Published in final edited form as:

Methods Mol Biol. 2015 ; 1254: 255–267. doi:10.1007/978-1-4939-2152-2_19.

Stem Cells, Neural Progenitors, and Engineered Stem Cells

Raj R. Rao and Shilpa Iyer

Abstract

Human pluripotent stem cells (hPSCs) have the unique potential to form every cell type in the body. This potential provides opportunities for generating human progenitors and other differentiated cell types for understanding human development and for use in cell type-specific therapies. Equally important is the ability to engineer stem cells and their derived progenitors to mimic specific disease models. This chapter will focus on the propagation and characterization of human neural progenitors (hNPs) derived from hPSCs with a particular focus on engineering hNPs to generate in vitro disease models for human neuro-mitochondrial disorders. We will discuss the methodologies for culturing and characterizing hPSCs and hNPs; and protocols for engineering hNPs by using a novel mitochondrial transfection technology.

Keywords

Human pluripotent stem cells; Neural progenitors; Mitochondria; Protofection; Genetic engineering

1 Introduction

Human pluripotent stem cells (hPSCs) that include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are expected to serve as useful models for studying human embryonic development in addition to their proposed potential for regenerative biomedical therapies [1–3]. Under specific culture conditions, hPSCs can be uniformly differentiated into multipotent human neural progenitors (hNPs) [4, 5]. The hNPs can also be differentiated into neurons and glial cells of the neural lineage [6–9]. These studies represent a first step toward generating a homogeneous, self-renewable and easy to propagate hNP cell population, committed to differentiation toward major cell types of the neural lineage. These cells not only provide healthy cell types for biomedical therapies; but serve as cell models for understanding the mechanisms of diseases progression in early human development.

We have developed methods for directed differentiation of hPSCs to generate uniform populations of hNPs with the potential for developing novel in vitro models to understand the mitochondrial genetics in many devastating childhood mitochondrial disorders. Recent work demonstrated the potential for use of a mitochondrial transduction technology to successfully introduce and express exogenous pathogenic mitochondrial DNA (obtained from a patient afflicted with Leber's hereditary mitochondrial neuropathy); into hNPs devoid of their own mitochondrial DNA. This technology termed “protofection” requires a recombinant engineered protein referred to as TFAM or mitochondrial transcription factor A for introduction of full-length human mtDNA into mitochondria [10, 11]. The “protofection”

protein TFAM has been engineered to contain an N-terminal protein transduction domain (PTD)—stretch of 11 arginines—a strong mitochondrial localization sequence (MLS) derived from mitochondrial superoxide dismutase 2 (*SOD2*) gene sequence, yielding the final recombinant protein rhTFAM [11–13]. Our encouraging findings demonstrated that the human mitochondria could be manipulated from outside the cell, and provide the basis for engineering the human neural progenitors to understand the role and effects of pathogenic mtDNA during human development and for providing cell type-specific drug screening platforms in specific childhood and adult neurodegenerative disorders affecting the mitochondria (Fig. 1).

2 Materials

2.1 Human Pluripotent Stem Cell Passaging and Subculture

1. NIH approved WA09 human embryonic stem cell line (<http://www.stemcells.nih.gov/research/registry>).
2. Human pluripotent stem cell (hPSC) medium (all chemicals from Life Technologies™, Grand Island, NY, unless otherwise labeled): Dulbecco's Modified Eagle's Medium (DMEM/F12) supplemented with 15 % fetal bovine serum (FBS, HyClone, Ogden, UT), 5 % knockout serum replacer (KSR), 1× non-essential amino acids (NEAA), 20 mM l-glutamine, 0.5 U/mL penicillin, 0.5 U/mL streptomycin, 0.1 mM β-mercaptoethanol (Sigma Chemicals, St. Louis, MO), 4 ng/mL fibroblast growth factor-2 (FGF-2) (Sigma Chemicals). Store at 4 °C and use within 1 week. Protect from light and wide swings in temperature.
3. Mouse embryonic fibroblast (MEF) medium (all chemicals from Life Technologies™, unless otherwise labeled): DMEM-High Glucose (DMEM-HiGlu) supplemented with 10 % FBS, (HyClone), 2 mM l-glutamine, 0.5 U/mL penicillin, 0.5 U/mL streptomycin. Store at 4 °C and use within 2 weeks.
4. 1 mg/mL collagenase: weigh out 10 mg collagenase type IV (Life Technologies™) and dissolve in 10 mL of DMEM/F12 medium supplemented with 15 % FBS, 5 % KSR at 37 °C. Filter sterilize and store at 4 °C and use within 1 week.
5. 0.05 % trypsin solution (Life Technologies™).
6. 1 mM ethylenediamine tetraacetic acid (EDTA) (Life Technologies™).
7. Trypan blue (Sigma Chemicals).

2.2 Derivation of Human Neural Progenitors from Human Pluripotent Stem Cells

1. 0.25 % trypsin (Life Technologies™).
2. 1 mM ethylenediamine tetraacetic acid (EDTA) (Life Technologies™).
3. Laminin-coated dishes: dissolve 5 µg/mL laminin (Sigma Chemicals) in cold, sterile deionized water. Add corresponding volume (96 well plate: 100 µL/well; 35 mm dish: 2 mL/dish; 6-well plate: 2 mL/well) of laminin solution to the plate

size being used. Swirl to ensure the entire surface of the plate or well is covered with the laminin solution. Allow dishes to incubate at room temperature for at least 30 min, but no longer than 1 h before use. Store coated dishes at 4 °C and use within 1 month. *See Note 1.*

4. Medium 1 (all chemicals from Life Technologies™): DMEM/F12 supplemented with 20 % KSR, 1 % NEAAs, 1 mM l-gluta-mine, 0.5 U/mL penicillin, 0.5 U/mL streptomycin. Store at 4 °C and use within 1 month. Protect from light and wide swings in temperature.
5. Medium 2 (all chemicals from Life Technologies™): DMEM/F12 with 1× N2 supplement, 4 ng/mL FGF-2, 2 mM l-glutamine, 0.5 U/mL penicillin, 0.5 U/mL streptomycin. Store at 4 °C and use within 1 month. Protect from light and wide swings in temperature.
6. Medium 3 (all chemicals from Life Technologies™, unless otherwise labeled): Neurobasal medium supplemented with 1× Pen-Strep and 1× B27, 2 mM l-glutamine, 20 ng/mL FGF-2 (Sigma Chemicals), 10 ng/mL LIF (Millipore, Billerica, MA). Store at 4 °C and use within 1 month. Protect from light and wide swings in temperature.

2.3 Passage and Subculture of Human Neural Progenitors

1. hPSC-derived hNPs. *See Note 2.*
2. hNP propagation medium (all chemicals from Life Technologies™, unless otherwise labeled): Neurobasal medium supplemented with 1× Pen-Strep and 1× B27, 2 mM l-glutamine, 20 ng/mL FGF-2 (Sigma Chemicals), 10 ng/mL LIF (Millipore). Store at 4 °C and use within 1 month. Protect from light and wide swings in temperature.
3. Poly-ornithine/Laminin (POLA)-coated dishes: Dissolve 20 µg/mL POLA (Sigma Chemicals) 5 µg/mL laminin (Sigma Chemicals) in cold, sterile deionized water. Use in sequential steps for coating dishes. Store coated dishes at 4 °C and use within 1 month. *See Note 3.*

2.4 Dideoxycytidine (ddC) Treatment of Human Neural Progenitors

1. 2',3' - Dideoxycytidine (ddC) (Sigma Chemicals). Store at -20 °C.
2. hNP medium during ddC treatment (all chemicals from Life Technologies™, unless otherwise labeled): Neurobasal medium supplemented with 1× Pen-Strep and 1× B27, 2 mM l-glutamine, 20 ng/mL FGF-2 (Sigma Chemicals), 0.1 mg/mL pyruvate (Sigma Chemicals), 0.05 mg/mL uridine (Sigma Chemicals), 10 ng/mL LIF (Millipore). Store at 4 °C and use within 1 month. Protect from light and wide swings in temperature. *See Note 4.*

2.5 Isolation and Introduction of Pathogenic mtDNA into ddC-Human Neural Progenitors

1. Qproteome Mitochondria Isolation Kit (Qiagen, Valencia, CA). *See Note 5.*

2. Mitochondrial DNA Extraction Kit (PromoKine, Heidelberg, Germany). *See Note 6.*
3. Plasmid-Safe ATP-dependent DNase (Epicentre Biotechnologies, Madison, WI). *See Note 7.*
4. UltraClean GelSpin DNA purification kit (MO BIO Laboratories, Carlsbad, CA). *See Note 8.*
5. DNA Quant-iT assay kit (Life Technologies™). *See Note 9.*
6. E-Gel® Precast Agarose Gels (Life Technologies™). Store at room temperature and protect from light.
7. Recombinant human TFAM (Gencia Corporation, Charlottesville, VA). *See Note 10.*
8. Roche Expand long template buffer 3 containing high Mg^{2+} (Roche Applied Sciences, Indianapolis, IN). Store at $-20^{\circ}C$.

2.6 Labeling Pathogenic mtDNA and rhTFAM

1. Rabbit anti-nestin antibody (Millipore).
2. Mitotracker Red or Mitotracker Green (Mitosciences, Eugene, OR, USA). *See Note 11.*
3. Appropriate AlexaFluor-488 secondary antibodies (Molecular Probes™, Invitrogen™, Carlsbad, CA).
4. Cy3 dye (Mirus, Madison, WI).

2.7 Immunocytochemistry

1. Chamber slides (ThermoFisher Scientific, Rochester, NY).
2. 10× Phosphate-buffered saline (PBS) $^{++}$ with Ca^{2+} Mg^{+} (Millipore).
3. 4 % (w/v) paraformaldehyde (PFA, e.g. Sigma Chemicals): Prepare fresh for each experiment. Work in a fume hood and wear gloves, as paraformaldehyde is toxic. Weigh out 4 g PFA and add to glass beaker. Add 75 mL distilled water and place on heated stirrer to dissolve. The solution needs to be carefully heated (use a stirring hot-plate at a temperature $\sim 56^{\circ}C$) to dissolve the PFA. Add 2 drops of 1 M sodium hydroxide and once all has gone into solution, add 10 mL of 10× PBS $^{++}$. Make sure that pH is between 7.2 and 7.4. Make up volume to 100 mL with distilled water. Store at $4^{\circ}C$ and use within 1 week.
4. Blocking solution: 3 % (w/v) goat serum (Hyclone Labs) in PBS $^{++}$. Store at $4^{\circ}C$ and use within 48 h.
5. High salt buffer (HSB): 50 mL 1 M Tris, pH 7.4, 950 mL deionized water, 0.25 M sodium chloride (14.61 g). Store at room temperature and use within 2 months.

6. Permeabilization buffer : add 25 μ L of Tween 20 (EMD Chemicals, Darmstadt, Germany) to 50 mL HSB.
7. DAPI.
8. Fluorescence-free mounting medium.
9. Nail varnish.

2.8 Restriction Enzyme Digestion

1. AllPrep DNA / RNA Mini Kit (Qiagen, Valencia, CA). *See Note 12.*
2. Quant-IT DNA and RNA assays (Life Technologies™). *See Note 13.*
3. iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA). *See Note 14.*
4. SfaN1 restriction enzyme (New England BioLabs, Ipswich, MA). Store at -20°C .
5. PCR primers for ND4 region of the mitochondrial genome. To detect the LHON mutation at G11778A: where G gets replaced by A:
 - a. Forward Primer: 11632-11651 (CAGCCACATAGCCCTCGTAG).
 - b. Reverse Primer: 11843-11862 (GCGAGGTTAGCGAGGCTTGC).

2.9 Differentiation of Engineered Human Neural Progenitors into Neurons

1. hNP differentiation medium (all chemicals from Life Technologies™): Neurobasal medium supplemented with $1\times$ Pen-Strep and $1\times$ B27, 2 mM l-glutamine. Store at 4°C and use within 1 month. Protect from light and wide swings in temperature.
2. Anti- β -tubulin III antibody (e.g. R&D Systems, Minneapolis, MN).

2.10 Equipment

1. Dissection microscope.
2. Cell culture incubator.
3. Centrifuge.
4. Hemocytometer.
5. Water bath.
6. Light microscope with phase contrast optics.
7. Aspirator for fluids.
8. DNA gel electrophoresis apparatus (an automated apparatus is preferable, e.g. Experion, BioRad).
9. Fluorescence microscope with confocal capabilities (e.g. Olympus IX70, Olympus, Center Valley, PA).

10. PCR thermocycler.

3 Methods

3.1 Human Pluripotent Stem Cell Passaging and Subculture

1. Use at least 3-day-old MEF plates (1.2×10^5 cells/cm²), aspirate off medium and replace with 2 mL of hPSC medium. Place dish at 37 °C until ready to plate out cells.
2. Add 1 mL of collagenase solution per 35 mm dish containing hESC colonies. Place on 37 °C stage for 2–3 min. Colonies can be observed rounding under dissection microscope.
3. Aspirate off collagenase solution and add 1 mL 0.05 % trypsin solution.
4. Allow trypsin to contact cells for no more than 40 s, then aspirate off the trypsin solution.
5. Add 1 mL of 10 % FBS in DMEM/F12 to 35 mm dish and begin to gently pipette up and down to dislodge or knock off and break up cell clumps, while continuously observing cells under microscope.
6. Place harvested cells in 15 mL tube containing 8 mL hPSC medium.
7. Add fresh medium to the dish and wash off and collect any remaining trypsinized cells, add to 15 mL tube.
8. Spin harvested cells for 4 min at $200 \times g$ at room temperature.
9. Resuspend trypsinized pellet in 2 mL of hPSC medium per 35 mm dish used.
10. Count cells using hemocytometer by taking 10 μ L cell suspension and mixing with 10 μ L Trypan blue.
11. Aspirate medium from pre-equilibrated dishes and plate cells at 150,000 cells per 35 mm dish in 2 mL medium. Place at 37 °C in a 5 % CO₂ incubator. Evenly distribute cells on the plate via a uni-directional quick movement of the plate and place it back on the shelf in the incubator (do not swirl cells in the plate).
12. Feed everyday with a 50 % medium change until ready to passage again in 3–4 days. The cells at this stage should exhibit distinct colony morphology (high nuclear to cytoplasmic ratio) characteristic of hPSCs.
13. hPSCs can be maintained in suspension prior to use in immunocytochemical and/or flow cytometry analysis.
14. For immunocytochemical analysis, hPSCs propagated under routine conditions are passaged and transferred into MEF-seeded chamber slides. Medium is changed every day prior to fixing for immunocytochemical analysis (*see* Subheading 3.5).

3.2 Derivation of Human Neural Progenitors from Human Pluripotent Stem Cells

1. Trypsinize hPSCs using 0.25 % trypsin in EDTA solution for 1 min.
2. Count cells using hemocytometer and transfer 3×10^5 hPSCs in fresh hPSC medium to a 35 mm laminin-coated plate.
3. After 24 h, replace hPSC medium with Medium 1, with medium change every day for a period of 7 days.
4. After 7 days in Medium 1, replace with Medium 2, with medium change every day for a period of 7 days.
5. After 7 days in Medium 2, replace with Medium 3, with medium change every day for a period of 7 days.
6. After 3 weeks of differentiation, hNPs can be sub-cultured in hNP propagation medium as detailed below.

3.3 Passage and Subculture of Human Neural Progenitors

1. Thaw hNPs by rotating the cryovial in a 37 °C water bath.
2. Swirling slowly, add 10 mL of pre-warmed hNP propagation medium to the cell suspension in a sterile 15 mL tube.
3. Spin cells for 4 min at $200 \times g$ at room temperature.
4. Aspirate supernatant and resuspend cells in 2 mL of hNP propagation medium.
5. Plate cells at 1×10^6 cells per 35 mm POLA-coated dish. Place at 37 °C in a 5 % CO₂ incubator. Replace medium everyday.
6. Once the hNPs reach 90 % confluence, carefully remove the media.
7. Add pre-warmed hNP propagation medium to the cells. Triturate the cells with a pipette to manually detach them from the dish. Alternatively, the cells can be detached using a cell scraper.
8. Observe the dishes under a bright field or a phase contrast microscope to ensure that all cells have been removed. If necessary, the cells can be centrifuged at $200 \times g$ for 4 min in order to resuspend the pellet at a specific concentration.
9. It is recommended to subculture the cells by passaging 1:2 or 1:3 to maintain a high density.
10. hNP cells can be maintained in suspension, prior to use in immunocytochemical analysis.
11. For immunocytochemical analysis, propagate hNPs in chamber slides coated with POLA to provide a similar culture condition as on regular dishes.

3.4 Dideoxycytidine (ddC) Treatment of Human Neural Progenitors

1. Treat hNPs with 10 μ M 2',3' ddC solution for reduction of endogenous mtDNA, for a period of 6 days. *See Note 15.*

2. Use immunocytochemical analyses to determine expression of hNP markers in ddC-treated hNPs (Subheading 3.5).

3.5 Immunocytochemistry

3.5.1 Fixation

1. Work in a fume hood. Wash once cells obtained after completing Subheadings 3.1 or 3.2, in PBS⁺⁺. Use aspirator to remove and a transfer pipette to add PBS.
2. Add enough PFA solution (~ 0.8 mL/ 1.8 cm²) to cover bottom of well of chamber slides or dish.
3. Let sit at room temperature for 15–20 min.
4. Wash cells 3 times in 1 mL of PBS⁺⁺.
5. Store fixed cells at 4 °C until ready to stain with chosen markers.

3.5.2 Localization of Specific Markers in Human Neural Progenitors by Immunocytochemical Analysis

1. Wash once fixed cells in wells of chamber slides (from Subheading 3.3) with 1 mL of PBS⁺⁺. Use aspirator to remove and transfer pipette to add PBS.
2. If staining for intracellular markers, wash cells with permeabilization buffer 3 times for 5 min each.
3. Add 0.8 mL blocking solution to each well. Incubate at room temperature for 45 min.
4. Dilute the primary antibody in 1 mL of blocking solution at supplier recommended dilution (for anti-nestin antibody 1:200).
5. Aspirate off blocking solution from wells and add 300 μ L of diluted primary antibody at optimal titer. Cover to prevent exposure to light and incubate 1 h at room temperature. This can be extended to overnight at 4 °C, if necessary.
6. Wash cells 4 times in HSB (5 min each) if staining for intracellular markers. Wash cells 4 times in PBS⁺⁺ (5 min each) if staining for cell surface markers.
7. Whilst completing washes in step 6, prepare the secondary antibody in blocking solution at supplier recommended dilution (for AlexaFluor 1:1,000).
8. Aspirate off last wash from wells and add 300 μ L of diluted secondary antibody at optimal titer. Incubate 1 h at room temperature. During incubation, cover sample with aluminum foil to prevent fluorescence bleaching.
9. Wash wells 4 times in 1 mL of PBS⁺⁺ for 5 min each wash.
10. Add 0.8 mL of a 1:10,000 dilution of DAPI in distilled H₂O to each well. Incubate for 5 min at room temperature. Cover with foil during incubation, to prevent exposure to light.
11. Wash cells 3 times in 1 mL of PBS⁺⁺.

12. Verify that staining has taken place under fluorescence microscope before mounting.
13. Gently remove sides of chamber and aspirate excess surrounding PBS.
14. Place one drop of mounting media directly in the center of each well area.
15. At an angle gently lower a cover slip onto the slide trying to avoid air bubbles where possible. Remove excess mounting media from slide and seal with nail varnish on all 4 sides.
16. Keep in dark storage until results are observed/documentated. It is recommended to document the same day. Examples of nestin expression in hNPs and ddC - treated hNPs are shown in Fig. 2.

3.6 Preparation of LHON Pathogenic mtDNA and Introduction of LHON Pathogenic mtDNA into ddC-Treated Human Neural Progenitors

1. Propagate LHON cybrid cells containing a >96 % homoplasmic G11778A mutation in the ND4 region of mitochondrial genome in DMEM containing 10 % FCS.
2. Isolate mitochondrial DNA from the LHON cybrid cells using Qproteome Mitochondria Isolation Kit and the Mitochondrial DNA Extraction Kit according to the manufacturer's instructions.
3. Treat the isolated mitochondrial DNA with limiting amounts of Plasmid-Safe ATP-dependent DNase, which selectively digests all forms of DNA but does not affect closed circular or nicked circular double-stranded DNA.
4. Purify the isolated mitochondrial DNA according to protocols for the UltraClean GelSpin DNA purification kit. *See Note 16.*
5. Quantify the DNA solution with DNA Quant-iT assay kit and visualize the linear band on a 0.8 % agarose gel.
6. For introduction into ddC-hNPs, the rhTFAM (+/- LHON mtDNA) prepare mix as follows: incubate at 37 °C for 30 min the purified rhTFAM to 3 mg of LHON mtDNA in PBS containing high salt Mg²⁺ buffer. *See Note 17.*
7. Incubate ddC-hNPs in growth medium containing rhTFAM complexed with LHON pathogenic mtDNA for 2 h at 37 °C. Controls involve incubating the hNPs in growth medium containing only rhTFAM or PBS.
8. After the incubation period, replace the medium with regular growth medium and use cells in different experimental analyses as outlined below.

3.7 Imaging of LHON mtDNA Entry into ddC-Treated Human Neural Progenitors

1. For real-time imaging of LHON mtDNA entry into ddC-treated hNPs, propagate cells to 80 % confluence on laminin-coated 35 mm glass bottom dishes.

2. Stain Mitochondria in hNPs using either Mitotracker Red or Mitotracker Green depending on whether tracking is being conducted for Alexa488-rhTFAM or Cy3 labeled LHON-mtDNA complexed with unlabeled rhTFAM, respectively.
3. Purify rhTFAM by dialyzing with 1× PBS buffer and label with Alexa 488 dye according to the manufacturer's instructions. *See Note 18.*
4. Label circular mtDNA carrying the LHON G11778A mutation using Cy3 dye according to the manufacturer's instruction.
5. Mix the labeled rhTFAM or the labeled LHON mtDNA complexed with MTD-TFAM in 2 mL DMEM and add to independent dishes of hNPs with labeled mitochondria. *See Note 19.*
6. Obtain real-time single plane-time lapse images every 5 min using a confocal microscope.

3.8 Restriction Enzyme Digestion Analysis

1. Amplify a PCR product in the ND4 gene spanning the SfaN1 site removed by the G11778A mutation from the cDNA of hNPs, and digest with SfaN1 to check for the presence or absence of the mutation.
2. Digest cDNA PCR products and analyze using an automated electrophoresis apparatus.

3.9 Differentiation Potential of Engineered hNP Cells into Neurons

1. Allow the engineered hNPs to attain 100 % confluency in regular growth medium.
2. Replace the propagation medium with differentiation medium (*see Subheading 2.8*) for a period of 14 days.
3. For immunocytochemical analysis, propagate hNPs in chamber slides coated with POLA to provide a similar culture condition as on regular dishes. After differentiation for 14 days.
4. Subject the neurons obtained to immunocytochemical analysis for β -tubulin-III expression.

4 Notes

1. Laminin-coated dishes provide the appropriate surface for derivation of hNPs.
2. It is recommended to use WA09 -derived [STEMEZ hNP™] human neural progenitors (<http://www.arunabiomedical.com>) during initial experimentation, and for comparison when deriving hNPs from hPSCs.
3. POLA-coated plates provide the appropriate surface for propagation of hNPs.
4. The pyruvate/uridine mixture is normally made at 1,000× in PBS and aliquoted as 500 μ L samples and stored at -20°C .

5. The Qproteome Mitochondria Isolation kit contains buffers and reagents for isolation of high-purity mitochondria preparations from cells. Lysis Buffer should be stored at -20°C upon arrival. All other buffers and Protease Inhibitor Solution (100 $\times$) should be stored at $2-8^{\circ}\text{C}$.
6. The Mitochondrial DNA Extraction kit contains 5 $\times$ Cytosol Extraction Buffer, Mitochondrial Lysis Buffer, Enzyme B Mix (lyophilized), TE Buffer. After opening the kit, store Enzyme B Mix at -70°C . Store all other buffers at 4°C . Make 1 $\times$ Cytosolic Extraction Buffer by mixing the 1 mL of the 5 $\times$ buffer with 4 mL ddH₂O. Add 275 μL of TE buffer to Enzyme B Mix. Mix well, aliquot and refreeze immediately at -70°C . Stable for up to 3 months at -70°C . All buffers should be placed on ice at all times during the experiment.
7. Plasmid-Safe DNase is supplied in a 50 % glycerol solution containing 50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 % Triton[®] X-100. One unit degrades 1 nmol of deoxynucleotides in linear dsDNA in 30 min at 37°C in 1 $\times$ Plasmid-Safe Reaction Buffer and 1 mM ATP. Plasmid-Safe 10 $\times$ Reaction Buffer contains: 330 mM Tris-acetate (pH 7.5), 660 mM potassium acetate, 100 mM magnesium acetate, 5.0 mM DTT. ATP is required for Plasmid-Safe DNase activity and should be added to a final concentration of 1 mM. Store only at -20°C in a freezer without a defrost cycle.
8. The UltraClean GelSpin DNA purification kit uses an economical silica binding particle method to purify DNA from agarose gels. Kit reagents and components should be stored at room temperature ($15-30^{\circ}\text{C}$).
9. The DNA Quant-iT assay kit provides concentrated assay reagent, dilution buffer, and pre-diluted DNA standards. Store in refrigerator ($2-8^{\circ}\text{C}$) and protect from light.
10. Store the purified protein in Tris-buffered 50 % glycerol at -20°C .
11. Use Mitotracker Red for staining and tracking mitochondria as Alexa488 is used for tracking MTD-TFAM. Use Mitotracker Green for tracking Cy3-labeled pathogenic mtDNA complexed with unlabeled MTD-TFAM.
12. The AllPrep DNA / RNA Mini Kit contains buffers and reagents and should be stored at room temperature ($15-25^{\circ}\text{C}$). It is stable for at least 9 months under these conditions.
13. The Quant-IT DNA and RNA assay kit provides concentrated assay reagent, dilution buffer, and pre-diluted DNA standards. Store in refrigerator ($2-8^{\circ}\text{C}$) and protect from light.
14. The iScript[™] cDNA Synthesis Kit is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. Store the enzyme at -20°C . Nuclease-free water can be stored at room temperature.
15. ddC is a potent inhibitor of mitochondrial DNA polymerase γ . Loss of cell viability is expected during ddC treatment of hNPs. Care should be taken in

replacing growth medium supplemented with pyruvate and uridine once every 24 h.

16. Treated DNA can be further purified by ethanol precipitation, spin columns, or organic extraction.
17. The presence of the high salt Mg^{2+} buffer is critical for facilitating protein binding to the DNA.
18. It is recommended to assess DNA-binding capacity of rhT-FAM by electrophoretic mobility shift assay (EMSA) that is based on maximum retardation of circular DNA.
19. A ratio of 500 ng of circular DNA complexed with 1 μ g of rhTFAM is recommended.

Acknowledgments

This work was supported in part by start-up funds provided to Dr. Iyer by the Center for the Study of Biological Complexity at Virginia Commonwealth University and a grant from the Presidential Research Innovation Program (PRIP) at Virginia Commonwealth University. The authors would also like to thank Gencia Corporation, Charlottesville for provision of the recombinant human TFAM. Mention of vendor names is for information only and does not imply endorsement.

References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998; 282:1145–1147. [PubMed: 9804556]
2. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318:1917–1920. [PubMed: 18029452]
3. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007; 131:861–872. [PubMed: 18035408]
4. Shin S, Mitalipova M, Noggle S, et al. Long-term proliferation of human embryonic stem cell-derived neuroepithelial cells using defined adherent culture conditions. *Stem Cells*. 2006; 24:125–138. [PubMed: 16100006]
5. Dhara SK, Hasneen K, Machacek DW, et al. Human neural progenitor cells derived from embryonic stem cells in feeder-free cultures. *Differentiation*. 2008; 76:454–464. [PubMed: 18177420]
6. Shin S, Dalton S, Stice SL. Human motor neuron differentiation from human embryonic stem cells. *Stem Cells Dev*. 2005; 14:266–269. [PubMed: 15969621]
7. Wilczynska KM, Singh SK, Adams B, et al. Nuclear factor I isoforms regulate gene expression during the differentiation of human neural progenitors to astrocytes. *Stem Cells*. 2009; 27:1173–1181. [PubMed: 19418463]
8. Iyer S, Alsayegh K, Abraham S, et al. Stem cell-based models and therapies for neurodegenerative diseases. *Crit Rev Biomed Eng*. 2009; 37:321–353. [PubMed: 20528730]
9. Wilson PG, Cherry JJ, Schwamberger S, et al. An SMA project report: neural cell-based assays derived from human embryonic stem cells. *Stem Cells Dev*. 2007; 16:1027–1041. [PubMed: 18078382]
10. Khan SM, Bennett JP Jr. Development of mitochondrial gene therapy. *J Bioenerg Biomembr*. 2004; 36:387–393. [PubMed: 15377877]
11. Iyer S, Thomas RR, Portell FR, et al. Recombinant mitochondrial transcription factor A with N-terminal mitochondrial transduction domain increases respiration and mitochondrial gene expression. *Mitochondrion*. 2009; 9:196–203. [PubMed: 19460293]

12. Iyer S, Bergquist K, Young K, et al. Mitochondrial gene therapy improves respiration, biogenesis, and transcription in G11778A Leber's hereditary optic neuropathy and T8993G Leigh's syndrome cells. *Hum Gene Ther.* 2012; 23:647–657. [PubMed: 22390282]
13. Iyer S, Xiao E, Alsayegh K, et al. Mitochondrial gene replacement in human pluripotent stem cell-derived neural progenitors. *Gene Ther.* 2012; 19:469–475. [PubMed: 21918550]

Fig. 1. Sequence of steps involved in creation of neural progenitor cell model based on mitochondrial gene replacement

Fig. 2. Immunostained images of **(a)** hNPs and **(b)** ddC-treated hNPs. The cultures were highly homogeneous and exhibit neural rosette morphology, that stain positive for Nestin (*green*). DAPI (*blue*) was used for staining the nuclei. Magnification 20×