Transferring isolated mitochondria into tissue culture cells

Yi-Wei Yang1 and Michael D. Koob1,2,*

1Graduate Program in Molecular Cellular Developmental Biology and Genetics and 2Department of Laboratory Medicine and Pathology, Institutes for Translational Neuroscience (ITN) & of Human Genetics (IHG), University of Minnesota, Medical Bioscience Building, 2101 6th St SE, Minneapolis, Minnesota 55455, USA

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ABSTRACT

We have developed a new method for introducing large numbers of isolated mitochondria into tissue culture cells. Direct microinjection of mitochondria into typical mammalian cells has been found to be impractical due to the large size of mitochondria relative to microinjection needles. To circumvent this problem, we inject isolated mitochondria through appropriately sized microinjection needles into rodent oocytes or single-cell embryos, which are much larger than tissue culture cells, and then withdraw a ‘mitocytoplast’ cell fragment containing the injected mitochondria using a modified holding needle. These mitocytoplasts are then fused to recipient cells through viral-mediated membrane fusion and the injected mitochondria are transferred into the cytoplasm of the tissue culture cell. Since mouse oocytes contain large numbers of mouse mitochondria that repopulate recipient mouse cells along with the injected mitochondria, we used either gerbil single-cell embryos or rat oocytes to package injected mouse mitochondria. We found that the gerbil mitochondrial DNA (mtDNA) is not maintained in recipient rho0 mouse cells and that rat mtDNA initially replicated but was soon completely replaced by the injected mouse mtDNA, and so with both procedures mouse cells homoplasmic for the mouse mtDNA in the injected mitochondria were obtained.

INTRODUCTION

Sequence variation in mammalian mitochondrial DNA (mtDNA) genomes can have major impacts on cell phenotypes, as was first demonstrated by an experiment in which resistance to chloramphenicol was stably transferred to previously sensitive mouse cells by fusing them to enucleated fragments (cytoplasts) generated from chloramphenicol-resistant mouse cells (1). Cybrids appeared at a rate of 2–8 per 10^4 cells plated when grown under conditions that selected for both the chloramphenicol-resistant mtDNA carried by the mitochondria in the cytoplasts and nuclear markers in the chloramphenicol-sensitive parent. The need for specific mitochondrial selection markers for the cybrid transfer of mitochondria was later eliminated by the development of recipient mammalian cell lines that were devoid of their own mtDNA genomes (rho0 cells) (2). Mammalian rho0 cells only grow in media supplemented with uridine and pyruvate, which allows rho+ cybrids made from these cells to be selected for their ability to grow in culture media without these supplements. More recent refinements of cybrid transfer procedures include the development of methods for chemical inactivation of the mitochondria in recipient cells (3) and for the chemical enucleation of donor cells (4). Cybrid transfer techniques are now widely used to generate transmitochondrial cell lines used to evaluate the functional importance of mtDNA sequence variants, particularly those thought to cause disease (5,6).

Direct injection of isolated mitochondria into mammalian tissue culture cells has been evaluated as an alternative to cybrid transfer techniques, but was found to be impractical (2,7). Since most mitochondrial fragments are too large to pass through the injection needles of the size needed to work with these cells, on average less than one mitochondrion could be injected per cell and transfer rates were found to be between 1 and 3 recipients per 1000 cells injected (7). Significant numbers of mitochondria can be injected into mouse oocytes or one-cell embryos because the large sizes of these cells are more amenable for injection with appropriately sized microinjection needles (8–10). However, oocytes injected with exogenous mitochondria have to date had a limited number of experimental applications, primarily because these cells contain more ‘background’ mitochondria than any other mammalian cell, have a limited number of cell

*To whom correspondence should be addressed. Tel: +1 612 626 4521; Fax: +1 612 626 2032; Email: koobx001@umn.edu

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cycles in culture, and are in general not good model cells for characterizing many of the biological activities of mitochondria.

In this report, we describe a practical method for delivering isolated mitochondria into mammalian tissue culture cells.

MATERIALS AND METHODS

Animals

B6D2F1 mice were bred by crossing C57B/6J females with DBA/2J males (purchased from Jackson Laboratory, Bar Harbor, Maine). Sprague Dawley rats were purchased from Charles River Laboratory (Wilmington, MA) and Harlan Sprague Dawley Inc. (Indianapolis, Indiana). Mongolian gerbils and Golden Syrian hamsters were purchased from Charles River Laboratory.

Superovulation

Mouse B6D2F1 mice at the age of 7–12 weeks were used for superovulation. 5IU of PMSG (EMD) were injected intraperitoneally between 1 p.m. and 2 p.m. Approximately 48 h later, 5IU of hCG (The National Hormone and Peptide Program) were injected intraperitoneally between noon and 1 p.m. The females were transferred to the male cages for mating (11,12).

Rat Sprague Dawley rats at the age of 28–35 days were used for superovulation. 15IU of PMSG were injected intraperitoneally between 11 a.m. and 1 p.m. Approximately 50 h later, 30IU of hCG were injected intraperitoneally. The females were housed in the original cages for oocytes production or transferred to the male cages in the late afternoon (~5 p.m.) for mating (modified from (13)).

Gerbil Mongolian gerbils at the age of 4–7 weeks were used for superovulation. 5IU of PMSG were injected intraperitoneally between 11 a.m. and 1 p.m. Approximately 50 h later, 5IU of hCG were injected intraperitoneally. The females were housed in the original cages for oocytes production or transferred into the male cages in the late afternoon (~5 p.m.) for mating (modified from (13)).

Oocyte and embryo isolation

The next morning after hCG injection, the donor females were sacrificed using CO2 euthanasia. Ovaries and oviducts were dissected and placed in modified human tubal fluid (mHTF) medium. Oocytes or embryos were released from the swollen ampulla of the oviducts by tearing the ampulla using pairs of forceps under a dissection microscope. The cell clumps were transferred to a dish containing 300 μg/ml Hyaluranaidase (Sigma, St. Louis, MO) in mHTF and incubated at room temperature for several minutes to dissociate the surrounding cumulus cells. Oocytes or embryos were washed by passing through three mHTF microdrops and cultured in HTF microdrops at 37°C with 5% CO2 (modified from (11)).

Preparing rodent eggs for mitochondrial injection

The zona pellucida of isolated mouse, rat and gerbil eggs were removed by incubating in Tyrode’s acidic solution (Sigma) or in modified Tyrode’s acidic solution (pH adjusted to 3.7) at room temperature until the zona disappeared. Zona-free eggs were washed by passing through three mHTF drops and incubated in mHTF drops containing 2 μg/ml cytochalasin B at room temperature for 10 min.

Cell culture

Mouse STO cell line, LL/2 cell line and rat YB2/0 cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA). Mouse STO cells with stable expression of mitochondria-targeted Green Fluorescent Protein (GFP) were generated by transfecting pcDNA6-Tfam-L-GFP plasmid and selecting in 6 μg/ml blasticidin (Invitrogen, Carlsbad, CA) in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplied with 10% fetal bovine serum (Invitrogen) and 1x penicillin/streptomycin (Invitrogen) for over 2 weeks. Stable clones were obtained by single cell culture of blasticidin-resistant cells in 96-well plates. Rat YB2/0 cells, LL/2-STOmt-rat and LL/2-STOmt-gerbil cell lines were cultured in DMEM with 10% fetal bovine serum and 1x penicillin/streptomycin (rho+ medium). LL/2 rho0 cells were generated as described in (16). LL/2rho0 cells, LL/2-rho0 cell lines and LL/2-gerbil cell lines were culture in DMEM with 10% fetal bovine serum and 1x penicillin/streptomycin supplied with 100 μg/ml pyruvate and 50 μg/ml uridine (rho0 medium).

Mitochondria isolation

Mammalian mitochondria isolation was performed as described (16) with modifications. In brief, mouse STO cells were harvested from 100 mm dishes using 0.05% trypsin/EDTA. Cell were washed with Cell Washing Buffer (1 mM Tris-HCl, pH 7.0, 0.13 M NaCl, 5 mM KCl and 7.5 mM MgCl2, pre-chilled on ice) twice and pelleted at 500 g for 10 min. The cell pellet was resuspended in 100–200 μl of 1/10x Isolation Buffer (4 mM Tris-HCl, pH 7.4, 2.5 mM NaCl and 0.5 mM MgCl2, pre-chilled on ice) and incubated on ice for 1–2 min. The cells were then broken using a Pellet Pestle® tissue grinder (Kontes Glass Co., Vineland, NJ) with approximate 50 strokes on ice. One-ninth of the original cell resuspension volume of 10x Isolation Buffer (400 mM Tris-HCl, pH 7.4, 250 mM NaCl and 50 mM MgCl2) was added to the homogenate. The homogenate was centrifuged at 500 g for 5 min at 4°C to move unbroken cells and cell debris. The mitochondria were harvested by centrifugation at 7000g for 10 min at 4°C and resuspended in 90 μl of 15% sucrose/TEN (15% sucrose, 10 mM Tricine-KOH, 0.2 mM EDTA and 50 mM NaCl, pH 7.5) with 1 unit/μl DNase I (Roche, Basel, Switzerland), 40 mM Tris-HCl, pH 8.0 and 2.5 mM
MgCl₂. The reaction was incubated on ice for 1 h to digest DNA from broken nuclei or broken mitochondria. After 1 h incubation, 1 μl of 0.5 M EDTA was added to the reaction to inactivate the DNase I. The mitochondria were pelleted at 7000 g for 10 min at 4°C, and washed with 100 μl of pre-chilled Mitochondria Resuspension Buffer (MRB, 225 mM Mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris and 5 mM KH₂PO₄, pH 7.2 (17)). The mitochondria were pelleted at 7000 g for 10 min at 4°C and placed on ice.

### Microinjection

Injection dishes were set up as illustrated in Supplementary Figure S1. Zona-free eggs were transferred from 2 μg/ml cytochalasin B/mHTF drops to the egg drops (E drop: 2 μg/ml cytochalasin B/3% PVP (polyvinylpyrrolidone)/mHTF). The mitochondria pellet was resuspended in pre-chilled MRB right before setting the dish. Microinjection was performed in the E drops using a mouse ICSI (intracytoplasmic sperm injection) microcapillary with 6 μm inner diameter (róż Eppendorf, Hamburg, Germany) driven by a Piezo-drill micromanipulator (Prime Tech Piezo Impact Drill PMM-150FU, Prime Tech LTD, Japan). Mitochondria were injected close to the left end of the eggs where the eggs were held by a homemade needle (with ~20 μm outer-diameter). A gentle aspiration was applied from the holding needle to pull cytoplasm along with the injected mitochondria into the nucleus as soon as the injection was finished. A mitocytoplast was generated by quickly pulled the holding needle away from the egg or by passing through the drop edges to seal the membrane. The mitocytoplasts were placed in the mitocytoplast drops (C drops: 3% PVP/mHTF) and incubated at 37°C with 5% CO₂ for at least 15 min.

### HVJ-E mediated cell fusion

LL/2 rho0 cells were harvested from dishes by gently pipetting and washed with phosphate buffered saline (PBS) once. Washed cells were transferred to the cell drops (3% PVP/mHTF) on the injection dish shown in Supplementary Figure S1. HVJ-E stock (GenomOne™ CF EX, Cosmo Bio Co., LTD, Japan) was diluted one to four in 1x Fusion Buffer. A homemade needle with ~30 μm outer diameter was used to transfer LL/2 rho0 cells to the HVJ-E drop. After brief incubation (1–2 min), cells were transferred to the cytoplast drops and attached to cytoplasts. The cell-cytoplast pairs were incubated in a 37°C, 5% CO₂ incubator until fusion was observed. Fused cells were transferred to 96-well plates containing 50 μl of rho0 medium supplied with 1x non-essential amino acid (Sigma) and 50 μl of HTF for single cell culture.

### Species-specific mtDNA PCR assays

Total DNA were prepared by incubating cells in 100 μg/ml proteinase K (Sigma)/1x colorless Go-Taq PCR buffer (Promega Co., Madison, WI) at 55°C for 1–2 h, followed by incubation at 90°C for 15 min. Primers used in the mtDNA assays were mouse mtDNA D-loop PCR (BglII-mus-H: 5’- ACTAGCCTCCATCTCATCTTCTTCT

AATC-3’/HSPtRNAph: 5’- ACCTTTGTTTATGG GATACAATTATCC-3’ or BglII-mus-For 2: 5’-TTCC CAAGCATATAAGCTAGT GACA-3’/BglII-mus-For 2: 5’-ACAGGACTTTGGCTGGACC-3’), and gerbil mtDNA PCR (3’-gerbilmtDNA-ND3-Forward: 5’-CTGCTTCTTCTCTCTTACT-3’/5’-gerbilmtDNA-ND4-Reverse: 5’-GATAAGAGGGGTG AGATTGGA-3’). The PCR condition used was: 95°C, 5 min; 95°C, 30 s, 55°C, 1 min; 72°C, 1 min, 30 cycles; 72°C, 5 min.

### Imaging mitochondria in recipient mouse cells

Mouse STO-rho0-DsRedmt cells were plated to Lab-Tek™II chambered cover glass (Nunc, Rochester, NY) 2 days before imaging. Mitochondria injection and cytoplasts fusion were performed as described above. Fused cells were cultured in phenol red-free medium for 2–3 h in a 37°C, 5% CO₂ incubator. Olympus Fluoview® FV1000 confocal laser scanning microscope with a UPLSAPO 100x oil 1.4 N.A. objective lens (Olympus America Inc., Center Valley, PA) was used for imaging fluorescence-labeled mitochondria in cells. The microscope was pre-warmed to 33–36°C and the temperature was kept through the whole imaging duration. The culture medium was supplied with 95% air, 5% O₂ during imaging. FV1000 ASW ver2.1.b software (Olympus America Inc.) was used to take time-lapse images with 30 s or 1 min intervals. The pictures were processed and extracted from the series of images using FV1000 ASW ver2.1.b software.

### RESULTS

#### Overview of the mitocytoplast cell fusion method

The method we developed to deliver isolated mitochondria into mammalian cells is a two-step process (Figure 1). The first step is generating artificial cytoplasts carrying injected mitochondria (i.e. mitocytoplasts), and the second step is fusing these mitocytoplasts to recipient cells. In brief, mouse mitochondria carrying wild-type, functional mitochondrial genomes were isolated from STO-mt-GFP cells and DNase I was added to remove any DNA contamination during the isolation procedure. The isolated mitochondria were injected into one-cell mouse embryos. A mitocytoplast containing part of the cytoplasm along with injected mitochondria was pulled out from the embryo at the opposite end from the entry point immediately after mitochondria injection and placed in PVP-mHTF microprops. The recipient LL/2 rho0 cells (lacking any mtDNA) were incubated briefly in the hemagglutinating virus of Japan envelope (HVJ-E) extract (Cosmo Bio Co., LTD., Japan) (18) and then attached to cytoplasts in PVP/mHTF microdrops. After incubating in a 37°C, 5% CO₂ incubator, fused cells were sorted to a 96-well plate for single cell culture (see Supplementary Figure S1).

Figure 2 contains images of mitocytoplasts being generated from mouse one-cell embryos and the subsequent fusion of the mitocytoplasts to the rho0 recipient.
cells (also see Supplementary Movies S1 and S2). We used 0.5 µm fluorescent beads (FluoSpheres, Invitrogen) as the injection marker to mimic mitochondria during the procedure shown in this figure. We used commercial needles made for mouse ICSI (D1 = 6 µm, Eppendorf) and were able to obtain good control while microinjecting mitochondria into mouse eggs. The injection was performed using a Piezo-driven micromanipulator (Prime Tech LTD., Japan), which uses electric pulses to control microvibrations that allow the needle to penetrate the zona pellucida, the protecting coats of mammalian eggs (19) and to break the cell membrane locally. This type of equipment has been used with great success in nuclear transfer and intra-sperm injection (20,21), mainly due to reduced stress and damage to the eggs during these procedures.

In our experiment, the zona pellucida was removed using Tyrode’s acidic solution (22) before injection so that mitocytoplasts could be pulled directly from the opposite end of the pipette entry point. After injection, the injected fluorescent beads were pulled into the mitocytoplast by gently aspirating with a holding needle with ~20 µm tip opening (Figure 2a). The junction of the membrane bridging cytoplast fraction and the egg was broken and sealed by passing through the edges of the medium drops. The fluorescent bead+-cytoplasts were pulled one by one from injected mouse eggs and placed in PVP/mHTF microdrops for recovery.

After briefly incubating in HVJ-E solution, recipient cells were brought to the cytoplast drops and attached to cytoplasts (Figure 2b). HVJ-E induces membrane–membrane fusion and the content of the donor cytoplast directly releases into the recipient cells (18). Close contact between the recipient cells and cytoplasts was ensured by pushing the recipient cells against the cytoplasts held by a blunt, sealed needle. The cytoplast-cell pairs were incubated in PVP/mHTF microdrops in a 37°C, 5% CO₂ incubator and were checked every 30 min or hourly. The fused cells were sorted to 96-well plates for single cell culture. Figure 2e shows several examples of cytoplast-fused cells. The presence of fluorescent beads in cells indicates the successful fusion of cells to cytoplasts and subsequent transfer of injected material.

**Imaging introduced mitochondria in mouse cells**

Figure 3 shows isolated mouse mitochondria that we delivered into a recipient mouse tissue culture cell via mitocytoplast fusion. GFP-labeled mouse mitochondria were isolated and injected into mouse embryos, and mitocytoplasts were generated from the mouse embryos. Mitocytoplasts carrying injected GFP mitochondria were fused to mouse rho0 cells with DsRed-labeled mitochondria attached on a chambered cover glass. The cells were incubated in a 37°C, 5% CO₂ incubator for ~3 h and were imagined using Olympus Fluoview® FV1000 confocal laser scanning microscope. In the cell shown in Figure 3, introduced mitochondria fused to the endogenous mitochondrial networks, as shown by the diffusion of the GFP signal and the co-localization of the GFP and DsRed signals (note that the mitochondria in the neighboring cells are only red). This result suggests that mitochondria delivered using this procedure retain biological activities that enable them to fuse to endogenous mitochondrial networks in recipient cells and demonstrates that we can inject and transfer large quantities of isolated mitochondria into mammalian tissue culture cells.

**Using eggs from different rodent species to generate mitocytoplasts**

Although mitocytoplasts generated from mouse embryos can support the transmission of isolated mitochondria into mouse tissue culture cells, large numbers of mouse mitochondria endogenous to the embryo are introduced into the recipient cells as well. We hypothesized that using...
eggs from different rodent species could solve this mitochondria-background issue due to nuclear-mitochondrial incompatibility, especially when applying selective pressure for mitochondrial function.

We tested eggs from three rodent species other than mouse (Sprague Dawley rats, Mongolian gerbils and Golden Syrian hamsters) for mitochondria injection, cell fusion and viability of cells after mitocytoplast fusion. We compared the quality of mitocytoplast generated from these rodent species using the method we developed by assessing cell growth after cytoplast fusion. We found that mitocytoplasts from gerbil embryos, rat oocytes and rat embryos can support fused cell growth as well as mitocytoplasts from mouse embryos. Figure 4 shows the results of species-specific PCR assays of using gerbil embryos and rat oocytes to deliver isolated mouse mitochondria into mouse rho0 cells. Only mouse mtDNA genomes were detected in the final cell lines produced by this procedure, and neither gerbil nor the rat mtDNA genomes were retained. Gerbil mtDNA genomes were not detected in the mouse cells even when they first assayed (as early as 10 days after mitocytoplast fusion; Figure 4a, top). On the other hand, rat mtDNA genomes were initially detected in the cybrid cells (Supplementary Figure S2), but this rat mtDNA background was lost after 20–30 days in culture. Figure 4b shows species-specific PCR assays of 18 subclones from LL/2-STOmt-rat clone B5 (Supplementary Figure S2). No rat mtDNA genomes were detected in all these clones assayed 30 days in culture whereas mouse mitochondrial genomes were maintained at high levels (Figure 4b).

We performed relative quantification PCR assays of five of these clones along with LL/2-STOmt-gerbil G1 cells, and found that these clones contained levels of mtDNA genomes 1.4-2.5-fold higher than LL/2 cells (Supplementary Figure S3).
Restoration of mitochondrial function of rho0 cells by introduced mouse mitochondria

We tested if LL/2-STOmt-gerbil clones have converted from mit- (no mitochondrial function) to mit+ (full mitochondrial function) by culturing cells in a growth medium that requires mitochondrial function (rho + medium). As shown in Supplementary Figure S4, cells from clone LL/2-STOmt-gerbil-G1 (shown as clone 1 in the figure, middle row) grew normally in rho + medium. On the other hand, the control, original LL/2 rho0 cells (bottom row) and a cytoplast-fusion control clone (LL/2-gerbil-F2, top row) did not grow in rho + medium. The respiration proficiency of these cybrid cell lines was further confirmed by a Seahorse XF mitochondrial respiration assay (Supplementary Figure S5).

Cybrid transfer of mitochondrial genomes into rho0 mouse cells

We performed cybrid transfer (23) to confirm the functionality and the cytoplasmic location of the mouse mitochondrial genomes in the LL/2-STOmt-gerbil and LL/2-STOmt-rat cell lines we generated from the mitocytoplast fusion procedure. We transferred mitochondria from LL/2-STOmt-gerbil and LL/2-STOmt-rat cell lines to an attached mouse cell line with stable expression of mitochondria-targeted DsRed (STO-rho0/cMyc/LTAg-mtDsRed cells, blasticidin-resistant) by HVJ-E mediated cell fusion. The blasticidin-resistant clones we obtained from 96-well single cell culture were able to grow in rho+ medium, indicating the restoration of mitochondrial function by cybrid transfer. The cells have an attached phenotype with mitochondrial-targeted DsRed expression, which are consistent with the properties of STO-rho0/c Myc/LTAg-mtDsRed. These features confirmed that the nuclei were originally from the blasticidin-resistant STO rho0 cells. Using a mouse mtDNA PCR assay, we confirmed the presence of mouse mtDNA in these clones generated from cybrid transfer (Supplementary Figure S6). These results support that our mitocytoplast fusion procedure can transfer isolated mitochondria into mammalian cells, and the transferred mitochondria and mitochondrial genomes remain functional.

DISCUSSION

We report a practical method for delivering isolated mitochondria into mammalian tissue culture cells.
This procedure involves injecting mitochondria into rodent oocytes or single-cell embryos, withdrawing a ‘mitocoplast’ cell fragment containing the injected mitochondria from a point opposite of the injection site, and then fusing these mitocytoplasts to recipient cells through viral-mediated membrane fusion. (Figures 1 and 2 and Supplementary Movies S1 and S2). By using oocytes or single-cell embryos from non-mouse species in this procedure, we were able to obtain mouse cells that were homoplasmic for the mouse mtDNA carried in the injected mitochondria (Figure 4).

Of the cells fused to rat oocyte mitocytoplasts, 70% survived and amplified quickly in rho0 medium and 80% of the surviving clones carried mouse mitochondrial genomes (Supplementary Figure S2). Although 100% of the surviving clones also carried rat mitochondrial genomes 10 days after mitocytoplast fusion, the rat mitochondrial genomes were spontaneously lost in these cells within 30 days of injection (Figure 4). This result suggests that in the presence of more than one mtDNA species, mouse cells favor mouse mitochondrial genomes over those from different species, and is consistent with the results from a study that looked at the ability of different rodent mtDNA genomes to replicate in mouse cells (24).

The mitocoplast transfer procedure that we have developed offers a viable alternative to the now standard cybrid transfer technology for generating cell lines that stably maintain exogenous mtDNA genomes. The primary advantage of the mitocytoplast approach is that isolated mitochondria rather than mitochondria already present within viable cytoplasts can be transferred into tissue culture cells. This new approach is the only method for generating transmitochondrial cell lines in those instances in which mitochondria can be isolated readily but viable cytoplasts containing those mitochondria cannot. This approach also allows us to readily mix mitochondria from varied sources in the same cell, and does not require any form of selection against donor cells. Most importantly, however, the mitocytoplast approach now allows us to introduce isolated mitochondria that have been modified outside of cells back into living cells.

Engineering mitochondrial genomes in mammalian mitochondria is not yet possible, primarily because proven techniques for directly transferring DNA molecules into mitochondria in living mammalian cells are not yet available (25). However, DNA constructs have been successfully introduced into isolated mammalian mitochondria by bacterial conjugation (26), electroporation (16,27) and other approaches (28). Previous papers have described methods to introduce isolated mitochondria into cells in culture (29,30). However, in our hands we have been unable to replicate those results. The mitocytoplast approach that we have developed allows mitochondria transformed by DNA constructs to be transferred back into cells in order to both fully evaluate and optimize the transformation procedure and to test the ability of the transformed DNA to either modify the mtDNA genome within the mitochondria or to replicate independently within the mitochondrial matrix.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Figures 1–6, Supplementary Movies 1 and 2 and Supplementary Materials and Methods.

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