that the pCMV-mtLuc (CGG) would be delivered to mitochondria by LFN 2000, and that the pDNA would be used to produce the Nluc luciferase protein *via* mitochondrial transcription/translation as shown in Figure S3A. As another possibility, we hypothesized that the cytosolic mRNA might be translated to the Nluc luciferase protein in the cytosol, because the CMV promoter can function in the nucleus to efficiently transcribe cytosolic mRNA (Figure S3B). To validate this point, we performed *in vitro* translation assays in a nucleo-cytoplasmic translation system, and confirmed that mtLuc (CGG)-mRNA was not translated to the protein *via* the cytosolic translation system (Figure 4). We also considered the possibility that cytosolic mtLuc (CGG)-mRNA might be delivered to mitochondria, and the mRNA would then be translated to the protein in mitochondria. This possibility can be rejected by the result showing that the inhibition of the nuclear transcription of pCMV-mtLuc (CGG) to decrease the mRNA-levels has no effect on the transgene expression of Nluc luciferase (Figure 5). Collectively, we conclude that the luciferase activities detected in Figure 2B can be attributed to the mitochondrial delivery of the pCMV-mtLuc (CGG) by LFN 2000.

Although LFN 2000 is a well-known nuclear transfection reagent, there have been no reports of mitochondrial transfection using LFN 2000. Here, we considered the reasons for why LFN2000 could achieve mitochondrial delivery of pDNA. As shown in previous reports [17, 23-25], positively charged carriers can bind to negatively charged mitochondria in living cells *via* electrostatic interactions. Thus, pDNA would be accessible to mitochondria by LFN2000, which is positively charged. We next examined the issue of how pDNA overcomes the mitochondrial membrane. It has been previously reported that isolated mitochondria can take up linear DNA in mammalian tissues [35] and furthermore linear turnip 11.6 kb mitochondrial pDNA was

internalized into human mitochondria [36]. These previous reports prompted us to hypothesize that pDNA localized near mitochondria might be internalized into mitochondria. On the other hand, Kolintchenko et al. reported that circular pDNA was not delivered into mitochondria in plants [37]. An alternative possibility is that the mitochondrial uptake of circular pDNA occurs during mitochondrial fusion and fission, since mitochondria are continually undergoing this process [38]. This important issue needs to be investigated in more detail in the future, since the details of the mechanism responsible for this are currently unclear.

We investigated the mitochondrial transgene expression when pCMV-mtLuc (CGG) was transfected by MITO-Porter system (Figure 6). As shown in Figure 6A, KALA-MITO-Porter showed the higher transfection activity than R8-MITO-Porter and LFN 2000. It is suggested that this high transfection activity by KALA-MITO-Porter was the result of the efficient cellular uptake and mitochondrial delivery as shown in Figures 6B and 6C. Transfection activity of LFN 2000 was lower than that of KALA-MITO-Porter, although the cellular uptake efficiencies were comparable as shown in Figure 6B. This fact indicates that mitochondrial targeting plays an important role for mitochondrial transgene expression, because mitochondrial localization of pCMV-mtLuc (CGG) was few in the case of transfection by LFN 2000 (Figure 6C). On the other hand, the transfection activity of R8-MITO-Porter was lower than that of KALA-MITO-Porter, although R8-MITO-Porter could deliver pCMV-mtLuc (CGG) to mitochondria in HeLa cells (Figure 6C). As the cellular uptake efficiency of KALA-MITO-Porter was higher than that of R8-MITO-Poter, the amount of pCMV-mtLuc (CGG) delivered to mitochondria by KALA-MITO-Porter would be larger than that of R8-MITO-Porter. This fact might contribute to highier mitochondrial transfection by KALA-MITO-Porter than R8-MITO-Porter. As another possibility, KALA might enhance membrane fusion between MITO-Porter and mitochondria, because KALA

can destabilize mitochondrial membrane [28]. Enhancement of membrane fusion by KALA might promote mitochondrial delivery of pCMV-mtLuc (CGG), resulting in high mitochondrial transgene expression. This important issue regarding membrane fusion activity of the carriers needs to be investigated in more detail in the future.

We compared cellular protein concentrations after the transfection of pCMV-mtLuc (CGG) using each of the carriers. The protein concentrations were determined using a BCA protein assay kit, and relative cellular protein concentration (%) was calculated by dividing the concentration of cellular protein in treated cells with carriers that in untreated cells. The values for the R8-MITO-Porter, KALA-MITO-Porter and LFN 2000 treatments were 101 ± 1%, 51 ± 5% and 109 ± 4% (n = 3). This result indicates that the KALA-MITO-Porter showed higher cytotoxicity than the R8-MITO-Porter and LFN 2000. The cytotoxicity of the KALA-MITO-Porter might be explained by the fact that KALA can destabilize the mitochondrial membrane. We conclude that the reduction of toxicity induced by the carrier is an important issue, although the KALA-MITO-Porter showed the highest mitochondrial transgene expression. We continue to direct our efforts toward constructing new carriers for mitochondrial transgene expression with low cytotoxicity.

In conclusion, we demonstrated the utility of an artificial mitochondrial DNA vector with a virus promoter on achieving mitochondrial transgene expression using pCMV-mtLuc (CGG). Moreover, we optimized the mitochondrial transfection of pCMV-mtLuc (CGG) using MITO-Porter system. Our mitochondrial transgene expression system has the potential to be a useful tool for nanoscience and nanotechnology to control intracellular microenvironment *via* regulating mitochondrial function and further open innovative research fields.

## ASSOCIATED CONTENT

### Supporting Information

Supporting Information includes **Supplementary Figure** (*Figures S1-S3*) and **Supplementary Vector sequences** (*Sequences S1-S6*).


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TABLE

**Table 1.** Positions of point mutations in Nluc luciferase gene to construct artificial mitochondrial reporter DNA vector

| Gene | Codon (position) | Amino acid encoded by the codon |
|---|---|---|
| **Nluc [Wild-type]** | tgg (34-36) | **Trp** for universal and mitochondrial codon |
| | agg (133-135) | **Arg** for universal codon / **Stop codon** for mitochondrial codon |
| | tgg (400-402) | **Trp** for universal and mitochondrial codon |
| | tgg (487-489) | **Trp** for universal and mitochondrial codon |
| **mtLuc (CGG)** | tgA (34-36) | **Stop codon** for universal codon / **Trp** for mitochondrial codon |
| | Cgg (133-135) | **Arg** for universal and mitochondrial codon |
| **mtLuc (TAG)** | tgA (34-36) | **Stop codon** for universal codon / **Trp** for mitochondrial codon |
| | TAg (133-135) | **Stop codon** for universal and mitochondrial codon |
| **mtLuc (CGG)/3xTGA** | tgA (34-36) | **Stop codon** for universal codon / **Trp** for mitochondrial codon |
| | Cgg (133-135) | **Arg** for universal and mitochondrial codon |
| | tgA (400-402) | **Stop codon** for universal codon / **Trp** for mitochondrial codon |
| | tgA (487-489) | **Stop codon** for universal codon / **Trp** for mitochondrial codon |

Positions of point mutation in Nluc luciferase gene are indicated by upper case.

**Table 2.** Characteristics of MITO-Porter encapsulating pCMV-mtLuc (CGG)

| **Carrier-type** | Lipid composition (mol ratio) | Diameter (nm) | ζ-potential (mV) |
|---|---|---|---|
| **R8-MITO-Porter** | DOPE/SM/CHEMS/STR-R8=9/2/1/1.2 | 726±24 | 56±3 |
| **KALA-MITO-Porter** | DOPE/SM/CHEMS/STR-KALA=9/2/1/1.2 | 747±64 | 53±1 |

All types of carriers contain condensed particles of pCMV-mtLuc (CGG). Data denote the mean ± S.D. (n=3).

FIGURE LEGENDS.

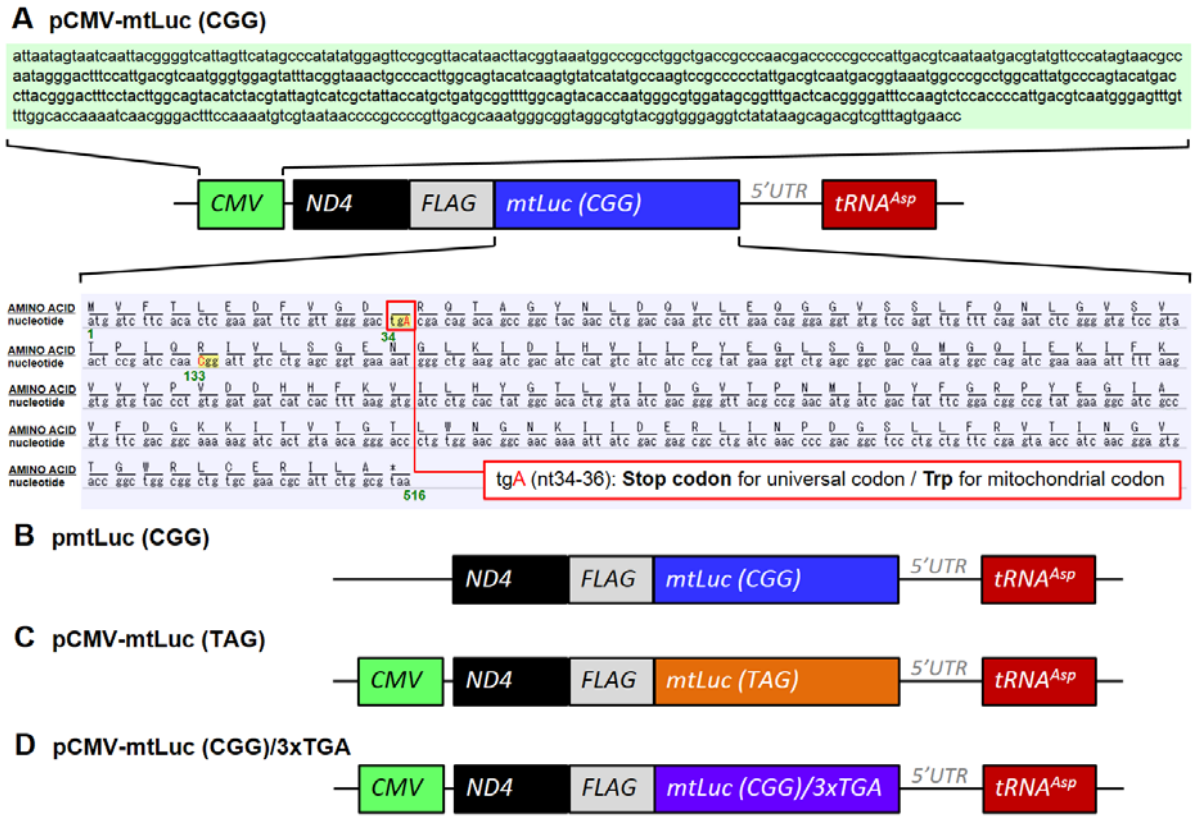


**Figure 1.** Design of pCMV-mtLuc (CGG) and the DNA vector analogues. The pCMV-mtLuc (CGG) (**A**) contains the CMV promoter, the mtLuc (CGG) gene that codes mitochondrial Nluc luciferase and $tRNA^{Asp}$ with the 5'UTR. If the Nluc luciferase was translated in the cytosol, translational arrest would be expected at codon "tgA" (nt34-36), a stop codon in the nuclear genetic codon. While, the full length version of mitochondrial Nluc luciferase was produced in mitochondrion, because "tgA" codon encodes for Typ in mitochondria. Positions of point mutation in Nluc luciferase gene to construct mtLuc (CGG) gene are indicated by upper case. The designs of plasmids including pmtLuc (CGG) (**B**), pCMV-mtLuc (TAG) (**C**) and pCMV-mtLuc (CGG)/3xTGA (**D**) used in this study are shown. The sequence information of these plasmids are shown in Supplementary Vector sequences (Sequences S1-S4).

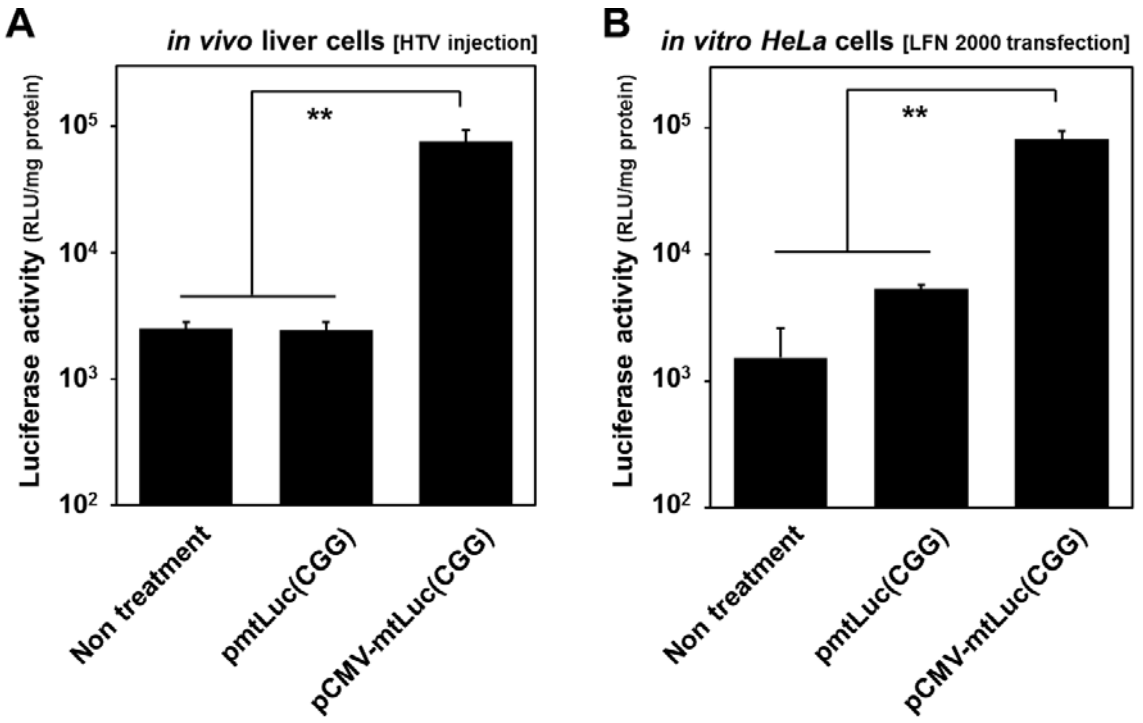


**Figure 2.** Evaluation the transgene expression of pCMV-mtLuc (CGG). (**A**) A 100 μg of pCMV-mtLuc (CGG) was administered *via* the tail vein of mice *via* HTV injection, and the livers were harvested at 6 hr postinjection and the Nluc luciferase activities were measured. In this experiment, we also administrated pmtLuc (CGG) without CMV promoter as a negative control. Bars represent the means±SD. (n=3). **Significant differences were calculated by one-way ANOVA, followed by bonfferoni test ($p<0.01$). (**B**) A 0.4 μg of pCMV- mtLuc (CGG) or pmtLuc (CGG) was transfected to HeLa cells using LFN 2000. After 24 hr transfection, the Nluc luciferase activities were measured. Bars represent the mean±SD. (n=3). **Significant differences were calculated by one-way ANOVA, followed by bonfferoni test ($p<0.01$).

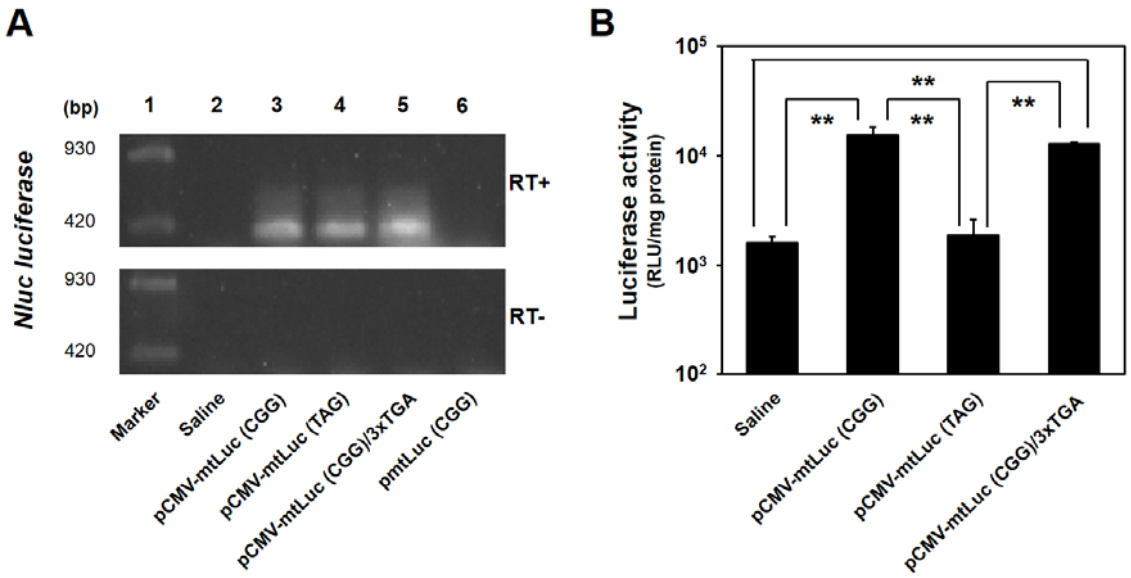


**Figure 3.** Validation of transgene expression of mitochondrial Nluc luciferase in liver tissue following the HTV injection of pCMV-mtLuc (CGG). A 100 μg of pCMV-mtLuc (CGG) or other sample pDNA was administered to the tail vein of mice *via* HTV injection, and the livers were harvested at 6 hr postinjection. For gel images of exogenous mRNA (**A**), total RNA was extracted from the harvested livers, and PCR was done with specific primers to detect exogenous mRNA coding *Nluc luciferase* gene (405 bp) after reverse transcription (RT (+), upper panel) and before reverse transcription (RT (-), lower panel). The PCR products were subjected to electrophoresis. Lane 1, DNA ladder marker; lane 2, saline administration; lane 3, pCMV-mtLuc (CGG) administration; lane 4, pCMV-mtLuc (TAG) administration; lane 5, pCMV-mtLuc (CGG)/3xTGA administration; lane 6, pmtLuc (CGG) administration. To evaluate transgene expression of mitochondrial Nluc luciferase (**B**), the luciferase activities of the harvested livers were measured following the HTV injection of pCMV-mtLuc (CGG), pCMV-mtLuc (TAG) and pCMV-mtLuc (CGG)/3xTGA. Bars represent the means±SD. (n=3). **Significant differences were calculated by one-way ANOVA, followed by the Bonfferoni test ($p<0.01$).

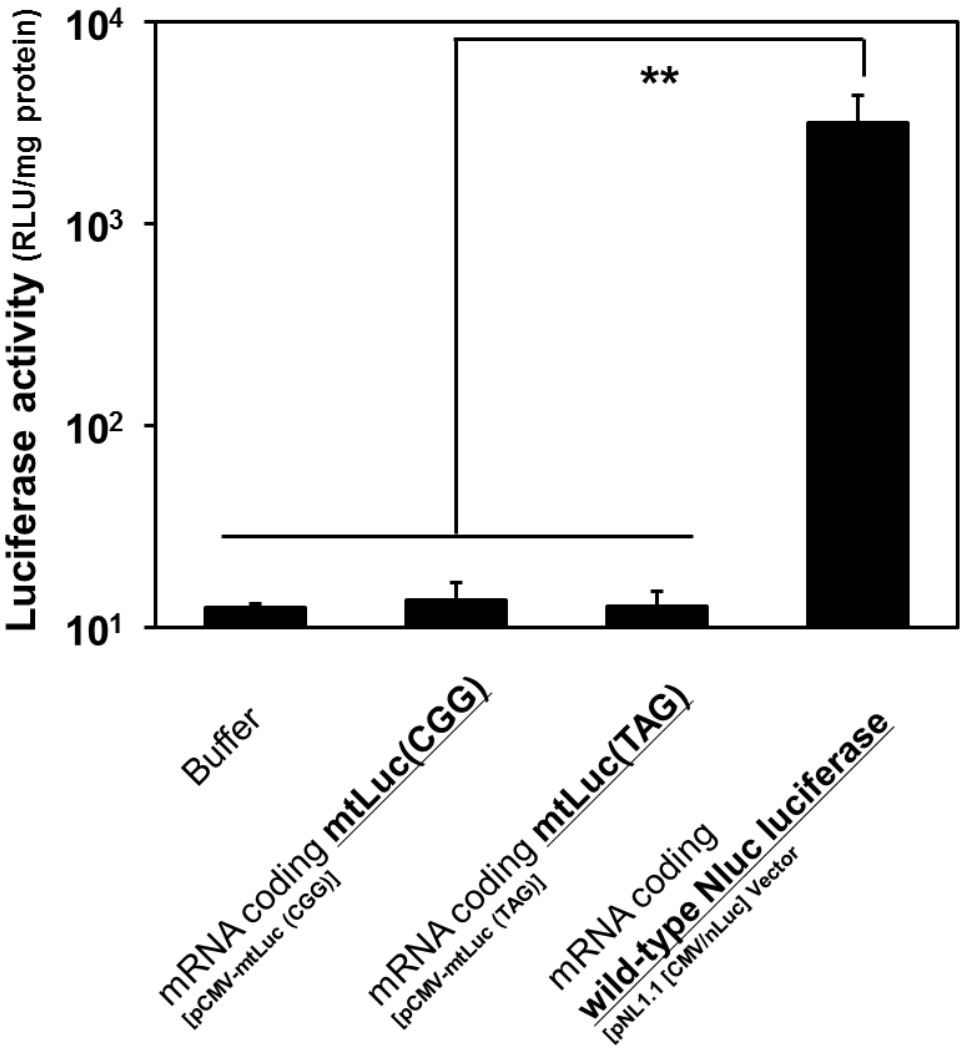


**Figure 4.** *In vitro* translation assay in nucleo-cytoplasmic translation system. The mRNAs coding mtLuc (CGG) or mtLuc (TAG) was added to the Rabbit Reticulocyte Lysate, followed by in vitro nucleo-cytoplasmic translation. The translational products (Nluc luciferase) were then detected using the Nano-Glo Luciferase Assay System. In this experiment, we also evaluated the translation efficiencies of mRNA containing wild type Nluc luciferase as a positive control. Structural information regarding the plasmids used in this experiment is summarized in Figure S2. Bars represent the mean±SD. (n=3). **Significant differences were calculated by one-way ANOVA, followed by bonfferoni test ($p<0.01$).

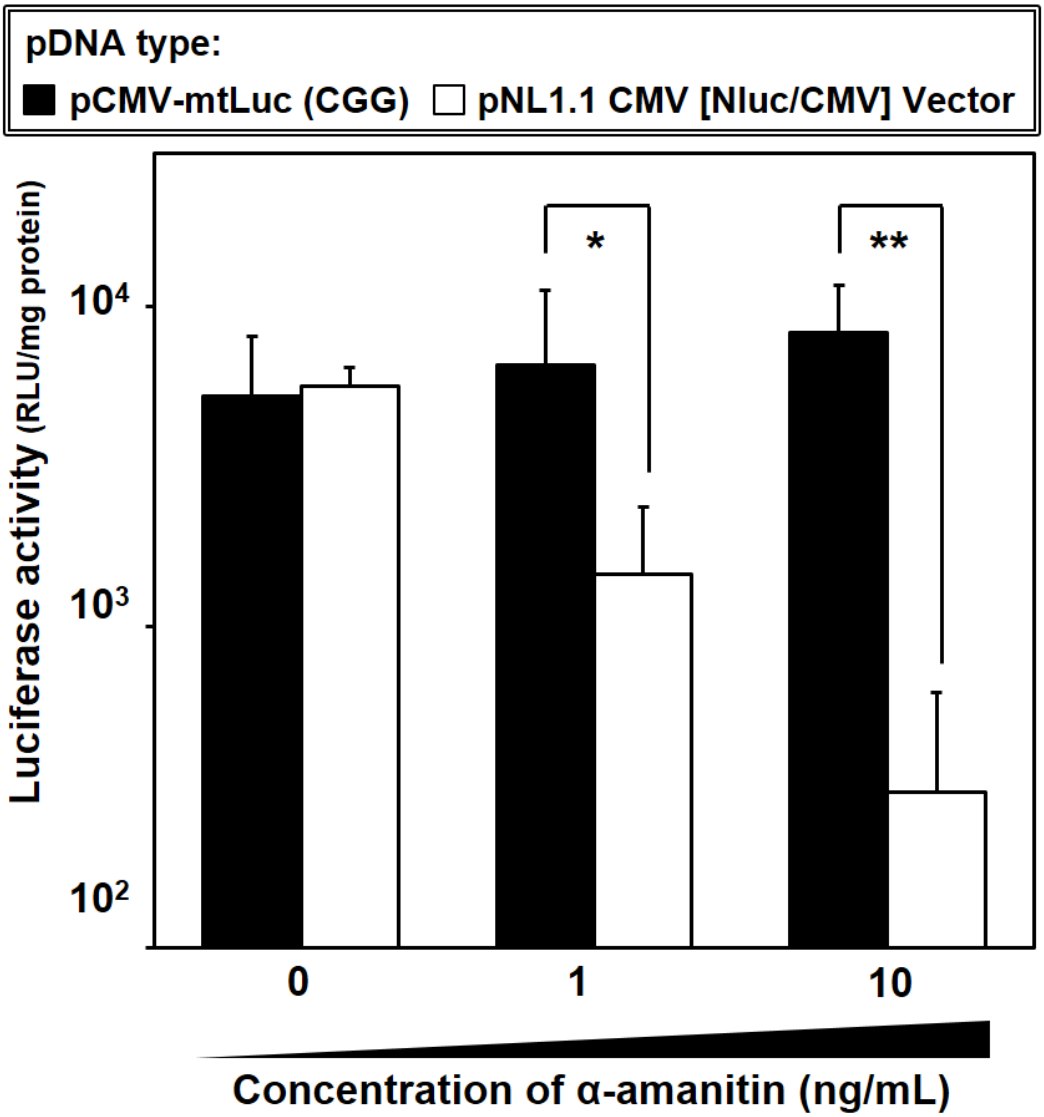


**Figure 5.** Investigation of the transcription of pCMV-mtLuc (CGG) in nucleo system. pCMV-mtLuc (CGG) or pNL1.1 CMV [Nluc/CMV] Vector that codes Nluc luciferase optimized for the nuclear genetic codon system were transfected to HeLa cells using LFN 2000, after treatment with α-amanitin, a nuclear transcription inhibitor. After a 24 hr transfection period, the Nluc luciferase activities were measured. Bars represent the means±SD. (n=3). Two-way ANOVA analysis was performed to compare the effect of two factors that are "pDNA type" and "applied dose of α-amanitin". No significant interactions between the two factors ($p$ = 0.054) were detected and no significant differences among the different "applied dose of α-amanitin" groups was found (p = 0.628). Significant differences between pCMV-mtLuc (CGG) group and pNL1.1 CMV [Nluc/CMV] Vector were detected (**p < 0.01, *p < 0.05 by two way ANOVA analysis, followed by Bonferroni's correction).

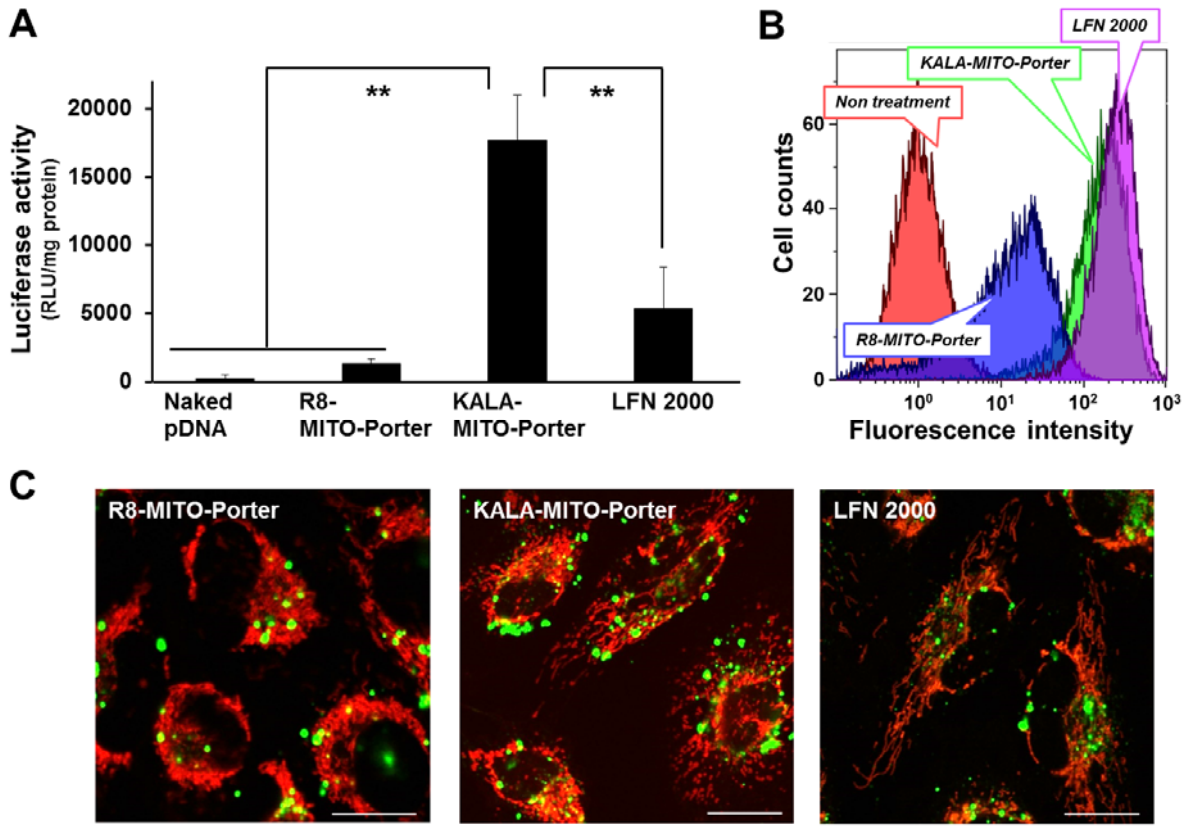


**Figure 6.** Investigation of mitochondrial transfection of pCMV-mtLuc (CGG) using MITO-Porter system. (**A**) Evaluation the transgene expression of pCMV-mtLuc (CGG). A 0.4 μg of pCMV- mtLuc (CGG) was transfected to HeLa cells using R8-MITO-Porter, KALA-MITO-Porter and LFN 2000. After 24 hr transfection, the Nluc luciferase activities were measured. Bars represent the mean±SD. (n=3). Significant differences were calculated by one-way ANOVA, followed by bonfferoni test (** $p<0.01$, * $p<0.05$). (**B**) Flow cytometry analysis to evaluate the cellular uptake of pCMV-mtLuc (CGG). The histogram plot shows the fluorescence intensities of Cy-5 labeled pCMV-mtLuc (CGG) taken up by HeLa cells, after transfection. (**C**) Intracellular observation of pCMV-mtLuc (CGG) using CLSM. Cy-5 labeled pCMV-mtLuc (CGG) (pseudo green color) is seen to colocalize with red-stained mitochondria in HeLa cells, observed as yellow signals in the merged image. Scale bars, 20μm.

# Supplementary data

**Supplementary Figures**

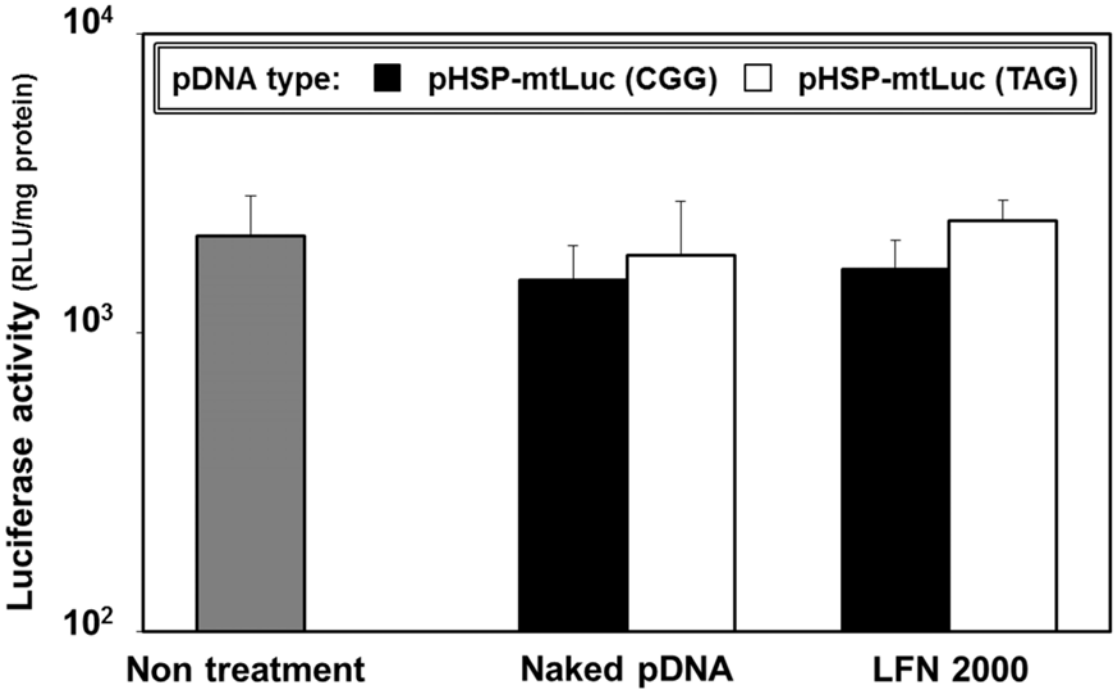


**Figure S1** Evaluation of the transgene expression of pHSP-mtLuc (CGG) in HeLa cells. A 0.4 μg sample of pHSP-mtLuc (CGG) that codes mitochondrial Nluc luciferase was transfected to HeLa cells using LFN 2000. In this experiment, we also used pHSP-mtLuc (TAG) that contains the mitochondrial/universal stop codon (TAG) in the mitochondrial Nluc luciferase gene. Sequence information regarding pHSP-mtLuc (CGG) and pHSP-mtLuc (TAG) [1] are shown in Supplementary Vector sequences (Sequences S5-S6). Nluc luciferase activities were measured after a 24 hr period of transfection. Bars represent the means±SD. (n=3). We performed two-way ANOVA analysis to compare the effect of two factors that are “pDNA type” and “carrier type” and found no significant ($p = 0.33$).

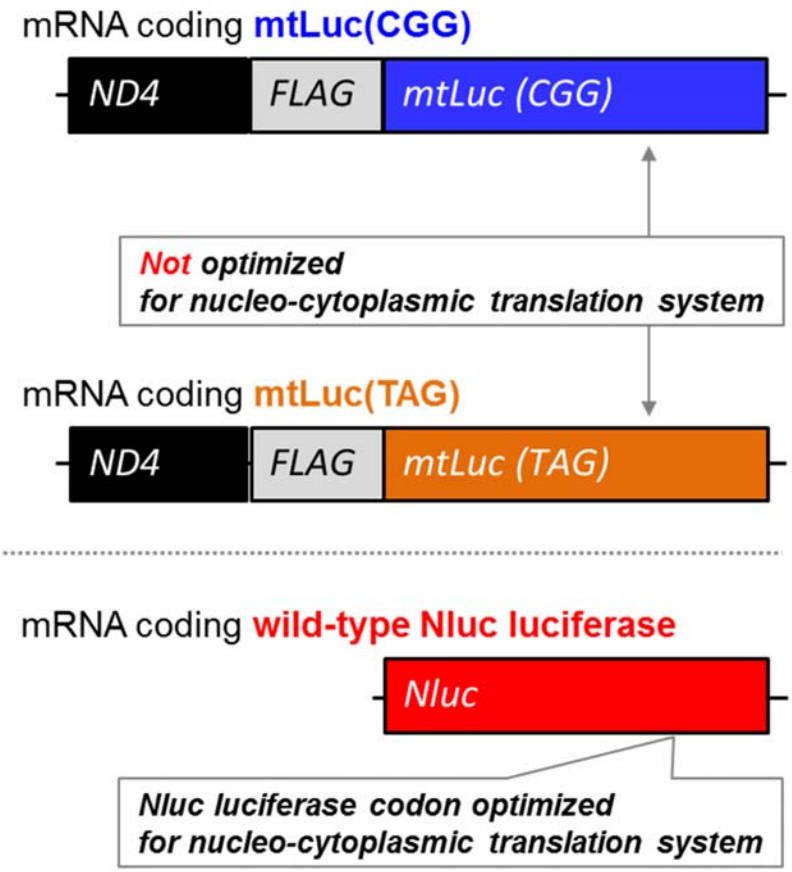


**Figure S2** Structural information of mRNAs for *in vitro* translation assay in nucleo-cytoplasmic translation system. For the experiment shown in Figure 4, the mRNAs coding mtLuc (CGG), mtLuc (TAG) and wild type Nluc luciferase were prepared using pCMV-mtLuc (CGG) (Sequence S1), pCMV-mtLuc (TAG) (Sequence S3) and pNL1.1 CMV [CMV/nLuc] Vector (Promega).

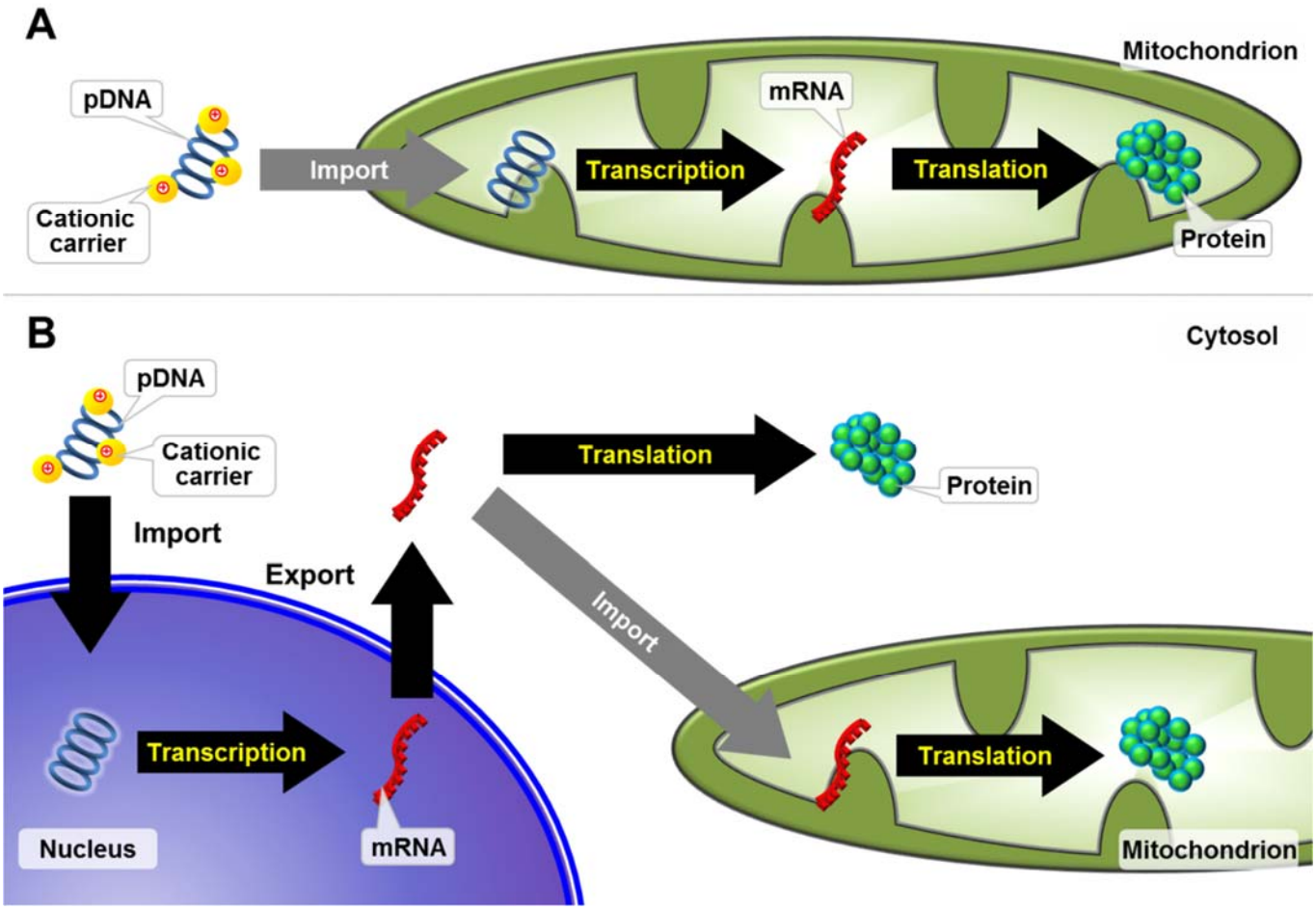


**Figure S3** Schematic image showing the intracellular fate of pDNA in mitochondrial transgene expression. Figure S3A shows a conceptual image of mitochondrial transfection where the pDNA is delivered to mitochondria, and the pDNA was transcribed to mRNA to produce the protein via translation inside a mitochondrion. Figure S3B shows a conceptual image of mitochondrial transgene expression by cytosolic mRNA. One possibility is that the cytosolic mRNA might be translated to the protein via the cytosolic translation system. Another possibility is that cytosolic mRNA is delivered to mitochondria, and the mRNA would be translated to protein in mitochondria.

**Supplementary Vector sequences**

The DNA vectors were designed by inserting the DNA fragment gene as shown below (**Sequences S1-S6**) into pBluescript SK (-) vector (Stratagene) between the multi cloning site (*EcoR* I and *Sma* I sites).

**Sequence S1** for pCMV-mtLuc(CGG). The DNA sequence contains the CMV promoter [highlighted in green], the ND4 gene [highlighted in gray], the sequence corresponding to FLAG [highlighted in yellow], the mtLuc (CGG) gene [highlighted in aqua] and the $tRNA^{Asp}$ gene with the 5'UTR [highlighted in pink]. The CMV promoter was derived from pTriEx-3 Neo Vector (Promega, Madison, WI, USA) to bases 1,021-1,596. The ND4 gene, $tRNA^{Asp}$ gene with the 5'UTR were derived from mouse mtDNA corresponding to bases 10,168-11,544 and bases 6,873-7,011 (GenBank: JF286601.1), respectively. The mtLuc (CGG) gene was synthesized by inserting a point mutation shown by UPPER CASE letters into Nanoluc luciferase gene of pNL1.1[Nluc] corresponding to bases 100-615 (GenBank: JQ437370.1).

*(5' EcoRI)* -

ctgcagattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggc
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**Sequence S2** for pmtLuc(CGG). The DNA sequence contains the ND4 gene [highlighted in gray], the sequence corresponding to FLAG [highlighted in yellow], the mtLuc (CGG) gene [highlighted in aqua] and the tRNA$^{Asp}$ gene with the 5'UTR [highlighted in pink]. The ND4 gene, the tRNA$^{Asp}$ gene with the 5'UTR were derived from mouse mtDNA corresponding to bases 10,168-11,544 and bases 6,873-7,011 (GenBank: JF286601.1), respectively. The mtLuc (CGG) gene was synthesized by inserting a point mutation, shown by UPPER CASE letters, into Nanoluc luciferase gene of pNL1.1[Nluc] corresponding to bases 100-615 (GenBank: JQ437370.1).

*(5' EcoRI)* -

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**Sequence S3** for pCMV-mtLuc(TAG). The DNA sequence contains the CMV promoter [highlighted in green], the ND4 gene [highlighted in gray], the sequence corresponding to FLAG [highlighted in yellow], the mtLuc (TAG) gene [highlighted in aqua] and the $tRNA^{Asp}$ gene with the 5'UTR [highlighted in pink]. The CMV promoter was derived from pTriEx-3 Neo Vector (Promega, Madison, WI, USA) to bases 1,021-1,596. The ND4 gene, $tRNA^{Asp}$ gene with the 5'UTR were derived from mouse mtDNA corresponding to bases 10,168-11,544 and bases 6,873-7,011 (GenBank: JF286601.1), respectively. The mtLuc (TAG) gene was synthesized by inserting a point mutation, shown in UPPER CASE letters into the Nanoluc luciferase gene of pNL1.1[Nluc] corresponding to bases 100-615 (GenBank: JQ437370.1).

*(5' EcoRI)* -

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**Sequence S4** for pCMV-mtLuc(CGG)/3xTGA. The DNA sequence contains the CMV promoter [highlighted in green], the ND4 gene [highlighted in gray], the sequence corresponding to FLAG [highlighted in yellow], the mtLuc (CGG)/3xTGA gene [highlighted in aqua] and the $tRNA^{Asp}$ gene with the 5'UTR [highlighted in pink]. The CMV promoter were derived from pTriEx-3 Neo Vector (Promega, Madison, WI, USA) to bases 1,021-1,596. The ND4 gene, $tRNA^{Asp}$ gene with the 5'UTR were derived from mouse mtDNA corresponding to bases 10,168-11,544 and bases 6,873-7,011 (GenBank: JF286601.1), respectively. The mtLuc (CGG) gene was synthesized by inserting a point mutation by UPPER CASE letters into Nanoluc luciferase gene of pNL1.1[Nluc] corresponding to bases 100-615 (GenBank: JQ437370.1).

*(5' EcoRI)* -

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tgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtg
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**Sequence S5** for pHSP-mtLuc(CGG). The DNA sequence contains the HSP promoter [highlighted in green], the ND4 gene [highlighted in gray], the sequence corresponding to FLAG [highlighted in yellow], the mtLuc (CGG) gene [highlighted in aqua] and the $tRNA^{Asp}$ gene with the 5'UTR [highlighted in pink]. We previously reported this sequence information in reference [1].

*(5' EcoRI)* -

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attagtaaaatcaattacataactttgtcaaagttaaattatagatcaataatctatatatcttatctgcag - *(3' Sma I)*

**Sequence S6** for pHSP-mtLuc(TAG). The DNA sequence contains the HSP promoter [highlighted in green], the ND4 gene [highlighted in gray], the sequence corresponding to FLAG [highlighted in yellow], the mtLuc (TAG) gene [highlighted in aqua] and the $tRNA^{Asp}$ gene with the 5'UTR [highlighted in pink]. We previously reported this sequence information in reference [1].

*(5' EcoRI)* -

ctgcagccctcctcttaatgccaaaccccaaaaacactaagaacttgaaagacatataatattaactatcaaaccctatgtcctgatcaattcta
gtagttcccaaaatatgacttatattttagtacttgtaaaaattttacaaaatcatgttccgtgaaccaaaactctaatcatactctattacgcaataa
acattaacaaatgctaaaaattattcttccctcactaatgctactaccactaacctgactatcaagccctaaaaaaacctgaacaaacgtaacct
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tcccaacacttattattattacccgatgagggaaccaaactgaacgcctaaacgcagggatttatttcctattttataccctaatcggttctattcc
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aacaacttactatggttggcatgcataatagcatttcttattaaaataccattatatggagttcacctatgactaccaaaagcccatgttgaagctc
caattgctgggtcaataattctagcagctattcttctaaaattaggtagttacggaataattcgcatctccattattctagacccactaacaaaatat
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cagacagccggctacaacctggacc**aagtccttgaacagggaggtgtgtccagtttgtttcagaatctcggggtgtccgtaactccga**
**tccaaTAgattgtcc**tgagcggtgaaaatgggctgaagatcgacatccatgtcatcatcccgtatgaaggtctgagcggcgaccaaatg
ggccagatcgaaaaaatttttaaggtggtgtaccctgtggatgatcatcactttaaggtgatcctgcactatggcacactggtaatcgacggg
gttacgccgaacatgatcgactatttcggacggccgtatgaaggcatcgccgtgttcgacggcaaaaagatcactgtaacagggaccctgt
ggaacggcaacaaaattatcgacgagcgcctgatcaaccccgacggctccctgctgttccgagtaaccatcaacggagtgaccggctggc
ggctgtgcgaacgcattctggcgtaagaaaggaaggaatcgaaccccctaaaattggtttcaagccaatctcatatcctatatgtctttctcaat
aagatattagtaaaatcaattacataactttgtcaaagttaaattatagatcaataatctatatatcttatctgcag - *(3' Sma I)*

## References

[1] Y. Yasuzaki, Y. Yamada, T. Ishikawa, H. Harashima, Validation of Mitochondrial Gene Delivery in Liver and Skeletal Muscle via Hydrodynamic Injection Using an Artificial Mitochondrial Reporter DNA Vector, Mol Pharm, 12 (2015) 4311-4320.