

Original Article

Fabrication of fusogenic and magnet-responsive cells for transplantation of an intact mitochondrial network

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Received 24 July 2025 Accepted 5 November 2025 Published 11 May 2026

Abstract

Mitochondrial transplantation is a promising treatment for many diseases associated with mitochondrial defects or aging; however, a reliable method for mitochondrial transfer remains urgently needed. In this study, we assemble fusogenic and magnet-responsive cells (FMRCs), which are enucleated stem cells loaded with Fe₃O₄ nanoparticles and further incorporated fusogenic vesicular stomatitis virus glycoprotein G (VSV-G). Mitochondrial transplantation from FMRCs via fusion in the presence of a magnetic force restores normal mitotic activity, mitochondrial membrane potential, ROS levels and ATP production in cells subjected to partial mtDNA depletion or in cybrids harboring mtDNA with a 4977-bp deletion. SNP tracing and qPCR analysis of the mitochondrial and nuclear genomes unequivocally demonstrate that exogenous mitochondria are able to reside stably and predominately. Mitochondrial transplantation stimulate autophagy and thus the clearance of defective endogenous counterparts, resulting in lower mtDNA heteroplasmy. These results suggest that FMRCs are excellent vehicles for mitochondrial transplantation and could be used for the treatment of aging and mitochondria-associated diseases.

Key words fusogenic and magnet-responsive cell, cell fusion, mitochondrial transplantation, autophagy, SNP analysis, VSV-G, 143B^{Δ4977} cell

Introduction

Mitochondria are semi-autonomous organelles that generate most of the chemical energy needed to power the cell's biochemical reactions. Mitochondria are also involved in many other fundamental cellular processes [1]. Mutations in mitochondrial DNA (mtDNA) can be Mendelian, maternally inherited, or sporadic and can occur at various time points during development, leading to mitochondrial dysfunction which plays a major role in aging, metabolic diseases, neurodegenerative diseases, neuromuscular disorders and cancers [2–4]. Consequently, mitochondria are now attracting increased research interest and are major targets for pharmaceutical companies.

Mitochondrial transfer offers a potential cure for diseases associated with mitochondrial dysfunction. Mitochondrial transfer has been observed naturally *in vivo* and in cell culture via open-ended tubular extensions called tunneling nanotubes (TNTs) [5–7].

In addition, membrane-bound extracellular vesicles (EVs) occasionally contain mitochondria and transfer them to surrounding cells [8]. Mitochondrial transfer via TNTs and EVs reportedly decreases reactive oxygen species (ROS) levels and apoptosis rates [9–11]. However, the low efficacy of these approaches limits their therapeutic value.

Currently, many reports claim the remarkable effects of isolated mitochondria in combating mitochondrial dysfunction, both in model systems and in clinical settings [12,13]. McCully and colleagues [14] reported that ischemic hearts transplanted with healthy mitochondria presented a reduced infarct size, decreased cardiomyocyte loss, and improved post-ischemic myocardial function. Injection of autologous mitochondria derived from muscle reportedly decreases the brain infarct volume and reverses neurological deficits in a stroke model in rats [15]. However, the underlying mechanism has not been elucidated in the above reports

and the use of isolated mitochondria has raised uncertainty [16,17].

Enucleated cells (cytoplasts) are traditionally used to form cybrids, cytoplasmic hybrids created by the fusing cells from patients harboring mtDNA mutation(s) with cells devoid of mtDNA (Rho0 cells) [18]. In a sense, enucleated cells are excellent vehicles for the transmission of mitochondria because in this case, the mitochondria are protected from the harsh extracellular environment and thus remain almost intact. We previously demonstrated that a type of miniature cytoplast, plasma membrane vehicles (PMVs) generated by mechanical extrusion, could be exploited for the transplantation of functional mitochondria [19]. Rho0 cells or acetaminophen-damaged HepG2 cells transplanted with functional mitochondria from mesenchymal stem cells were protected from cell death and regained mitotic activity [19,20]. However, these approaches rely heavily on membrane fusion, which is notoriously difficult and often leads to cell death.

Membrane fusion is a fundamental biological process, ranging from membrane biogenesis and intracellular traffic to hormone secretion and viral infection. Membrane fusion proceeds through a series of well-defined intermediate steps, characterized as the so-called hemifusion model [21,22]. These steps are governed by energy barriers, which can be overcome by disparate fusogens, including proteins, peptides, lipids and ions [23]. Energy barriers can also be reduced *in vitro* using mechanical and chemical techniques, such as polyethylene glycol (PEG) induction, electrical stimulation and optically-induced cell fusion [24–26]. However, these approaches are flawed by either cytotoxicity or low efficiency.

In this manuscript, we report a breakthrough in VSV-G-mediated membrane fusion. Fusogenic and magnet-responsive cells (FMRCs), which are enucleated stem cells loaded with Fe₃O₄ nanoparticles and further incorporated with fusogenic vesicular stomatitis virus glycoprotein G (VSV-G), were generated and used for mitochondrial transfer via fusion. A magnetic force was used to bring FMRCs into close proximity with target cells, enabling efficient membrane fusion. Consequently, the transplantation of functional mitochondrial networks from FMRCs to recipient cells, such as cells with partial mtDNA depletion or cybrids harboring a 4977-bp mtDNA deletion, was achieved, leading to restored mitotic activity and reduced mtDNA heteroplasmy.

Materials and Methods

Preparation of the VSV-G conditioned medium

Ad293 cells (Agilent, Santa Clara, USA) were cultured in a complete medium containing 90% DMEM (Sigma-Aldrich, Shanghai, China), 10% FBS (Biowest, Shanghai, China), and penicillin/streptomycin (Beyotime, Shanghai, China). The culture was incubated at 37°C in a humid environment containing 5% CO₂. Ad293 cells were seeded in 6-well plates, cultured overnight, and transfected with 0.8 µg/well pLV-VSVG plasmid via PolyJet reagent (SigmaGen Laboratories, Ijamsville, USA) according to the instruction manual. Condition medium (CM) was collected at 48 h. Ad293 cells were harvested and lysed by three freeze-thaw cycles. The lysate and CM were pooled into 2-mL tubes, and the cell debris was removed via differential centrifugation at 300, 2000, and 10,000 g for 10 min at each step. The clear CM was then condensed by super-centrifugation (Type 100Ti fixed angle, 344619; Beckman Coulter, Fullerton, USA) at 100,000 g for 70 min. Approximately 200 mL of concentrated VSV-G CM was collected from 6 mL of debris-free CM. The total protein concentration was then measured via the BCA assay kit (Thermo

Fisher Scientific, Shanghai, China).

Generation of enucleated BMSCs

Cell enucleation was carried out according to a previously reported method with modifications [27]. Approximately 5×10^5 /well of cells were seeded in an ECM (extracellular matrix)-coated 24-well plate. After 24–48 h of cultivation, the cells reached approximately 90% confluence. The 24-well flask was then fitted upside-down into a 50-mL conical tube containing 10 mL of medium for enucleation: calcium-free DMEM (MeilunBio, Shanghai, China) supplemented with calcium chloride (100 ng/mL; Damao, Tianjin, China), cytochalasin B (10 µg/mL; Aladdin, Shanghai, China), colchicine (5 µg/mL; Aladdin), 2% sucrose (w/v; Damao), and N-acetyl-L-cysteine (2 mM; Aladdin). The 24-well plates were spun in a warm Eppendorf centrifuge at 4500 g and 35°C for 60 min. After incubation, the medium was replaced by fresh culture medium and the cells were allowed to recover in the incubator for at least 1 h before being used for the fusion experiments.

Generation of cells with partial mtDNA depletion

BALB/c and KM mice (8- to 12-week-old) were purchased from the Shanghai Laboratory Animal Company (Shanghai, China). Myoblast cell line derived from BALB/c mice (C2C12 cells) or BMSCs derived from KM mice were treated with dideoxy-cytidine (ddC, 10 mM; MedChemExpress, Shanghai, China) and ethidium bromide (EB, 5 mM; MedChemExpress) for 4–7 d, which generated cells with partial mtDNA depletion (PMD). The remaining mitochondria in cells with partial mtDNA depletion (PMD cells) were defective due to ddC and EB treatment. The medium was then replaced by normal culture medium supplemented with uridine (50 mg/mL; Sigma-Aldrich) and pyruvate (0.1 mM; Sigma-Aldrich). Mitochondrial deficiency was verified by TMRE staining (500 nM; Abcam, Cambridge, USA). The content of mtDNA was examined via qPCR.

Fabrication and fusion of fusogenic and magnet-responsive cells

To optimize the loading conditions and visualize the distribution in the cells, rhodamine-conjugated Fe₃O₄ nanoparticles (a gift from Professor Haibao Zhu) were used. Different amounts of nanoparticles were incubated with the cells for 3–24 h on top of a magnet with a strength of 1000–5000 Gauss (Gs). To prepare FMRCs, enucleated cells (Ad293 or BMSCs) were loaded with Fe₃O₄ nanoparticles (200 ng, 747327; Sigma-Aldrich) in a 24-well plate and cultured for 12 h on top of a magnet of 1500 Gs, and then incubated with VSV-G conditioned medium (5 mg/mL) for 30 min.

To increase one-to-one cell fusion, target cells were seeded in a 24-well plate and cultured overnight at 15% confluency and then labeled with DiI (red; Beyotime). FMRCs (2×10^5) were labeled with Calcein-AM (green; Beyotime). Then cells were mixed and cultivated for 30 min in the presence of a magnetic force (1500 Gs). pH-dependent fusion was carried out by adding pre-warmed (45°C) fusion buffer (pH = 4.5) for 1 min. After a 30-min incubation, the cells were stained with Hoechst (33342; Solarbio, Beijing, China) and examined via confocal microscopy (LSM 800, Excitation: 346 nm, Emission: 460 nm; ZEISS, Jena, Germany) in a glass-bottom dish.

Measurement of calcium transient

Fluo4-AM (5 mM; Thermo Fisher Scientific) in calcium-free HBSS (Solarbio) was added to FMRCs. After incubation for 20 min, the

mixture was supplemented with 1% FBS (Beyotime) in HEPES saline buffer (Beyotime) and incubated for an additional 20 min. FMRCs in glass-bottom 24-well plates were washed with HBSS three times and then examined via confocal microscopy. Six pictures were taken from each well. The relative fluorescence intensity was calculated via ZEN 2 software.

Detection of cytoskeleton formation

The formation of the cytoskeleton was detected via immunofluorescent staining. FMRCs were fixed in 3.7% paraformaldehyde at room temperature for 10 min, and then treated with 0.1% Triton X-100 for 10 min. After washing with PBS three times, the microtubules were revealed by staining with Tubulin-Tracker Green (1:50 dilution in 5% BSA; Beyotime). For actin filaments, FMRCs were stained with FITC-conjugated phalloidin (Beyotime). For the detection of myosin, FMRCs were incubated with rabbit anti-mouse myosin (Sigma-Aldrich) at room temperature for 2 h, and then stained with Alexa Fluor 555-conjugated anti-rabbit IgG (1:200 dilution; Beyotime) for another 1 h. The cells were washed between the incubation of the primary and secondary antibodies. Finally, the cells were stained with Hoechst before being subjected to confocal microscopy.

Mitochondrial transplantation into cells with partial mtDNA depletion

To examine mitochondrial transplantation via cell fusion, PMD cells (using C2C12 cells) were used as recipient cells, in which the transplanted mitochondria could be easily detected because endogenous mitochondria are defective and faintly displayed after dye staining. After fusion with FMRCs [using enucleated BMSCs (eBMSCs)], PMD cells (prestained with CFSE, MCE, Shanghai, China) were cultivated in normal medium without supplementation with uridine or pyruvate. The cells were stained with TMRE and examined by confocal microscopy on day 1. Mitotic activity was evaluated by counting the number of cells on days 2 and 3.

The relative amount of mtDNA was determined via qPCR with a pair of mtDNA-specific primers for the *Nd6* gene on day 3 [28]: forward/mtDNA, 5'-CCCAGCTACTACCATCATTCAGT-3', reverse/mtDNA, 5'-GATGGTTTGGGAGATTGGTTGATGT-3'. The cycle threshold (Ct) was normalized to the reference gene *beta-2 M*: forward/*beta-2 M*, 5'-ATGGGAAGCCGAACATACTG-3', reverse/*beta-2 M*, 5'-CAGTCTCAGTGGGGGTGAAT-3'. The qPCR was carried out using SYBR Green qPCR Master Mix (TransGen Biotech, Beijing, China). The thermocycle parameters were: 95°C for 3 min, followed by 95°C for 10 s, 60–65°C for 30 s and 72°C for 1 min for a total of 30 cycles.

Experiments involving mitochondrial transplantation into recipient cells were approved by the Animal and Human Experiment Ethical Committee of Shantou University on March 4, 2024 (establishment of a novel mitochondrial transplantation technique No. 202401007).

Mitochondrial transplantation into cells harboring a common mtDNA deletion

143B^{A4977} and control 143B cells were gifts from Dr. Lei Liu (Institute of Zoology, Chinese Academy of Sciences, Beijing, China). Enucleation of Ad293 cells was performed as reported previously [19,20]. The cells were mechanically extruded through a filter membrane with pores of 5 µm. After fusion, the cell viability was

assayed via a CCK8 kit (Beyotime) at days 3 and 5. The ATP content was measured via an ATP assay kit (Beyotime) according to the instructions provided by the company. The levels of ROS, cardiolipin content, and SDHA in the mitochondria were determined by staining with MitoSOX (Invitrogen, Carlsbad, USA), 10-nonyl acridine orange (NAO; MCE), and anti-SDHA (GeneTex, Irvine, USA), respectively. The heteroplasmy of mtDNA was evaluated via qPCR of three fragments of mtDNA. The D-loop maintained in both wild-type and mutant mtDNA was used for standardization of the mtDNA mass. The *ND4* gene included in the deleted region of 4977-bp was used for the measurement of wild-type mtDNA. The TRNK-ND5 region bordering the deletion was used for the assay of mutant mtDNA. The specific primers used were as follows: forward/D-loop, 5'-CACAGGTCTATCACCTATTAAC-3', reverse/D-loop, 5'-CAGAGATGTGTTAAGTGTGTG-3'; forward/*Nd4*, 5'-CGCTCATTGCATACCTTCAATC-3', reverse/*Nd4*, 5'-GTTACTAGCACAGAGAGTTCTCC-3'; and forward/*TRNK*, 5'-CTAGAGCCCACTGTAAAGCTAAC-3', reverse/*Nd5*, 5'-GTTGACCTGTAGGGTGAGAAG-3'. The cycle threshold (Ct) was normalized to the reference gene as above. qPCR was carried out via the use of SYBR Green qPCR Master Mix. The thermocycle parameters were: 95°C for 3 min, followed by 95°C for 10 s, 60–65°C for 30 s and 72°C for 1 min for a total of 30 cycles.

SNP analysis of mtDNA and nDNA

FMRCs (using eBMSCs from BALB/c mice) were fused with partial mtDNA-deleted cells (BMSCs from KM mice). After fusion, the mt-tRNA^{Arg} or mt-*Nd4* genes were amplified at days 1, 3, 5 and 10 with a pair of primers reported previously [29,30]: mt-tRNA^{Arg}, 5'-CTACTTCCACTACCATGAGC-3' (F) and 5'-GTATGGAGCTTATGGAGTTGG-3' (R). The PCR products were sequenced for SNP analysis. Since the enucleation efficiency is approximately 95%, we cloned the cells immediately after fusion. Three clones were generated in about 2 weeks. SNP analysis of the mt-tRNA^{Arg} gene was carried out as described above. Furthermore, the SNP site rs27250976 was chosen to distinguish the nDNA of BALB/c or KM mice [31]. The SNP site was amplified with the following primer pairs: 5'-CATCTTAAGTCCTCAGCACC-3' (F) and 5'-GGCTACCTGAAACG-TCAAAAT-3' (R). The resulting PCR products were subsequently sequenced by Sangon Biotech (Shanghai, China).

Autophagy detection by Cyto-ID staining

To measure autophagy flux, partial mtDNA-depleted cells (BMSCs from KM mice) transplanted with or without mitochondria were stained with Mito-Tracker Red CMXRos (200 nM; Beyotime), or LysoTracker-Red (50 nM; Enzo Life Science, New York, USA) together with CYTO-ID Green (Enzo Life Science) in the presence of chloroquine (CQ, 60 µM; MedChemExpress) at 37°C for 20 min 48 h after fusion. The R6G group and normal BMSCs were used as controls. After washing, the cells were examined via confocal microscopy.

Autophagy detection by western blot analysis

The following antibodies were used for western blot analysis of autophagy: anti-LC3B (ab192890; Abcam), anti-β-actin (AF5003; Beyotime), anti-Beclin1 (HA721216; HUABIO, Hangzhou, China), anti-p62 (HA721171; HUABIO), and anti-p62 (A21020; Abbkine, Wuhan, China) antibodies. Western blot analysis was carried out as described previously [32]. Briefly, the cells were harvested and

lysed in RIPA buffer supplemented with PMSF (1 mM, Beyotime) and phosphatase inhibitors (MCE). Supernatant was collected, and equal amount of protein was used for SDS PAGE electrophoresis. After protein transferring, the PVDF (polyvinylidene fluoride) films were blocked with western blot blocker buffer (QuickBlock, Beyotime), washed with TBST (Beyotime), and then incubated with appropriate antibodies overnight at 4°C. The membrane was developed using the enhanced chemiluminescence (ECL) solution (ChemStudio SA, Thermo Fisher Scientific). The protein band intensity was quantified using Image-Pro Plus software (Media Cybernetics, Rockville, USA).

Statistical analysis

All the statistical analyses were performed via GraphPad Prism 7 (GraphPad Software, San Diego, USA). All data are presented as the mean $\pm$ standard deviation. The experiments were repeated at least twice. The results were subjected to a two-tailed Student's *t*-test and a one-way ANOVA with Tukey's *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Fabrication of fusogenic and magnet-responsive cells (FMRCs)

The idea of fabricating FMRCs and using them for cell fusion and mitochondrial transplantation is depicted in Figure 1. BMSCs were loaded with Fe_3O_4 nanoparticles (Supplementary Figure S1), while fusogenic VSV-G glycoproteins were incorporated into the plasma membrane, forming FMRCs, which were fused with target cells in the presence of a magnet. The magnetic force overcame the energy barrier that forms during hemifusion. Consequently, mitochondria were transferred from FMRCs into recipient cells after the completion of fusion. The mitochondrial network was not exposed to the harsh environment and thus remained almost intact.

Furthermore, endogenous defective mitochondria could be eliminated by autophagy.

To examine the loading of the nanoparticles, rhodamine-conjugated Fe_3O_4 nanoparticles was added to the culture in the presence of a magnet (Figure 2A). The Fe_3O_4 nanoparticles appeared in the cytoplasm within 3 h. The intensity of rhodamine was correlated with incubation time, magnet strength, and nanoparticle amount (Figure 2A and Supplementary Figure S2). VSV-G-conditioned media were prepared from Ad293 cells transfected with a VSV-G expression cassette (Supplementary Figure S2). Cells loaded with Fe_3O_4 nanoparticles were incubated with VSV-G and then examined for incorporation by immunofluorescence staining. The results revealed that VSV-G appeared on the cell surface (Figure 2B). The staining intensity increased with the amount of VSV-G added to the culture (Figure 2B and Supplementary Figure S3). The magnet responsiveness of FMRCs was assayed by the speed of sedimentation in the presence or absence of a magnet (Figure 2C). More cells reached the bottom of the well in the presence of a magnet, whereas only a few cells were detected at a specific point in time when the magnet was absent (Figure 2C and Supplementary Figure S3).

The magnetic force stimulates the fusion of FMRCs

It has been reported that mechanical tension at the fusogenic synapse drives cell membrane fusion to overcome energy barriers under physical conditions [33]. We leveraged the static force from a magnet and fabricated FMRCs as described above to promote fusion. Indeed, the efficiency of fusion increased from 40% (VG) to approximately 60% (VG+MP, Figure 3A–C). Notably, the majority of fusion occurred between 2 cells with a diameter of 20–25 μm . A decrease in cell density favors the formation of two-cell fusion. Furthermore, fusion could be improved by increasing the amount of nanoparticles and the strength of the magnetic force, reaching

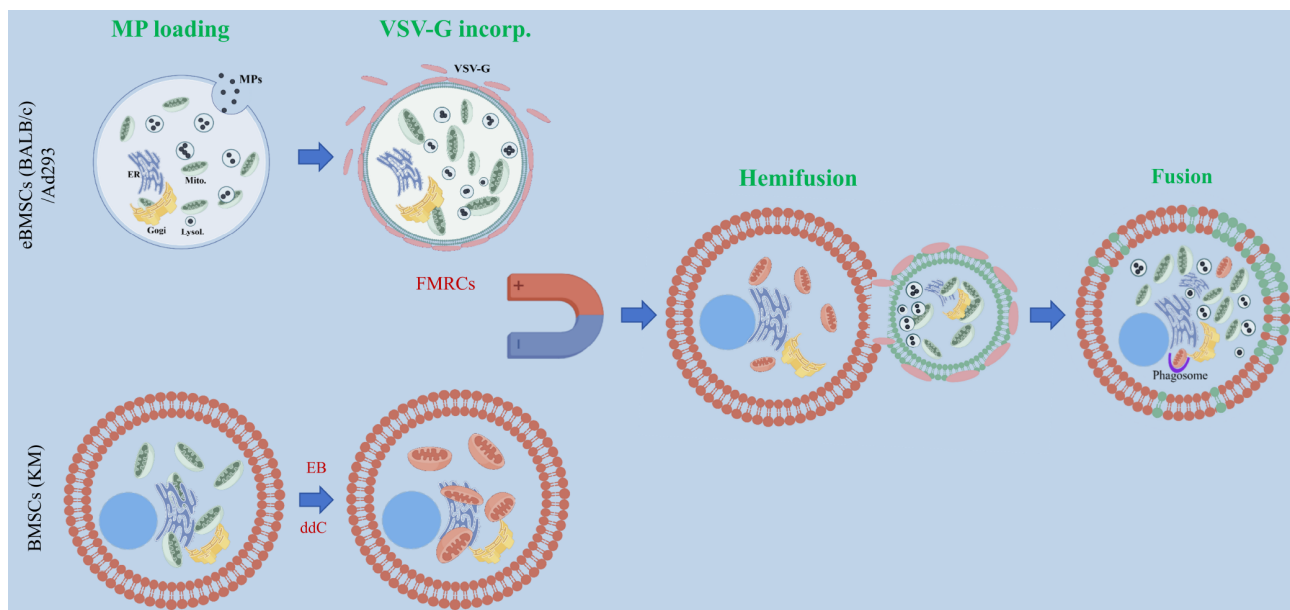


Figure 1. Schematic of the methods used for FMRC, fusion and mitochondria transplantation Cells (eBMSCs from BALB/c mice) were loaded with Fe_3O_4 nanoparticles (MPs) and incorporated with VSV-G to form FMRCs, which were then fused with target cells (partial mtDNA-depleted BMSCs from KM mice by treatment with ddC and EB). The presence of a magnetic force improved the formation of hemifusion by overcoming the energy barriers, leading to full fusion and mitochondrial transplantation and, subsequently, the elimination of defective endogenous mitochondria by autophagy. The figure was created via Figdraw (www.figdraw.com).

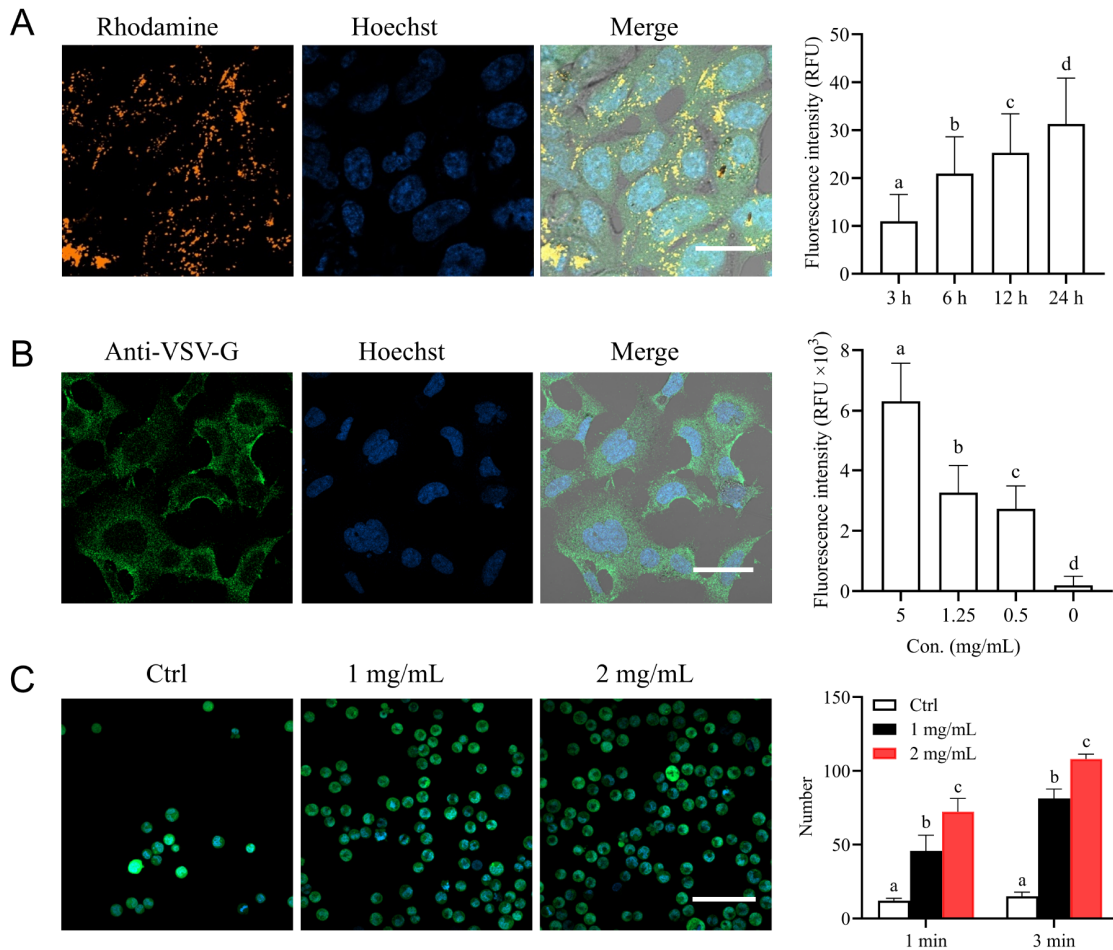


Figure 2. Loading of magnetic nanoparticles and incorporation of VSV-G (A) Ad293 cells (labeled with Calcein-AM) were incubated with 50 ng of Fe_3O_4 nanoparticles (rhodamine conjugated, red) on top of a magnet (1500 Gs) for 12 h. The cells were stained with Hoechst 33342 (blue) before being examined via confocal microscopy. The intensity of the loaded nanoparticles was quantified. Scale bar = 50 μm . $n = 3$. (B) Ad293 cells were incubated with different concentrations of VSV-G for 2 h. The cells were then stained with anti-VSV-G (green) and Hoechst 33342 (blue) before being examined via confocal microscopy. The intensity of the incorporated VSV-G protein was quantified. Scale bar = 50 μm . $n = 3$. (C) Ad293 cells were loaded with Fe_3O_4 nanoparticles (0, 1, or 2 mg/mL) and stained with calcein-AM. The cells were then harvested and added to a 24-well plate on top of a 1500-Gs magnet. The cells that sedimented to the bottom within 1 and 3 min were examined via confocal microscopy. The number of cells was counted manually. Scale bar = 100 μm . $n = 3$. Bars denoted with different letters at the top are significantly different ($P < 0.05$).

approximately 70% at a magnetic intensity of 1500 Gs and a nanoparticle concentration of 2 mg/mL (Figure 3D,E).

In addition, we found that FMRCs fusion was more favorable at higher temperatures. Actually, temperature-dependent cell fusion has been reported [34], showing that HIV envelope glycoprotein-mediated syncytium formation is more favorable at 37°C than at 4°C. In the present study, FMRC fusion was induced for 1 min in buffer pre-warmed to 37–55°C. The highest fusion rate was obtained when induction was performed at 50°C (Supplementary Figure S4A,B). However, FMRCs formed larger aggregates at 50°C, whereas more two-cell fusion was observed at 45°C. A similar phenomenon was observed in the fusion of FMRCs derived from various cell types (Supplementary Figure S4C). Notably, only a few cells showed cytotoxic effects during the induction of fusion.

The magnetic force stimulates calcium transient and cytoskeleton formation

To understand the mechanism underlying magnetic-force-stimu-

lated fusion, calcium transients were imaged using fluorescence. Magnetic force triggered an increase in calcium transients (Figure 4A,B), which could be dampened by inhibitors of voltage-gated calcium channels (fluoaxetine) or selective intracellular or extracellular calcium chelators (BAPTA/EDTA) (Figure 4C,D). Furthermore, inhibition of the calcium transients completely blocked the effect of magnetic force on fusion (Figure 4E). These results are consistent with earlier reports of the calcium transient's involvement in various fusion models [35].

Next, the effect of magnetic force on cytoskeleton formation was examined. The cytoskeleton has been demonstrated to be critical for cell fusion. Indeed, magnetic force significantly promoted the formation of actin filaments and myosin II, whereas microtubule formation appeared not affected (Supplementary Figure S5A,B). Consistently, the action of the magnetic force on fusion was abolished by the inhibitor (cytochalasin B) of actin filaments (Supplementary Figure S5C,D). However, a similar effect was detected with a microtubule inhibitor (colchicine). The discrepancy could be due to the method used to evaluate microtubule formation

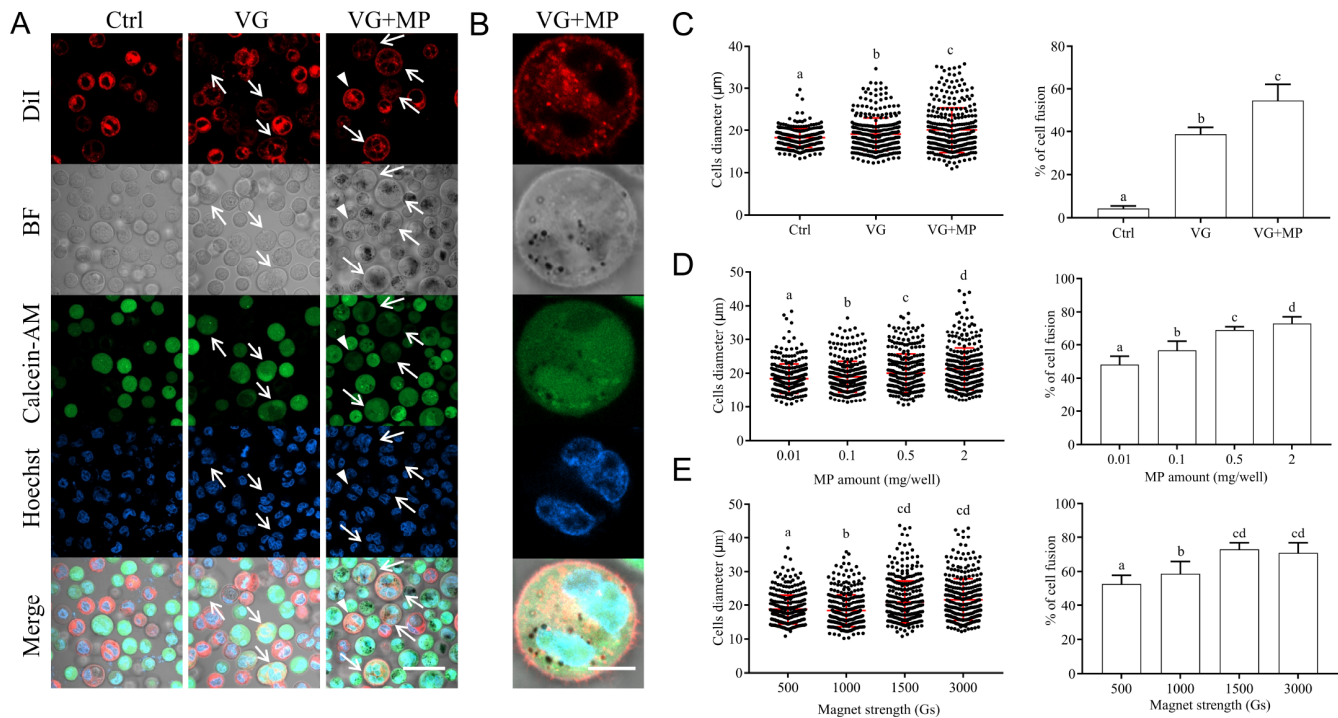


Figure 3. Magnetic force-stimulated fusion of Ad293 cells (A) Recipient cells (Ad293) were preseeded in a 96-well plate overnight and then labeled with DiI (red). Donor cells (Ad293 cells loaded with MPs containing the VSV-G protein) were labeled with calcein-AM (green) and then added to a 96-well plate. pH-dependent fusion was induced under magnetic force (1500 Gs) after the addition of VSV-G-containing medium for 30 min. Ctrl: control, no VSV-G; VG: with VSV-G-containing medium; MP: FMRCs loaded with Fe_3O_4 magnetic nanoparticles (MPs, 2 mg/mL). Hoechst: blue. Scale bar = 50 μm . Arrows indicate fusion between donor and recipient cells. The cell indicated with an arrowhead was enlarged in (B). (C) The cell diameter was measured, and the fusion efficiency was determined. Cells with a diameter larger than 20 μm were arbitrarily considered fused cells. $n = 8$. The middle red horizontal lines represent the median, and the error bars represent the inter-quartile range (top and bottom of the box). (D) Fusion efficiency was affected by the amount of Fe_3O_4 magnetic nanoparticles. $n = 8$. (E) Fusion efficiency was affected by the strength of the magnetic force. $n = 8$. Dot plots or bars denoted with different letters on top are significantly different ($P < 0.05$).

(immunofluorescence), which was not highly quantitative.

Mitochondria transplantation restores the mitotic activities of cells with partial mtDNA depletion

After establishing optimal conditions, we prepared FMRCs from enucleated BMSCs (Supplementary Figure S6), which contained predominantly rod-shaped mitochondrial networks. Compared with their nucleated counterparts, the mitochondria in FMRCs (eBMSCs) had a slightly lower (approximately 80%) membrane potential. In contrast, the isolated mitochondria were round in shape and had a reduced membrane potential to barely above background levels.

FMRCs (eBMSCs) can also be fused with a variety of target cells, including BMSCs, C2C12 cells, as well as C2C12-PMD cells. Fusion occurred with high efficiency, leading to mitochondrial transplantation, which displayed a network structure and maintained a membrane potential at approximately normal levels (Figure 5A–C). A large percentage of C2C12-PMD cells (compared to the uridine plus group) regained their ability to proliferate even when uridine was withdrawn, while cells without mitochondrial transplantation started to die (Figure 5D). The fused C2C12-PMD cell count declined on day 2 but quickly rebounded on day 3 (Figure 5E). Finally, we quantified the amount of mtDNA in C2C12-PMD cells before and after mitochondrial transplantation via qPCR of the *Nd6* gene (Figure 5F). The results revealed that mtDNA increased more than 100-fold after transplantation (Figure 5G).

Elimination of endogenous mitochondria in BMSC-PMD by autophagy

Currently, no reliable methods exist for long-term labeling and tracing of mitochondria. To unequivocally demonstrate the transfer of exogenous mitochondria, we took advantage of a single-nucleotide polymorphism (SNP) in the mt-tRNA^{Arg} gene, which has a stretch of different numbers of adenosines [29]. We confirmed this polymorphism via PCR amplification followed by sequencing. Consistent with a previous report, the mt-tRNA^{Arg} genes in the BMSCs generated from the BALB/c and KM mice included 10 and 9 adenosines, respectively (Figure 6A). FMRCs (eBMSCs) derived from BALB/c mice were subsequently fused with BMSC-PMDs from KM mice.

After fusion, the mt-tRNA^{Arg} gene was amplified and sequenced at days 1, 3, 5, and 10. We found that the contribution of the transplanted mt-tRNA^{Arg} gene increased from approximately 20% (T:A ratio) on day 1 to above 90% on day 10 (Figure 6A). Furthermore, we determined the origin of the cells via SNP analysis of the nuclear genome [31]. The cells were cloned and inserted into 96-well plates immediately after fusion. A total of 3 clones were obtained and used for SNP analysis of mtDNA and nDNA. The results revealed that 2 of the clones were hybrids of mtDNA and nDNA derived from BALB/c and KM mice, respectively (Supplementary Figure S7). The other group was contaminated with some BALB/c mouse BMSCs that were not

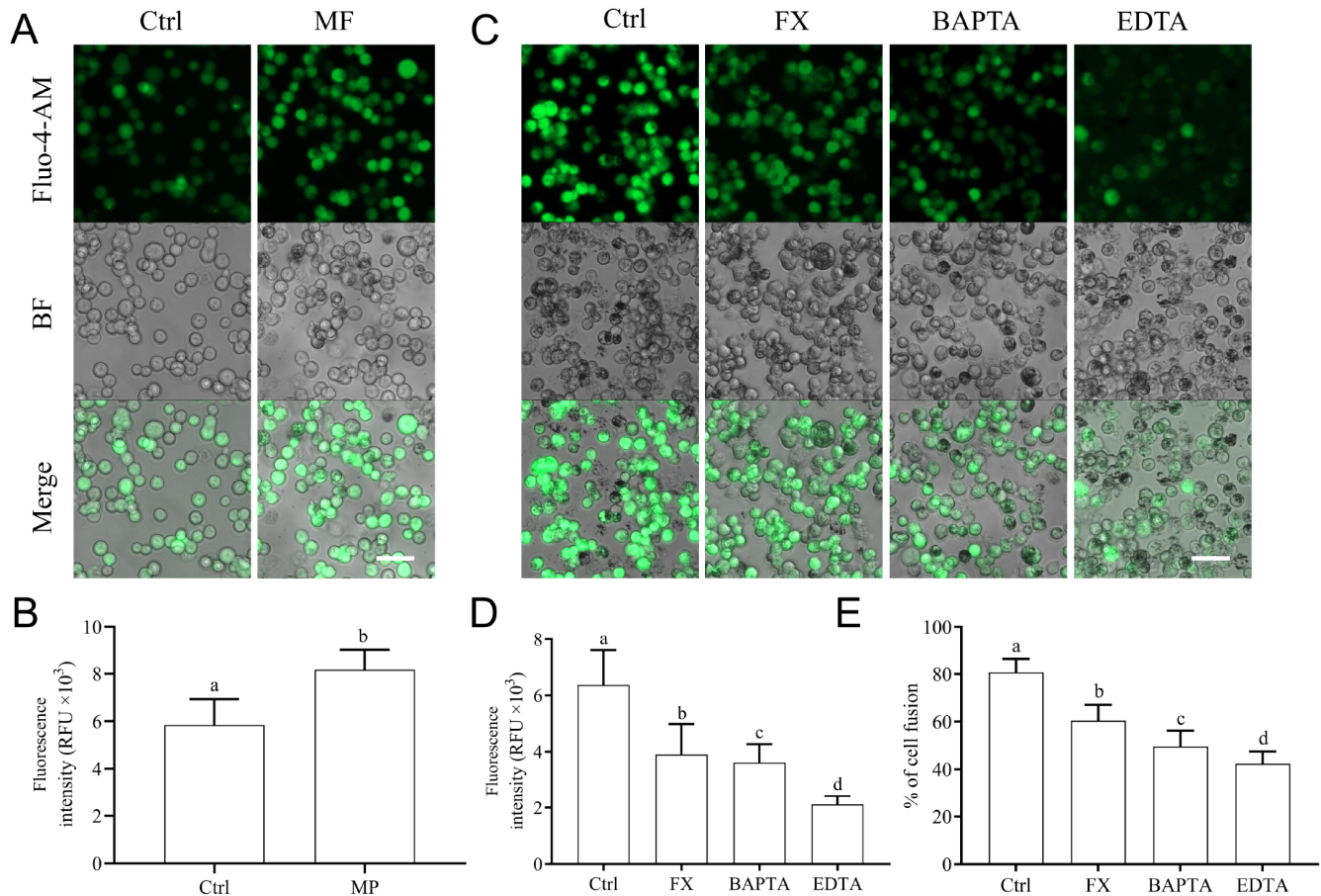


Figure 4. Calcium transients are stimulated by a magnetic force Ad293 cells loaded with Fe_3O_4 nanoparticles (2 mg/mL) and Fluo-4-AM (5 mM) were added to an ECM (extracellular matrix)-coated glass-bottom dish. The cells were incubated on top of a magnet (1500 Gs) for 10 min, and then examined via confocal microscopy. (A) Photomicrographs of cells loaded with Fluo-4-AM (green). Ctrl: control cells; MP: cells loaded with Fe_3O_4 nanoparticles. Scale bar = 50 μm . (B) Quantification of the Fluo-4 fluorescence intensity of the cells in (A). $n = 8$. *** $P < 0.001$. (C) Photomicrographs of cells treated with FX (fluoxetine-HCL, 10 μM), BAPTA (3 mM), EDTA (2.5 mM) or DMEM (Ctrl) for 2 h before loading of Fluo-4-AM. Scale bar = 50 μm . (D) Quantification of the Fluo-4 fluorescence intensity of the cells in (C). $n = 8$. (E) A cell fusion assay was performed in the presence or absence of FX (fluoxetine-HCL, 10 μM), BAPTA or EDTA. $n = 8$. Bars denoted with different letters on top in (D) and (E) are significantly different ($P < 0.05$).

enucleated successfully. Nonetheless, the results demonstrated that exogenous mitochondria could colonize stably in BMSCs-PMD.

To confirm that autophagy is responsible for the clearance of some defective mitochondria in the PMD-BMSC group, we carried out an autophagy flux test via the staining of Cyto-ID and LysoTracker Red DND-99 in the presence of chloroquine (CQ). The results revealed that mitochondrial transplantation significantly increased the level of autophagy in the BMSC-PMD group back to the levels of normal cells (Figure 6B), suggesting that restored energy production due to mitochondrial transplantation was essential to the restoration of autophagy process. In contrast, when FMRCs were pretreated with R6G, which compromises the normal function of mitochondria, the autophagy levels in the BMSC-PMD did not change after fusion. Furthermore, Mito-Tracker Red CMXRos and Cyto-ID revealed that significantly more mitochondria co-localized with autophagosomes 48 h postfusion (Figure 6C). In contrast, the BMSC-PMD and R6G groups presented little colocalization.

Moreover, we measured the protein levels of LC3, p62, and

Beclin1 in the PMD-BMSCs transplanted with or without mitochondria (Figure 6D). Consistent with previous reports [36], LC3-II and Beclin1 decreased in the PMD-BMSCs but returned to normal levels after fusion. On the other hand, p62 decreased in the PMD-BMSCs group but increased after fusion. As a control, the R6G group did not show the same trend as described above.

Reduction in oxidative stress and heteroplasmy in 143B cells harboring mtDNA deletion after mitochondrial transplantation

To investigate whether this technology can be used as a medical treatment for mitochondrial diseases, we tested the effect of mitochondrial transplantation in cybrids harboring a high percentage of mutant mtDNA with a 4977-bp deletion (143B $\Delta 4977$ cells) [37]. For technical reasons, the enucleation of human kidney embryonic cells (Ad293 cells) was achieved via mechanical extrusion through a filter with 5- μm pores [19,20], thus generating plasma membrane vesicles (PMVs), which are miniature cytoplasts with a diameter of approximately 5 μm (Supplementary Figure S8). Cytoplasts generated via extrusion contain segregated mitochondria

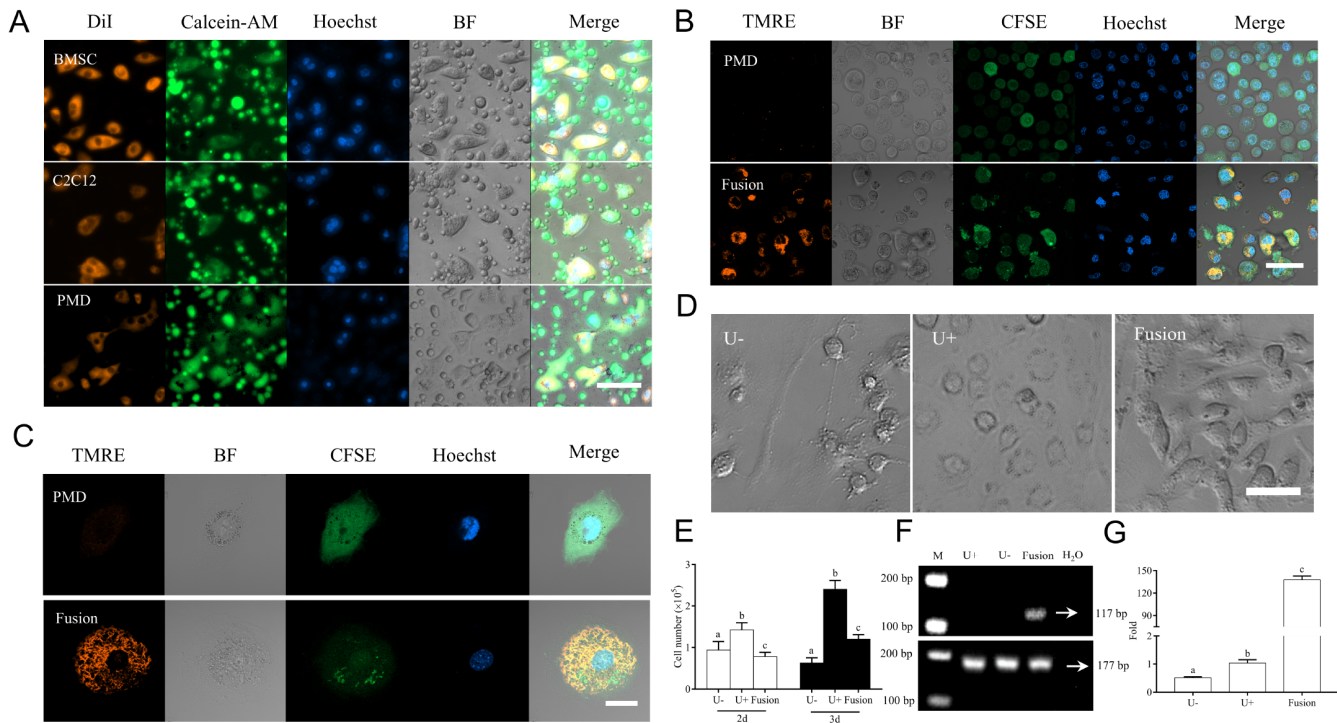


Figure 5. Transplantation of mitochondria stimulates the mitotic activity of cells with partial mtDNA depletion (A) Fusion between FMRCs (enucleated BMSCs loaded with MP and calcein AM and supplemented with VSV-G protein) and DiI-labeled C2C12 or C2C12-PMD cells. Scale bar = 50 μ m. (B) Confocal images of TMRE staining. Fusion was carried out between FMRCs and C2C12-PMD cells (prestained with CFSE, MCE). After fusion, the cells were stained with TMRE and examined via confocal microscopy. Scale bar = 50 μ m. (C) Enlarged image showing rod-shaped mitochondria in C2C12-PMD cells after fusion. Scale bar = 20 μ m. (D) Bright-field images of C2C12-PMD cells 3 days post-fusion. Cells with or without uridine are presented as controls. Scale bar = 50 μ m. U: uridine. (E) Cell numbers were counted at days 2 and 3 after mitochondrial transplantation. (F) Electrophoresis showing PCR bands for B2M (Beta 2 M, bottom, 177 bp) and mitochondrial DNA (upper, 117 bp). (G) Mitochondrial copy number was evaluated via relative qPCR. mtDNA levels in normal BMSCs, and C2C12 cells were provided as references for evaluating mitochondrial transplantation efficiency. $n = 3$. Bars denoted with different letters at the on top are significantly different ($P < 0.05$).

rather than an intact mitochondrial network. Nonetheless, the FMRCs derived from enucleated Ad293 cells efficiently transferred functional mitochondria to 143B^{Δ4977} cells via fusion.

The transplantation of mitochondria restored the normal mitochondrial membrane potential and the tubular network in 143B^{Δ4977} cells (Figure 7A,B and Supplementary Figure S8). In addition, 143B^{Δ4977} cells presented increased mitochondrial ROS levels, cardiolipin contents and SDHA levels due to mtDNA deletion. The levels of these genes returned to normal after mitochondrial transplantation (Figure 7C–E). These effects were not detected if the mitochondria in Ad293 cells were damaged by R6G before transplantation. Furthermore, ATP production and cell mitotic activity increased to levels comparable to those in control 143B cells after mitochondrial transplantation (Figure 7F,G).

Finally, heteroplasmy was evaluated via qPCR of three mtDNA fragments (Figure 7H,I). The D-loop region, which is maintained in both wild-type and mutant mtDNA, was used to standardize mtDNA mass. The *ND4* gene, which is within the deleted region of 4977-bp, was used for the measurement of wild-type mtDNA. The *TRNK-ND5* region, which borders the 4977-bp deletion, was used for the assay of mutant mtDNA. The results demonstrated that 143B^{Δ4977} cells harbor approximately 70% mutant mtDNA. In contrast, 143B cells contain a very low percentages of mutant mtDNA (approximately 0.12%). In any event, heteroplasmy in 143B^{Δ4977} cells was reduced to approximately 19% 5 d after

mitochondrial transplantation (Figure 7J,K).

Discussion

The shape of mitochondria varies in living cells and can range from punctuated structures to tubular networks. Maintaining these dynamic interconnected networks is crucial for both mitochondrial and cellular functions [38]. Techniques reported in the literature mostly utilize purified mitochondria, which inevitably break the branched network into dot-shaped mitochondria. This mechanical process is harmful because membrane breakage could result in the exchange of solutions in and out of the mitochondrial matrix and thus the dissipation of the proton gradient, eventually leading to a severe reduction in membrane potential (Supplementary Figure S6).

Moreover, isolated mitochondria in the medium were exposed to a calcium concentration approximately 10,000–20,000 fold greater than that in the cytosol. Calcium uptake through the mitochondrial calcium uniporter (MCU) leads to calcium overload, and Ca^{2+} accumulation can impair mitochondrial function, resulting in reduced ATP production and increased reactive oxygen species (ROS) production [39]. Furthermore, it has been reported that isolated mitochondria have low respiration activities and increased mitochondrial permeability, resulting in the release of pro-apoptotic proteins [40–42]. Therefore, although the success of transplantation using isolated mitochondria in cell cultures, animal models, and even clinical settings has been reported numerous times [43–45], the

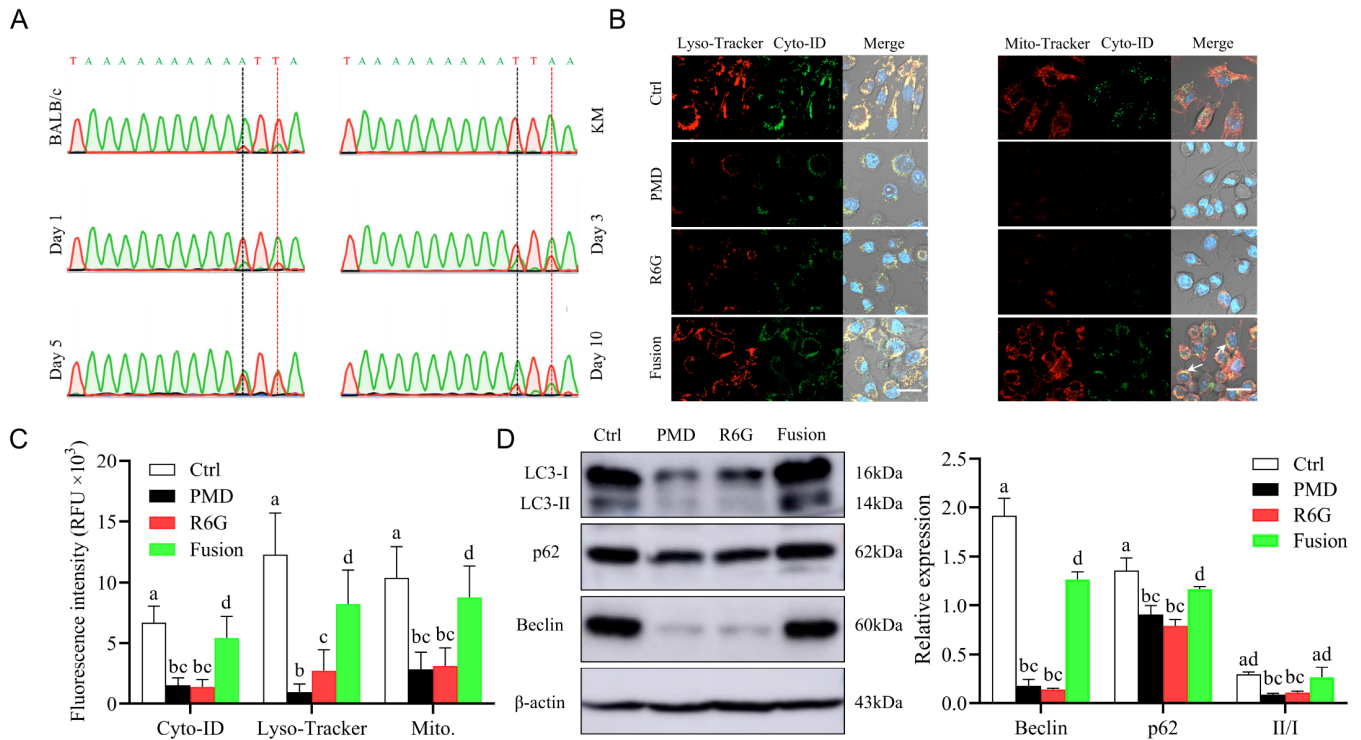


Figure 6. Clearance of endogenous mitochondria in PMD cells after mitochondrial transplantation (A) FMRCs of eBMSCs (BALB/c mice) were fused with BMSC-PMDs (KM mice). The cells were harvested at days 1, 3, 5, and 10 for SNP analysis of the mt-tRNA^{Arg} gene. (B) Cells were stained with Cyto-ID (green) and LysoTracker Red DND-99 or Mito-Tracker Red CMXRos at 48 h post cell fusion and examined via confocal microscopy. The arrows indicate that the mitochondria possibly underwent autophagy. Scale bar = 20 μ m. (C) The relative fluorescence intensities of Cyto-ID, LysoTracker Red DND-99 or Mito-Tracker Red CMXRos were evaluated. $n = 3$. (D) Western blot analysis of autophagy markers, including LC3-II, p62 and Beclin1 (left). Beta-actin was used as a loading control. The relative protein band intensity was determined (right). $n = 3$. In (B–D), Ctrl: normal BMSCs (KM) cells; PMD: BMSC-PMD cells (KM) without mitochondria transplantation; R6G: BMSC-PMD cells (KM) were fused with FMRCs pretreated with R6G (rhodamine 6G); fusion: BMSC-PMD cells (KM) were fused with FMRCs. Bars denoted with different letters at the top are significantly different ($P < 0.05$).

evidence and purported mechanisms remain controversial [16,17].

Mitochondria are occasionally found in extracellular vesicles [46]; however, their usage in mitochondrial transplantation is questionable. Micro-manipulation techniques including nuclear transfer and the FluidFM-based approach are suitable only for the transplantation of mitochondria into germ cells or a small number of cultured cells [47,48]. In contrast to the techniques mentioned above, we reported a novel approach for mitochondrial transplantation using FMRCs, which is fusogenic and magnet-responsive (Figure 1,2). FMRCs could be versatile in terms of the incorporation of different fusogens [23] and the delivery of a multitude of cargo [49]. Importantly, FMRCs were fabricated in a bio-friendly environment, which is extremely suitable for maintaining the bioactivities of therapeutics. In addition, the loading of large amounts of Fe₃O₄ nanoparticles was apparently tolerated. We did not detect noticeable cytotoxic effects, suggesting the superior biocompatibility of the Fe₃O₄ nanoparticles (Supplementary Figure S1).

The fusion of FMRCs with target cells depends critically on the magnetic force to overcome energy barriers (Figure 3). Increasing the strength of the magnet was favorable, but seemingly a plateau was reached at approximately 3000 Gs. However, the cells were not aligned directly on top of each other; thus, the force did not push the cell against the other. Instead, the force accelerated the spread, as indicated by the increase in cytoskeleton formation and calcium transients (Figure 4 and Supplementary Figure S5). Conceivably, a

stronger magnetic force applied to FMRCs in the capillary of microfluidics would stimulate fusion even better. Fusion with FMRCs was further optimized by increasing the fusion temperature, which is important for cells that are extremely refractory to fusion (Supplementary Figure S3). Higher temperatures probably disturb the lipid crystallinity of the plasma membrane. Notably, both the magnetic force and the higher temperature were important only during the induction of fusion but not after, indicating that once a hemifusion is formed, the development into full fusion is automatic.

The majority of fusion occurred between 2 cell types, which is favorable for cells that are still in the cell cycle because the formation of multinucleated aggregates would probably lead to cell death (please note that one cell is enucleated in the present study). To ensure one-to-one fusion, the target cells were pre-seeded overnight at approximately 15% confluency, while FMRCs were added at a ratio of approximately 3:1. However, owing to the uneven distribution (especially around the edge), a small fraction of the fusion was formed with 3–4 cells. FMRCs (eBMSCs) were then prepared and used for fusion and mitochondrial transplantation to cells with partial mtDNA depletion (PMD) (Figure 5A and Supplementary Figure S6). The transplanted mitochondria maintained the tubular network structure with approximately normal membrane potential (Figure 5B,C). Transplantation of mitochondria was further confirmed by qPCR amplification with a pair of mitochondria-specific primers for the *Nd6* gene, which revealed a

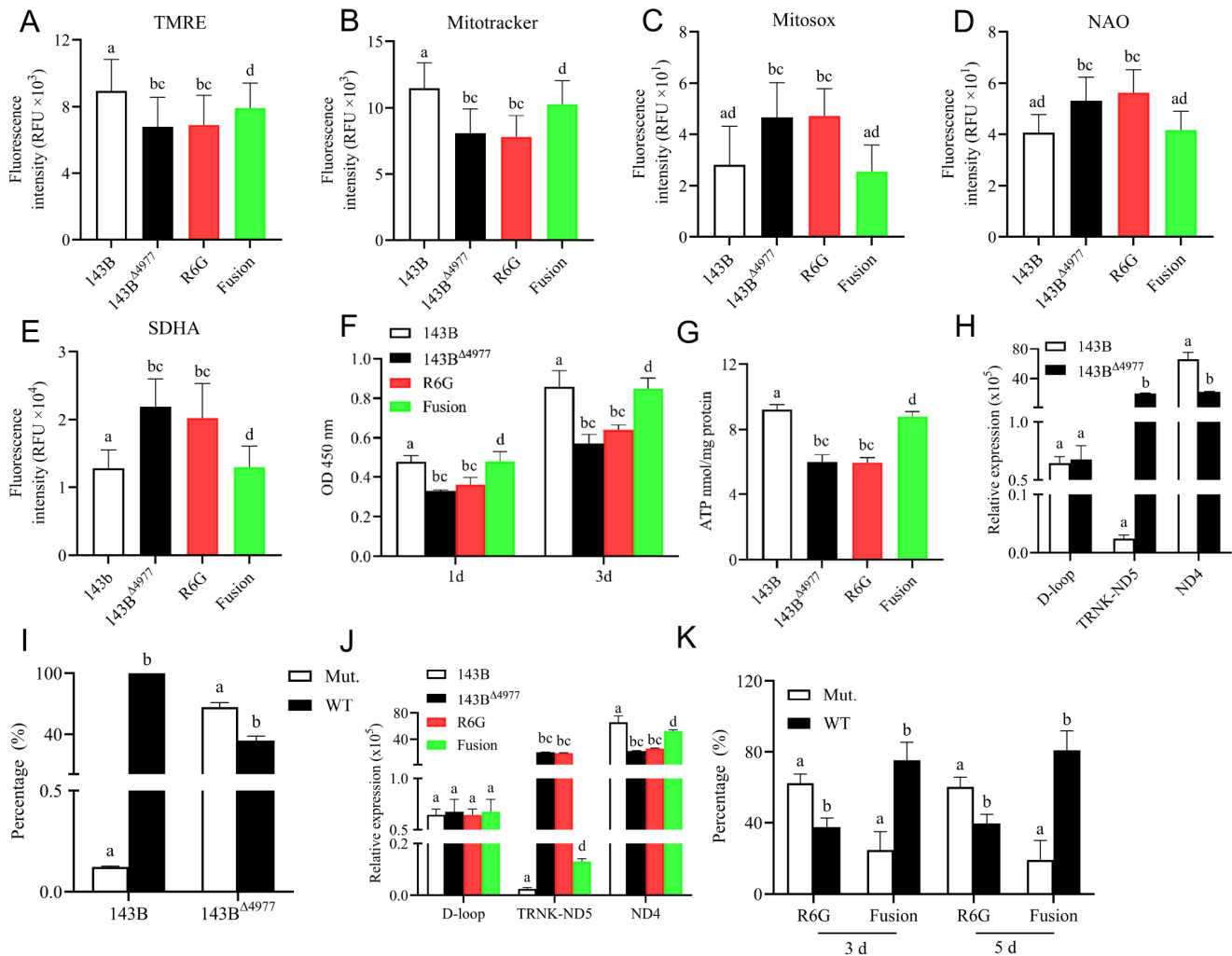


Figure 7. Transplantation of mitochondria restores normal mitochondrial functions in 143B^{Δ4977} cells FMRCs of Ad293 cells (cytoplasts generated via extrusion) were fused with 143B^{Δ4977} cells. The cells were subjected to fluorescence staining, ATP measurement, viability determination and qPCR at the indicated time points after fusion. (A–E) Fluorescence intensity of cells stained with TMRE, Mito-Tracker Red CMXRos, mito-SOX, NAO, and anti-SDHA. (F,G) Measurement of cell mitotic activity via the CCK8 assay and ATP production. (H) The heteroplasmy of mtDNA was assayed by qPCR analysis. (I) Percentage of wild-type (WT) and mutant mtDNA (Mut.) was calculated. (J) Assay of heteroplasmy of mtDNA 5 d after mitochondrial transplantation. (K) Changes in the percentages of wild-type (WT) and mutant mtDNA (Mut.) 3 or 5 d after mitochondrial transplantation. Ctrl: 143B control cells; ^{Δ4977}: 143B^{Δ4977} cells; R6G: 143B^{Δ4977} cells fused with FMRCs pretreated with R6G; Fusion: 143B^{Δ4977} cells fused with FMRCs. *n* = 3. Bars denoted with different letters at the top are significantly different (*P* < 0.05).

more than 100-fold increase in the number of mtDNA copies (Figure 5F,G). PMD cells transplanted with mitochondria restored mitotic activity within 3 days without uridine supplementation (Figure 5D, E). Notably, the number of cells under the fusion conditions was still lower than that under the uridine condition. This can likely be explained by several reasons: cells at the edge formed multi-nucleated aggregates, PMD cells did not fuse with FMRCs, and some of the PMD cells might not be rescued even with transplanted mitochondria in the absence of uridine. Alternatively, considering that the mitochondria in eBMSCs constituted only approximately 80% of the normal membrane potential (Supplementary Figure S6), it is possible that exogenous mitochondria might not be able to function properly immediately after transplantation.

SNP analysis of the mt-tRNA^{Arg} gene further confirmed the transplantation of exogenous mitochondria (Figure 6A). The percentage of the exogenous portion increased from approximately

20% to more than 90% (T:A ratio). Moreover, fusion cells were cloned and tested to determine for hybrids of mtDNA and nDNA via SNP analysis (Supplementary Figure S7). Indeed, 2 of the 3 clones tested had a pure KM nuclear genome, but had a combination of mtDNA from both BALB/c and KM mice. The remaining endogenous mitochondria could be the result of incomplete mitophagy, or the PMD cells generated in the study still maintained some functional mitochondria. In any event, the results demonstrated that exogenous mitochondria could stably (if not permanently) colonize PMD cells.

This result was consistent with the findings of the autophagy flux analysis (Figure 6B). PMD cells with defective mitochondria apparently could not support the energy-consuming autophagy process, whereas the transplantation of a large amounts of intact mitochondria is essential for triggering the restoration pathways, leading to the levels of autophagy back to those of normal cells.

Western blot analysis of LC3-II and Beclin1 further confirmed the increased autophagy levels in PMD cells after the transplantation of mitochondria (Figure 6C,D). The change in p62 levels was not expected, which decreased in PMD cells and increased back to normal after mitochondrial transplantation. p62 is a multifunctional stress-inducible scaffold protein involved in diverse cellular processes, suggesting that homeostatic regulation may affect p62 levels independent of autophagy. It has been reported that p62/SQSTM1 could be regulated at transcriptional, translational, or post-translational levels, such as degradation by the ubiquitin–proteasome system (UPS) system [50–52].

Moreover, the applicability of this technology for medical purposes was tested via cybrids harboring a high percentage of mutant mtDNA (143B^{Δ4977} cells), which includes a 4977-bp deletion ranging from 8470–13447 bp in human mtDNA [37,53]. This 4977-bp common deletion affects genes encoding 7 polypeptide components of the mitochondrial respiratory chain, and 5 of the 22 tRNAs necessary for mitochondrial protein synthesis. The accumulation of truncated mtDNA leads to three related mtDNA diseases: Pearson's syndrome, Kearns–Sayre syndrome and chronic progressive external ophthalmoplegia.

For technical reasons, the enucleation of Ad293 cells was achieved via mechanical extrusion. Nucleated cells cannot pass through the membrane filter; therefore, cytoplasts generated in this way cannot be contaminated by nucleated Ad293 cells. The results from this study clearly demonstrated that exogenous mitochondria from Ad293 cells were able to reside stably in 143B^{Δ4977} cells, leading to normalization of mitotic activity, ROS levels and mitochondrial functions (Figure 7 and Supplementary Figure S9). In addition, the transfer of mitochondria apparently reduced the amount of mutant mtDNA and thus decreased the ratio of heteroplasmy (Figure 7C).

The above results demonstrated that FMRCs are a versatile platform suitable for transplantation of intact mitochondrial networks. This technique could be invaluable in treating diseases associated with mitochondrial dysfunction and ageing. For example, we found that mitochondrial transplantation was effective in reversing the senescent phenotype and its associated secretory phenotype in a variety of cellular models, including the SH-SY5Y neuronal cell model for Parkinson's disease (Manuscript under review). Mitochondrial transfer further restored mitotic activity, glucose metabolism and ATP production. Recently, Dong *et al.* [54] demonstrated that transdermal mitochondrial transfer from enucleated MSCs to fibroblasts and endothelial cells in diabetic rats promoted chronic wound healing by reversing the hyperglycemia-induced cellular senescence. Furthermore, it is conceivable to transfer mitochondria to the substantia nigra via a stereotaxic intracranial delivery approach [55]. Moreover, immune cells could be collected from peripheral blood and transferred back to patients after *in vitro* mitochondrial transplantation, which is similar to the method used in CAR-T and NK cell therapy for cancer [56].

In summary, we report a breakthrough in cell fusion and mitochondrial transplantation by using the fabricated FMRCs. The transplanted mitochondria remain stable and provide energy for the clearance of damaged mitochondria, thus restoring normal metabolic processes in cells harboring mtDNA with oxidative damage, depletion or deletion. Materials other than mitochondria in FMRCs may affect many aspects of cellular activities; however, the transplantation of an intact mitochondrial network is indispensable.

Therefore, this study provides a potential cure for age-related diseases and other disorders associated with mitochondrial dysfunction.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

Data can be accessed upon reasonable request from the corresponding author(s).

Author Contributions

Liqun Xu: conceptualization, investigation, methodology, validation, writing—original draft, writing—review & editing. Xiao Li: investigation, methodology, validation, writing—original draft, writing—review & editing. Xing Fan: investigation, methodology, validation. Wei Yan: investigation, methodology, validation. Wanfei Wu: investigation, methodology, validation. Junwei Li: investigation, methodology, validation. Ronghao Deng: investigation, methodology, validation. Haibao Zhu: funding acquisition, methodology, supervision, writing—review & editing. Aihua Mao: funding acquisition, methodology, supervision, writing—review & editing. Pingnan Sun: methodology, resources, writing—review & editing. Xin Zhang: methodology, resources, writing—review & editing. Wencan Xu: methodology, resources, writing—review & editing. Chi-ju Wei: conceptualization, funding acquisition, methodology, resources, supervision, validation, writing—original draft, writing—review & editing.

Funding

This work was supported by the grants from the Natural Science Foundation of Guangdong (Nos. 2019A1515011547, 2023A1515011906, and 2023A1515012586), the Guangdong High-Level University Project “Green Technologies for Marine Industries”, and Li Ka Shing Foundation (No. 2020LKSG10C), and a Scientific Research Initiation Grant (Nos. NTF20030 and NTF20225). Informed consent was obtained from all individual participants included in the study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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