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An in vitro approach to study mitochondrial dysfunction: a cybrid model

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TITLE:

An *In Vitro* Approach to Study Mitochondrial Dysfunction: A Cybrid Model

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SUMMARY:

Transmitochondrial cybrids are hybrid cells obtained by fusing mitochondrial DNA (mtDNA)-depleted cells (rho^0 cells) with cytoplasts (enucleated cells) derived from patients affected by mitochondrial disorders. They allow the determination of the nuclear or mitochondrial origin of the disease, evaluation of biochemical activity, and confirmation of the pathogenetic role of mtDNA-related variants.

ABSTRACT:

Deficiency of the mitochondrial respiratory chain complexes that carry out oxidative phosphorylation (OXPHOS) is the biochemical marker of human mitochondrial disorders. From a genetic point of view, the OXPHOS represents a unique example because it results from the complementation of two distinct genetic systems: nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Therefore, OXPHOS defects can be due to mutations affecting nuclear and mitochondrial encoded genes.

The groundbreaking work by King and Attardi, published in 1989, showed that human cell lines depleted of mtDNA (named rho^0) could be repopulated by exogenous mitochondria to obtain the so-called “transmitochondrial cybrids.” Thanks to these cybrids containing mitochondria derived from patients with mitochondrial disorders (MDs) and nuclei from rho^0 cells, it is possible to verify whether a defect is mtDNA- or nDNA-related- related. These cybrids are also a powerful tool to validate the pathogenicity of a mutation and study its impact at a biochemical level. This paper presents a detailed protocol describing cybrid generation, selection, and characterization.

INTRODUCTION:

Mitochondrial disorders (MDs) are a group of multisystem syndromes caused by an impairment

in mitochondrial functions due to mutations in either nuclear (nDNA) or mitochondrial (mtDNA) DNA[1]. They are among the most common inherited metabolic diseases, with a prevalence of 1:5,000. mtDNA-associated diseases follow the rules of mitochondrial genetics: maternal inheritance, heteroplasmy and threshold effect, and mitotic segregation[2]. Human mtDNA is a double-stranded DNA circle of 16.6 kb, which contains a short control region with sequences needed for replication and transcription, 13 protein-coding genes (all subunits of the respiratory chain), 22 tRNA, and 2 rRNA genes[3].

In healthy individuals, there is one single mtDNA genotype (homoplasmy), whereas more than one genotype coexists (heteroplasmy) in pathological conditions. Deleterious heteroplasmic mutations must overcome a critical threshold to disrupt OXPHOS and cause diseases that can affect any organ at any age[4]. The dual genetics of OXPHOS dictates inheritance: autosomal recessive or dominant and X-linked for nDNA mutations, maternal for mtDNA mutations, plus sporadic cases both for nDNA and mtDNA.

At the beginning of the mitochondrial medicine era, a landmark experiment by King and Attardi[5] established the basis to understand the origin of a mutation responsible for an MD by creating hybrid cells containing nuclei from tumor cell lines in which mtDNA was entirely depleted (rho^0 cells) and mitochondria from patients with MDs. Next-generation sequencing (NGS) techniques were not available at that time, and it was not easy to determine whether a mutation was present in the nuclear or mitochondrial genome. The method, described in 1989, was then used by several researchers working in the field of mitochondrial medicine[6–9]; a detailed protocol has been recently published[10], but no video has been made yet. Why should such a protocol be relevant nowadays when NGS could precisely and rapidly identify where a mutation is located? The answer is that cybrid generation is still the state-of-the-art protocol to understand the pathogenic role of any novel mtDNA mutation, correlate the percentage of heteroplasmy with the severity of the disease, and perform a biochemical investigation in a homogeneous nuclear system in which the contribution of the autochthonous nuclear background of the patient is absent[11–13].

This protocol describes how to obtain cytoplasts from confluent, patient-derived fibroblasts grown in 35 mm Petri dishes. Centrifugation of the dishes in the presence of cytochalasin B allows the isolation of enucleated cytoplasts, which are then fused with rho^0 cells in the presence of polyethylene glycol (PEG). The resulting cybrids are then cultivated in selective medium until clones arise. The representative results section shows an example of molecular characterization of the resulting cybrids to prove that the mtDNA is identical to that of the donor patients' fibroblasts and that the nDNA is identical to the nuclear DNA of the tumoral rho^0 cell line.

PROTOCOL:

NOTE: The use of human fibroblasts may require ethical approval. Fibroblasts used in this study were derived from MD patients and stored in the Institutional biobank in compliance with ethical requirements. Informed consent was provided for the use of the cells. Perform all cell culture procedures under a sterile laminar flow cabinet at room temperature (RT, 22–25 °C). Use sterile-

filtered solutions suitable for cell culture and sterile equipment. Grow all cell lines in a humidified incubator at 37 °C with 5% CO_2. Mycoplasma tests should be conducted weekly to ensure mycoplasma-free cultures. 143BTK$^-$ rho^0 cells can be generated as previously described[5].

1. Culture of cells

1.1. Before starting any procedure, verify the presence of the mutation in fibroblasts derived from MD patient(s) and quantify the percentage of heteroplasmy or homoplasmy by Restriction Fragment Length Polymorphism (RFLP) and/or whole mtDNA sequencing analyses[14].

1.2. Seed fibroblasts in four 35 mm Petri dishes, each containing 2 mL of Complete Culture Medium (**Table 1**). Let the cells grow until 80% confluent (48 h).

1.3. Grow 143BTK$^-$ rho^0 cells in 8 mL of Supplemented Culture Medium (**Table 1**) in a 100 mm Petri dish.

1.4. Maintain the cells in an incubator at 37 °C with 5% CO_2.

1.5. Check the absence of mtDNA in rho^0 cells by sequencing techniques[14].

2. Enucleation of fibroblasts

2.1. Sterilize four 250 mL centrifuge-suitable bottles by autoclave sterilization at 121 °C for a 20 min cycle. Dry them in a laboratory oven or at RT.

2.2. Prewarm the centrifuge at 37 °C.

2.3. Wash the 35 mm dishes containing the fibroblasts twice, using 2 mL of 1x phosphate-buffered saline (PBS) without (w/o) calcium and magnesium.

2.4. Clean the outer surface of the dishes with 70% ethanol and wait until the alcohol evaporates.

2.5. Remove the lids from the dishes and the screw caps from the bottles. Place each dish, without the lid, upside down on the bottom of each 250 mL centrifuge bottle.

2.6. Slowly add 32 mL of Enucleation Medium to each bottle (**Table 1**), allowing the medium to enter the dish and come into contact with the cells. Remove any bubbles from the dishes using a long glass Pasteur pipette, curving the tip in a Bunsen flame.

NOTE: It is important to remove the bubbles to allow the medium to enter the dish and come in contact with the cells.

2.7. Close each bottle with the screw cap and transfer them to the centrifuge.

2.8. Centrifuge 20 min at 37 °C and 8,000 × *g*, acceleration max, deceleration slow. Pay attention to balance the centrifuge: counterweight each bottle. If necessary, adjust the weight by adding a suitable volume of Enucleation Medium.

2.9. During centrifugation, use vacuum or a 10 mL serological pipette to aspirate and discard the medium from the 143BTK$^-$ rho^0 culture plates and wash them twice using 4 mL of 1x PBS w/o calcium and magnesium.

2.10. Add 2 mL of trypsin to cover the cell monolayer completely.

2.11. Place the dishes in a 37 °C incubator for ~2 min.

2.12. Remove the dishes from the incubator, observe cell detachment using an inverted microscope for live cells (objectives 4x or 10x), and inhibit the enzyme activity by adding 2 mL of Supplemented Culture Medium.

2.13. Aspirate the 4 mL of the cell suspension in the dish with a 10 mL pipette and transfer it to a 15 mL conical tube.

2.14. Count the cells using a Burker hemocytometer chamber or an automated counter.

2.15. At the end of centrifugation, aspirate the medium from the bottles and discard it.

2.16. Remove the dishes by inverting the bottles on a sterile gauze previously sprayed with 70% ethanol. Clean the outer surface of the dishes and their lids with 70% ethanol. Wait until the alcohol evaporates, and then close the dishes.

2.17. Before proceeding, check for cytoplast (ghost) formation using an inverted microscope for live cells (objective 4x or 10x). Look for extremely elongated fibroblasts due to the extrusion of their nuclei induced by cytochalasin B.

2.18. To each 35 mm dish, add 1 × 10^6 of 143BTK$^-$ rho^0 cells resuspended in 2 mL of 143BTK$^-$ rho^0 culture medium supplemented with 5% fetal bovine serum (FBS).

2.19. Leave the dishes for 3 h in a humidified incubator at 37 °C and 5% CO_2 and let the 143BTK$^-$ rho^0 cells settle on the ghosts. Do not disturb the dishes.

3. Fusion of the enucleated fibroblasts with rho^0 cells

3.1. After 3 h of incubation, aspirate and discard the medium from the dishes.

3.2. Wash the adherent cells twice with 2 mL of Dulbecco's Modified Eagle Medium (DMEM) high glucose w/o serum or with Minimum Essential Medium (MEM).

3.3. Aspirate and discard the medium.

3.4. Add 500 µL of PEG solution (see the **Table of Materials**) to the cells and incubate for exactly 1 min.

3.5. Aspirate and discard the PEG solution.

3.6. Wash the cells three times using 2 mL of DMEM high glucose w/o serum or with MEM.

3.7. Add 2 mL of Fusion Medium (**Table 1**) and incubate overnight in the incubator at 37 °C with 5% CO_2.

4. Cybrid selection and expansion

4.1. After overnight incubation, remove the plates from the incubator, trypsinize the cells as described above (steps 2.9–2.13), and transfer the content of each 35 mm dish into a 100 mm dish.

4.2. Add 8 mL of Selection Medium (**Table 1**) and place the plates in the incubator at 37 °C with 5% CO_2.

4.3. Change the medium every 2–3 days.

4.4. Wait for ~10–15 days of selection until colonies of cells appear.

4.5. Freeze one of the four Petri dishes by collecting all the clones and generating a "massive" culture as a backup of the cybrids, which can be eventually recloned and used for further investigations.

4.6. Trypsinize the cells in the remaining culture dishes, count, and seed into one or more Petri dishes at 50–100 cells/dish in the Supplemented Culture Medium (**Table 1**) until clones appear. Let them grow for some days.

4.7. Pellet the remaining cells by centrifugation at 1,200 × *g* for 3 min at RT and discard the supernatant.

4.8. Extract DNA from the pellet (see the **Table of Materials**).

4.9. Perform genotyping by variable number of tandemly repeated (VNTR) analysis as previously reported[15].

4.10. Pick up clones from the Petri dish with cloning cylinders or a pipette tip, using a stereomicroscope to avoid pooling of different clones, and transfer them to a 96-well plate, each

well containing 200 µL of Supplemented Culture Medium (**Table 1**).

4.11. Expand every clone until there are enough cells for freezing and extracting DNA.

4.12. Verify the mutation percentage of each clone by RFLP or other sequencing methods. Ideally, try to obtain both clones with wild-type mtDNA (0% mutation) and clones with different mutation percentages, both adding up to homoplasmic mutant mtDNA (100% mutation). See **Figure 1** for a schematic diagram of the cybrid generation protocol.

REPRESENTATIVE RESULTS:

Generating cybrids requires 3 days of laboratory work plus a selection period (~2 weeks) and additional 1–2 weeks for the growth of clones. The critical steps are the quality of cytoplasts and the selection period. The morphology of cybrids resembles that of the rho^0 donor cells. Assignment of the correct mtDNA and nDNA in the cybrids is mandatory to confirm the identity of the cells. An example is given in **Figure 2**. In this case, we generated cybrids starting from fibroblasts derived from a patient carrying the heteroplasmic m.3243A>G, one of the most common mtDNA mutations associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Analysis of VNTR showed that cybrid nDNA is identical to that of the rho^0 cells (**Figure 2A**), confirming the replacement of the patient's nDNA with the 143B genome. RFLP and/or sequencing analyses can be used to assess the presence of the mtDNA mutation and the heteroplasmy percentage (**Figure 2B,C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the cybrid generation protocol. Patient-derived fibroblasts are treated with cytochalasin B and centrifuged to obtain cytoplasts (enucleated cells). Cytoplasmic fusion of cytoplasts and mtDNA-depleted cells ($143BTk^-$ rho^0) allows the generation of cybrids that can be isolated after selection in the appropriate medium. Picking up and amplification of single clones yields different heteroplasmy percentages, which in theory can vary from 100% wild-type to 100% mutated. Abbreviation: mtDNA = mitochondrial DNA.

Figure 2: Molecular characterization of cybrids. (**A**) Analysis of Apo-B microsatellites shows that the nDNA extracted from the generated cybrids is identical to the nuclear DNA of the rho^0 cell line. (**B**) *HaeIII* restriction maps of the PCR product spanning the MELAS-associated m.3243A>G, used to quantify the amount of WT) and Mut mtDNA species. (**C**) Representative results of RFLP analysis showing m.3243A>G heteroplasmy levels in different cybrid clones (c1, c2, c3). Abbreviations: M = marker; bp = base pairs; WT = wild-type; Mut = mutant; RFLP = restriction fragment length polymorphism; MELAS = mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.

Table 1: Details of media used for cybrid generation.

DISCUSSION:

The mtDNA has a very high mutation rate compared to nDNA because of the lack of protective

histones and its location close to the respiratory chain, which exposes the molecule to damaging oxidative effects not efficiently counteracted by the repair systems[16]. The first pathogenic mtDNA mutations were identified in 1988[17, 18], and since then, a large number of mutations have been described. NGS technology is a relevant approach to screen the entire mitochondrial genome and easily identify variants[14]. However, assessing the pathogenic role of a never-described mtDNA mutation can be challenging and still relies on "old-fashioned" methods such as the generation of cybrid cells described in this protocol[11–13]. The cybrid generation described here recapitulates the original methods described by King and Attardi[5]. However, other protocols contain minor modifications mainly related to systems for cell enucleation[10]. In other instances, enucleation is unnecessary, for example, when the patient-derived biological material consists of platelets, which do not contain a nucleus[19].

Transmitochondrial cybrids possess several important features, making them a relevant research tool even when high-throughput molecular technologies can profile DNA and RNA at the single-cell level. In pioneering works, cybrids were used to establish or confirm the genetic origin of disorders clinically and biochemically defined as mitochondrial disorders[6–9,20]. Indeed, the system exclusively allows the study of the contribution of the mtDNA mutation without the influence of the nuclear genes of the proband and in a homogeneous nuclear background of the rho^0 cells.

In addition, a quantitative genotype-to-phenotype correlation can be performed, thanks to the isolation of different cybrid clones carrying different percentages of the mutations. The different clones were selected using Selection Medium (**Table 1**) supplemented with dialyzed FBS and not containing uridine and pyruvate, allowing the growth of rho^0 cells fused with cytoplasts only. Thus, rho^0 cells that had not fused or had fused with intact fibroblasts (bi- or polynucleated cells), as well as any residual non-enucleated intact fibroblasts, are eliminated. Indeed, rho^0 cells are completely depleted of mtDNA by long-term exposure to low concentrations of ethidium bromide, a potent inhibitor of the mitochondrial gamma-polymerase[21].

Lacking a functional respiratory chain, rho^0 cells rely exclusively on glycolysis for their energy requirements and become pyruvate-dependent. Additionally, rho^0 cells have become auxotrophic for pyrimidines (uridine is a pyrimidine precursor) because of the deficiency of the dihydroorotate dehydrogenase, an enzyme functioning within mitochondria and involved in the pyrimidine biosynthesis. While rho^0 cells are derived from human osteosarcoma 143B thymidine kinase-deficient (TK^-) cells, fibroblasts, cytoplasts, and polynucleated hybrids are TK^+. Therefore, TK^+ cells are removed due to exposure to 5-bromo-2'-deoxyuridine. In fact, this uridine analog is recognized by TK catalyzing the phosphorylation of deoxythymidine, which is then used in DNA biosynthesis. This process results in lethal mutations causing cell death. Another advantage of cybrids is the possibility to freeze and/or reuse them for long culture times without alterations. Moreover, like tumor cells, their high growth rate reduces the experimental study duration.

The molecular prerequisite to make this system useful and reliable is to verify the correct genetic contribution of the nuclear and mitochondrial DNA and the mtDNA amount in the repopulated cybrids. Nowadays, this can be easily performed by using NGS techniques allowing the analysis of the entire mtDNA molecule to define haplogroups and continuously verifying the presence of

any other unwanted pathogenic variants in addition to the variant under investigation. In the case of heteroplasmic conditions, the isolation of clones with different mutation loads, ranging ideally from 0% to 100%, can be used to correlate the mutation percentage with the severity of the mitochondrial impairment.

Possible pitfalls hidden in these cybrid generation procedures are linked to the tumor origin of the rho^0 cells used as nuclear donors. These cells are aneuploid, and it is not clear how this could eventually affect mitochondrial functions and the translation and assembly of the different respiratory chain subunits encoded by the nuclear and mitochondrial DNA, respectively. Generally, it would be advisable to generate cybrids using rho^0 cells of different origins and verify that the clones obtained display comparable phenotypes. Still another limitation is that tumor cells are mainly glycolytic while mitochondrial disorders rely massively on OXPHOS. Therefore, the impact of a glycolytic nucleus (rho^0 cells) on the consequences of an mtDNA mutation must be carefully established.

Despite the above limitations, the generation of cybrids has revolutionized the mitochondrial medicine field and is still used to establish the pathogenic role of novel mtDNA mutations. Moreover, cybrid technology is used to investigate the mitochondrial contribution to different diseases, ranging from common neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's disease[22–26], to cancer and anticancer treatment[27,28].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

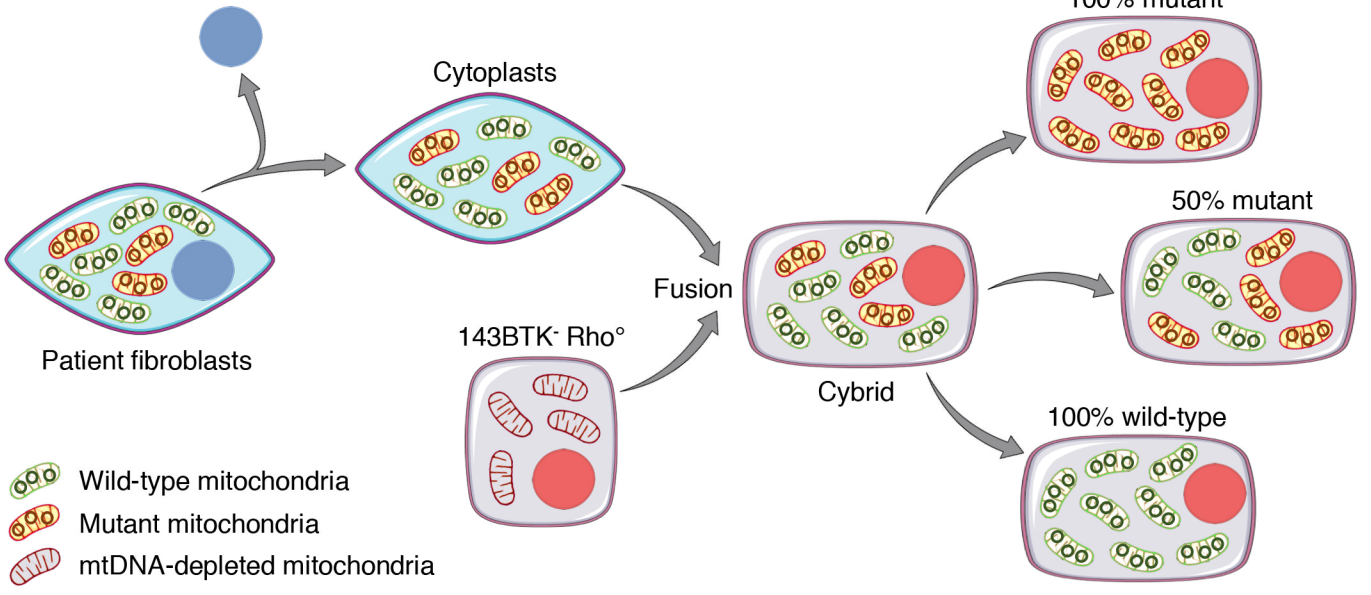
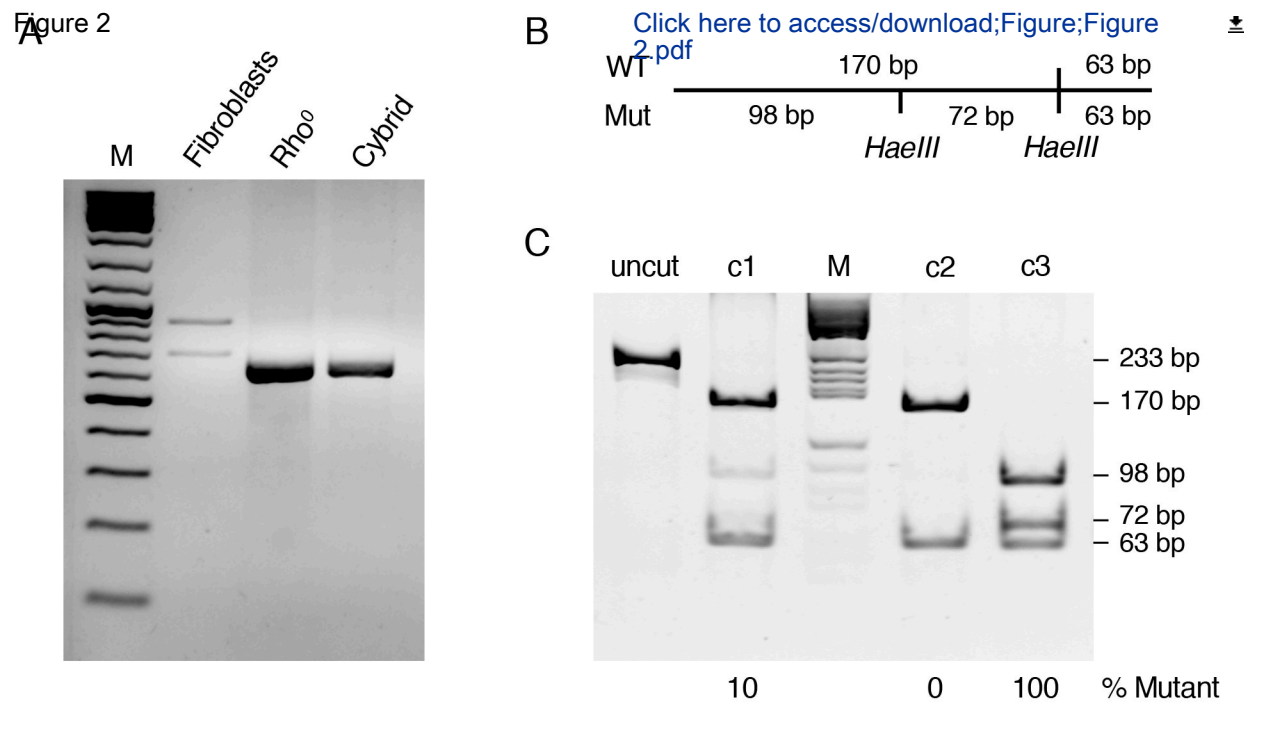


Figure 2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c2
c3
233 bp
170 bp
98 bp
72 bp
63 bp
10
0
100
% Mutant

Media Composition	
Complete culture medium	**Final conc.**
DMEM High Glucose (w/o L-Glutamine W/Sodium Pyruvate)	[1:1]
FetalClone III (Bovine Serum Product)	10%
Sodium Pyruvate 100 mM	1 mM
Penicillin-Streptomycin (solution 100x)	2%
L-Glutamine 200 mM (100x)	4 mM
Supplemented culture medium	**Final conc.**
DMEM High Glucose (w/o L-Glutamine W/Sodium Pyruvate)	[1:1]
FetalClone III (Bovine Serum Product)	10%
Sodium Pyruvate 100 mM	1 mM
Penicillin-Streptomycin (solution 100x)	2%
L-Glutamine 200 mM (100x)	4 mM
Uridine	50 µg/mL
Enucleation medium	**Final conc.**
DMEM High Glucose (w/o L-Glutamine W/Sodium Pyruvate)	[1:1]
FetalClone III (Bovine Serum Product)	5%
Sodium Pyruvate 100 mM	1 mM
Penicillin-Streptomycin (solution 100x)	1%
L-Glutamine 200 mM (100x)	2 mM
Cytochalasin B from Drechslera dematioidea	10 µg/mL
Fusion medium	**Final conc.**
DMEM High Glucose (w/o L-Glutamine W/Sodium Pyruvate)	[1:1]
FetalClone III (Bovine Serum Product)	5%
Sodium Pyruvate 100 mM	1 mM
Penicillin-Streptomycin (solution 100x)	1%
L-Glutamine 200 mM (100x)	2 mM
Selection medium	**Final conc.**
DMEM High Glucose (w/o L-Glutamine W/Sodium Pyruvate)	[1:1]
Dialyzed FBS	5%
Sodium Pyruvate 100 mM	1 mM
Penicillin-Streptomycin (solution 100x)	1%
L-Glutamine 200 mM (100x)	2 mM
5-Bromo-2'-Deoxyuridine	100 µg/mL

Milan, 9th December 2021

Dear Editor,

we would like to thank you and the Reviewers for the helpful comments and suggestions to improve our manuscript entitled "**In vitro approach to study mitochondrial dysfunctions: a cybrid model".**

Please find below a point-by-point response to the questions that have arisen during the revision process, starting from the Editorial comments.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. The language of the manuscript is difficult to understand. Please use professional copyediting services for improving the manuscript.

Authors reply the manuscript has been revised by a professional English Translator to correct any spelling or grammar issues, and to clarify any unclear sentences.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

Authors reply: we corrected the tense in the protocol section and used imperative throughout this section

4. Please define all abbreviations upon first use. For example, DMEM, FBS, etc.

Authors reply: all the abbreviations have been defined

5. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Please move the discussion about the protocol to the Discussion.

Authors reply: we have reduced whenever possible the notes

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Authors reply: we have included an ethical statement at the beginning of the protocol

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Please provide volume of all the media added. For example, selective medium, growth medium, etc.

Step 1.4: How was the mtDNA absence confirmed? Please provide all the associated steps.

Step 2.7, 4.1: How was trypsinization done? How were the cells counted? Please provide all the associated steps.
Step 2.10: How were the cells examined? What are the microscope settings and image parameters used? Please provide all the associated steps.
Step 4.5: How were the unused cells collected and pelleted? How was DNA extracted?
Step 4.7: How were the cells expanded?
Authors reply: for the above points we have detailed as much as possible the different steps also providing references to published material

8. Please include a single line space between each step, substep, and note in the protocol section. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Authors reply: we have highlighted in yellow the essential steps of the protocol for the video.

9. As we are a methods journal, please include in the Discussion the following in detail along with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
Authors reply: we have implemented the Discussion following the above indication. All the modifications are present as track-changes in the manuscript.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This is an interesting topic that covers a technology developed over 30 years ago. In 2021, the application of cybrid technology is important in the development of mitochondrial models and new therapeutic interventions. The modeling effort, while covered in quite a few review and methods papers to date, is important toward our understanding of mitochondrial and cellular biology and in the study of perturbations in mitochondrial function.

Major Concerns:
A good body of literature in the use of cybrids and rho-zero cells is omitted -- giving the work the appearance of something entirely new. It would be beneficial to compare and contrast published methodology and methods papers dating back to the 1990 and 2000s. In particular, the work of Trounce and Wallace should be included among others (the current Wallace reference is insufficient in characterizing the outstanding literature).
Authors reply: we would like to thank this reviewer for the suggestions. We have now cited additional literature concerning cybrids and rho zero cells. Searching PubMed by using the "cybrids" term, generated more than 950 papers a huge number which is above the limit for the bibliography and also behind the scope of this methodological manuscript. Nevertheless, the most relevant papers are now present in the cited literature highlighted in green.

King and Attardi's work is seminal but also more historical than "several years ago."
Answer: we have rephrased this sentence in : *The groundbreaking work by Michael King and Giuseppe Attardi, published in 1989, showed that human cell lines depleted of mtDNA (named rho0) could be repopulated by exogenous mitochondria to obtain the so called "trans-*

mitochondrial cybrids". Thanks to these cybrids, where mitochondria derive from MD patients and the nucleus from rho0 cells, it is possible to verify whether a defect is mtDNA or nDNA related.

Additionally, I would recommend extensive text editing as conventions, grammar and syntax need review for 1) telling a more comprehensive story, 2) highlighting specifics new to this group's work, and 3) clarity and greater purpose/utility throughout. For example, the current title "In vitro approaches to study mitochondrial dysfunctions: the cybrids model" should be revised to "In vitro approach to study mitochondrial dysfunction: a cybrid model."
Authors reply: a professional English Translator revised the manuscript to correct any spelling or grammar issues. See also response to Editorial Comments.
We have implemented the discussion to present a more comprehensive view of the cybrids technology and their utility during time of great technological improvement. We have also cited recent literature, in which cybrids are still used as a system to demonstrate pathological role of mtDNA variants.
We have also modified the title according to reviewer's indication.

Reviewer #2:
Manuscript Summary:
The authors present a detailed protocol describing cybrids' generation, selection, and characterization.

Minor Concerns:
The authors should point out that the use of different clones is advisable to normalize the effect of the nuclear background.
The authors should also point out that is important to identify the mtDNA haplogroup both in their controls as in their mutant cybrids
mtDNA complete sequence in controls and mutants could be desirable to discard the effect of any other additional potential pathogenic genetic variants present in the mtDNA
Authors reply: we would like to thank this reviewer for these helpful suggestions. We have now discussed the importance to analyse the whole mtDNA molecule in controls and mutant cybrids either to define the haplogroup and to identify possible unwanted variants.

Reviewer #3:
Manuscript Summary:
The authors describe the protocol for generating and establishing the cybrids in good enough detail however some small adjustments should be made to the protocol and to the table of used materials as it needs to contain more details on the media, PBS, PEG, etc that was used and catalog numbers.

Major Concerns:
N/A

Minor Concerns:
Line 100. Mycoplasma testing should be performed in an antibiotics-free growth medium.
Line 102 it should state humidified incubator
line 103 section 1.4 should be incorporated in section 1.1
Section 2 Please indicate which steps should be performed in a laminar flow cabinet.
Line 108 Please specify what kind of PBS, the same comment could be made for line 133 regarding the PEG.
Line 109 Shouldn't it state 70% etOH as 100% only fixes the cell wall instead of destroying it in respect to contamination, similar to line 121.

Line 126 Omit instead of 10% FBS
Line 131 Containing how much serum? give the complete formulation between brackets.
Line 133 What kind of PEG please specify.
Line 131 and 134 Cells also can be washed with MEM.
Line 135 DMEM also can contain 10% serum. So please specify why only 5% was used. If only 5% can be used omit instead of 10%.
Line 144 The selective medium, supplemented... it should state is supplemented with...
Authors reply: we have now amended all the above points as requested by the reviewer. They are visible as track changes in the revised manuscript. In order to make reading easier, we included a Table 1 explaining media composition.

Line 171 Also polyclonal cultures can be generated as long as the mutation and its percentage are regularly checked. Which always should be done also for the monoclonal. The mutation percentages should be established from the selected clones only which will be used for further passaging and experiments.
Authors reply: we checked percentages of the mutation in the massive cybrids cell culture before proceeding to generated clones. After isolation, each clone is verified for the percentage of the mutation. So a double check is performed.

Line 238. Also one of the major pitfalls in tumors generated from cybrids is that they lose their mutation percentage upon establishment of the tumor in vivo. See e.g.10.3389/fonc.2020.00770
Authors reply: we have never used cybrids to generate tumors, but only to establish the pathogenic role of an mtDNA variants. We can't comment on this.

Reviewer #4:
Manuscript Summary:

Major Concerns:
JoVE63452
In vitro approach to study mitochondrial dysfunctions: the cybrids model
For my taste, the video is much too long

The technique is known for years.
What is the purpose of the manuscript?
11/21

Authors reply: This is a methodological manuscript aiming at visualised a protocol for which a video has been not realised yet. It is important to highlight that despite 30 years of research, the cybrids technology is still used in the field of mitochondrial research as witnessed by papers recently published in biomedical journals, which are now cited in our manuscript.

We do hope that the revised version of the manuscript will be considered as a suitable contribution and accepted for publication in JoVE.

Sincerely,

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