

MITOCHONDRIA TRANSFER INTO FIBROBLASTS: LIPOSOME-MEDIATED TRANSFER OF LABELED MITOCHONDRIA INTO CULTURED CELLS

Mitochondria are organelles that play a key role in cellular energy metabolism through oxidative phosphorylation and the production of ATP. Mutations in mitochondrial DNA (mtDNA) that negatively affect the function of oxidative phosphorylation enzyme subunits or the mitochondrial protein translational mechanisms can result in severe impairment in tissues with high energy requirements (central nervous system, heart, skeletal muscle, etc).¹ Animal models of mtDNA diseases would be useful, yet techniques for efficient transfer of mitochondria are limited.² We tested whether isolated, viable mitochondria can be efficiently transferred into cultured mouse fibroblasts using synthetic liposomes, which are used routinely to deliver molecules such as DNA and RNA into cells.³

This study targets liposome-mediated transfer of labeled mitochondria into cultured NIH/3T3 mouse fibroblasts using a mitochondrial tracking dye. Mitochondria were isolated from the mouse liver and labeled with MitoTracker Red[®] CMXRos, a probe that binds selectively to the mitochondrial membrane and can be visualized using fluorescence microscopy.⁴ Transfer of mitochondria into cultured cells was performed using the synthetic liposome, Lipofectin (from Invitrogen). Controls consisted of MitoTracker Red[®]-labeled mitochondria without liposome transfection reagent, untreated cells, and cells labeled with MitoTracker[®] Red only. Under fluorescence microscopy, transferred mitochondria were observed in fibroblasts (throughout the cytoplasm around the nucleus). Proof that viable mitochondria can be transferred into cultured cells using synthetic liposomes paves the way for adapting this technique to the transfer of genetically engineered mitochondria into embryonic stem cells for development of live heteroplasmic mice that will serve as models of human mtDNA diseases.^{5,6}

INTRODUCTION

The goal of this study was to develop a delivery system to introduce isolated mitochondria into cells grown in culture. Microinjection of isolated mitochondria into fertilized eggs is feasible because of the large size of ova and adaptation of techniques already in use for microinjection of DNA to produce transgenic mice.⁷ Cultured fibroblasts or embryonic stem (ES) cells are, however, significantly smaller than ova and it would be extremely difficult, if not impossible, to microinject with isolated mitochondria preparations. Using liposome-mediated transfer, the aim of this study was to test the efficacy of cationic unilamellar lipid vesicles for transfer of isolated mitochondria into cultured cells.

METHODS

Cells (NIH/3T3 fibroblasts) were first plated in 6-well dishes under sterile conditions (with cover slips inserted into each well), so that they were approximately 50% confluent the following day. The cells were incubated overnight at 37°C, with 5% CO₂ in air.

On day two, mitochondria were isolated from the liver of a mouse.⁸ Livers were obtained and rinsed in SME (Sucrose, MOPS, EDTA) buffer on ice. Tissues were minced into samples no larger than 2 mm³ with scissors. Livers were homogenized with a glass teflon Dounce homogenizer in 30 to 40 mL of buffer. The mixture was centrifuged twice, each for 10 minutes at 750 × g at 4°C, discarding the pellets. The supernatant was then centrifuged at 9800 × g at 4°C for 15 minutes to

collect the mitochondria. Microsomes and blood pigments were removed from the pellet with the use of vacuum aspiration (sterile Pasteur pipette) and the solution was resuspended in STE (Sucrose, Tris, EDTA) buffer by stirring with a glass rod or pestle. Samples were re-homogenized with a glass-glass Dounce homogenizer and centrifuged at 9800 × g at 4°C for 15 minutes. Again, microsomes and excess blood pigments were removed from the pellet with vacuum aspiration. The pellet (mitochondria) was resuspended and MitoTracker Red[®] CMXRos (50 nm) was added and incubated at room temperature for 15 minutes. Another low speed centrifugation step was performed at 750 × g at 4°C for 10 minutes (pellet discarded). We centrifuged the supernatant for 15 minutes at 9800 × g at 4°C. The purified, MitoTracker Red[®]-labeled mitochondria were resuspended in culture medium (serum-free). Labeled mitochondria (100 μL) were added to 100 μL of liposome solutions (20 μL liposome stock solution plus 80 μL medium, incubated 30 minutes at room temperature) and incubated for 15 minutes at room temperature. In the final experiment, the liposome, Lipofectin[®], was used.

In six-well plates, the medium was removed from the cells and washed with serum-free medium. Then, 100 μL liposome-mitochondria complex solution was added to a volume of 1.9 mL serum-free medium, mixed gently, and added to the cells. The cells were incubated at 37°C in the CO₂ incubator overnight (≈16 hours).

After 16 hours, the transfection medium was aspirated and each well was washed 2× with 2.0 mL medium

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(with serum) to remove any debris and dead cells. Lastly, 2.0 mL medium was added to each well again and they were returned to the incubator for 2 to 4 hours. Then, cover slips were removed for fluorescence microscopy and the 6-well plates were returned to the incubator for possible follow-up observations.

RESULTS

Under the fluorescent microscope with 600× oil immersion magnification, faint labeling of mitochondria was observed in the control group without liposomes, suggesting that minute amounts of free dye diffused into the cells and bound to mitochondria originally in the cell. In the experimental group, bright fluorescent mitochondria were observed within the fibroblast cytoplasm around the nucleus. The fluorescence of the mitochondria was brighter than that in the control group; however, quantification was not performed. While some mitochondria and their liposomes appeared to aggregate,

possibly outside the cell, our results suggest that the majority successfully entered past the cell membrane and clustered toward the nucleus. It is, however, possible that mitochondria had not entered the cytoplasm as the liposome might have simply expelled dye instead of mitochondria into the fibroblasts.

DISCUSSION

These results strongly suggest that mitochondria can be delivered into fibroblast cells with the use of Lipofectin®. Future research will test to make sure that the transferred mitochondria are functional and not selectively eliminated by various cellular mechanisms. It will also be used to transfer mitochondria with a different mtDNA sequence to be able to analyze the viability of the transplanted organelles. This study provided an initial step in discovering an efficient method to deliver mitochondria into small cells and calls for further research in altering mtDNA in stem cells.

REFERENCES

1. Pinkert CA, Trounce IA. Production of transmitochondrial mice. *Methods*. 2000;26:348–57.
2. Pinkert CA, Trounce IA. Animal modeling: From transgenesis to transmitochondrial models. In: Berdanier CD ed. *Mitochondria in Health and Disease. Oxidative Stress and Disease Series*. Vol. 15, Boca Raton: CRC Press; 2005;559–80.
3. Malone RW, Felgner PL, Verma IM. Cationic liposome-mediated RNA transfection. *Proc Natl Acad Sci USA*. 1989;86(16):6077–6081.
4. Poot M, Zhang YZ, Kramer JA, et al. Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. *J Histochem Cytochem*. 1996 44:1363–1372.
5. McKenzie M, Trounce IA, Cassar CA, Pinkert CA. Production of homoplasmic xenomito-chondrial mice. *Proc. Natl. Acad. Sci USA*. 2004;101:1685–1690.
6. Pinkert CA, Trounce IA. Generation of transmitochondrial mice: Development of xenomito-chondrial mice to model neurodegenerative diseases. *Methods Cell Biol*. 2007;80:549–569.
7. Irwin MH, Johnson LW, Pinkert CA. Isolation and microinjection of somatic cell-derived mitochondria and germline heteroplasmy in transmitochondrial mice. *Transgen Res*. 1999; 8:119–123.
8. Pinkert CA, Irwin MH, Johnson LW, Moffatt RJ. Mitochondria transfer into mouse ova by microinjection. *Transgen Res*. 1997;6:379–383.