Differentiation of Human Neural Stem Cells into Motor Neurons Stimulates Mitochondrial Biogenesis and Decreases Glycolytic Flux

Laura C. O'Brien,¹ Paula M. Keeney,² and James P. Bennett, Jr.^{1-4,*}

Differentiation of human pluripotent stem cells (hPSCs) in vitro offers a way to study cell types that are not accessible in living patients. Previous research suggests that hPSCs generate ATP through anaerobic glycolysis, in contrast to mitochondrial oxidative phosphorylation (OXPHOS) in somatic cells; however, specialized cell types have not been assessed. To test if mitobiogenesis is increased during motor neuron differentiation, we differentiated human embryonic stem cell (hESC)- and induced pluripotent stem cell-derived human neural stem cells (hNSCs) into motor neurons. After 21 days of motor neuron differentiation, cells increased mRNA and protein levels of genes expressed by postmitotic spinal motor neurons. Electrophysiological analysis revealed voltage-gated currents characteristic of excitable cells and action potential formation. Quantitative PCR revealed an increase in peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α), an upstream regulator of transcription factors involved in mitobiogenesis, and several of its downstream targets in hESC-derived cultures. This correlated with an increase in protein expression of respiratory subunits, but no increase in protein reflecting mitochondrial mass in either cell type. Respiration analysis revealed a decrease in glycolytic flux in both cell types on day 21 (D21), suggesting a switch from glycolysis to OXPHOS. Collectively, our findings suggest that mitochondrial biogenesis, but not mitochondrial mass, is increased during differentiation of hNSCs into motor neurons. These findings help us to understand human motor neuron mitobiogenesis, a process impaired in amyotrophic lateral sclerosis, a neurodegenerative disease characterized by death of motor neurons in the brain and spinal cord.

Introduction

MITOCHONDRIAL BIOGENESIS (MITOBIOGENESIS) is the mechanism by which cells increase their mitochondrial components, ultimately increasing bioenergetic capacity. This process includes transcription of genes encoded by both the mitochondrial and nuclear genomes and is modulated based on the energy needs of the cell. Peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α) is an upstream regulator of transcription factors involved in mitobiogenesis and respiration and is encoded by the gene *PPARGC1A* [1,2]. PGC-1 α is thought to be the master regulator of mitobiogenesis.

PGC-1 α interacts directly with nuclear respiratory factors 1 and 2 (NRF1, NRF2) and estrogen-related receptor α (ERR α), which then translocate to the nucleus [3–5]. This results in increased transcription of genes, including those encoding electron transport chain (ETC) subunits and mitochondrial transcription machinery, which are then localized to the mitochondria [6]. Mitochondrial DNA (mtDNA)

is transcribed by a mitochondrial DNA-directed RNA polymerase (POLRMT) with the help of mitochondrial transcription factors A and B2 (TFAM and TFB2M) [7,8]. Mitochondrial DNA polymerase gamma (POLG) performs mtDNA replication [9]. Although only 13 mitochondrial genes encode proteins, all of them are essential for proper ETC function [10,11].

Recent studies in our laboratory and others implicate mitochondrial dysfunction as a significant pathology in neurodegenerative disorders. One of these disorders is amyotrophic lateral sclerosis (ALS), which is characterized by death of motor neurons in the brain and spinal cord. Survival time after diagnosis of ALS is an average of 3–5 years and current treatments do not stop progression of the disease. Despite extensive study, the mechanisms of sporadic ALS (sALS), which accounts for up to 95% of cases [12], are unknown; however, mitobiogenesis appears to be impaired in postmortem tissues of patients with sALS. PGC-1 α and its downstream targets showed decreased expression in the postmortem spinal cord and muscle from sALS patients

¹Department of Physiology and Biophysics, ²VCU Parkinson's and Movement Disorders Center, Departments of ³Neurology and ⁴Psychiatry, Virginia Commonwealth University, Richmond, Virginia.

*Current affiliation: Neurodegeneration Therapeutics, Inc., Charlottesville, Virginia.

[13]. Additionally, there was decreased activity of ETC subunits and increased mtDNA deletions in isolated post-mortem spinal motor neurons [14–16] and skeletal muscle [17] of sALS patients. Postmortem tissue studies provide information on end-stage disease states, but assessment of isolated human motor neurons in living patients is not currently possible.

The use of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), may provide insight into human cell biology. By differentiating hPSCs from ALS patients and healthy controls into motor neurons, one could potentially understand the physiological mechanisms underlying mitobiogenesis and ultimately what causes neurodegeneration in ALS. This may allow for the identification of targets that prove beneficial in the designing of drugs for neurodegenerative and motor neuron diseases. The first report of motor neuron differentiation from ALS patient iPSCs was in 2008 [18]. Since then, multiple groups have generated iPSC lines from patients containing genetic mutations associated with ALS [19–27]. While these studies further our understanding of familial ALS, patients with known genetic mutations comprise only 5%–10% of ALS cases. It is important to know if findings from these studies are also relevant in sporadic disease.

iPSCs from patients with sALS have been shown to recapitulate disease pathology [28]; however, the mitobiogenesis profile of ALS cells remains to be described. By studying cells from living patients with associated clinical data, we hope to learn more about sporadic disease. Toward this goal, a recent study from our laboratory showed decreased mitochondrial-encoded gene expression in peripheral blood mononuclear cells (MNCs) of ALS patients compared with control, suggesting a systemic bioenergetic impairment [29]. If motor neurons differentiated from ALS iPSCs display the same mitochondrial dysfunction seen in postmortem tissue, they may be used as a model of sALS.

Respiration analysis suggests that hPSCs produce ATP mainly through anaerobic glycolysis compared with mitochondrial oxidative phosphorylation (OXPHOS) in somatic cells [30–33]. However, the details of this reversible switch remain unclear. Initial studies reported that hPSCs had few underdeveloped mitochondria [34–36]. Recent studies have shown that while they rely on glycolysis for ATP production, hPSCs have active respiratory chain complexes and respire at maximal capacity [30,37]. The switch to OXPHOS during differentiation would require increased mitochondrial respiratory capacity. There is evidence to suggest that this is achieved by an increase in mitochondrial mass, ATP, and subsequent reactive oxygen species production during spontaneous differentiation of hPSCs [38,39]. However, more recent studies suggest that when normalized to total protein levels, hPSCs and fibroblasts have a similar mitochondrial mass [37,40]. Mitochondrial function during human motor neuron differentiation has not yet been assessed.

Normal cell culture conditions grow cells in room air containing ~20% oxygen. This is much higher than what neural cells are exposed to in the developing embryo or nervous system. Low oxygen conditions (2%–5%) have been shown to enhance proliferation, differentiation, and survival of stem cells and neurons [41–44], including spinal motor neurons [43]. For this study, we grew and differen-

tiated all cultures in 5% oxygen conditions. Neurons are high-energy-requiring postmitotic cells that rely heavily on their mitochondria. Based on the abundant literature supporting the switch from anaerobic glycolysis to mitochondrial OXPHOS during spontaneous differentiation of hPSCs, we hypothesized that mitobiogenesis is increased during motor neuron differentiation and that this would be accompanied by an increase in OXPHOS.

In this report, we differentiated commercially available human neural stem cells (hNSCs) and iPSCs derived from blood into electrically active cells with multiple markers of postmitotic spinal motor neurons. During this process, cells upregulated transcription of genes involved in mitobiogenesis signaling and protein expression of respiratory subunits. Respiration analysis revealed a shift from glycolysis in hNSCs to OXPHOS in motor neurons with a trend toward increased coupling to ATP synthesis. These findings help us to understand human motor neuron mitobiogenesis, a process impaired in ALS.

Materials and Methods

iPSC generation and neural induction

Peripheral blood was collected according to a Virginia Commonwealth University IRB-approved protocol. Integration-free iPSCs were generated from human MNCs using a previously described protocol [45], with modifications. Briefly, MNCs were isolated immediately from peripheral blood and expanded by culturing for 2 weeks in MNC medium. The pEB-C5 and pEB-Tg plasmids were obtained from Addgene and grown in the VCU Macromolecular Core Laboratory.

Three $\times 10^6$ MNCs were electroporated with the plasmids using an Amaxa Nucleofector 4D system (Lonza). Immediately following electroporation, an equal volume of 37°C RPMI medium was added to the cuvette and the cells were allowed to recover for 10 min at 37°C before returning to culture in MNC medium. Transfected cells were cocultured on mouse embryonic fibroblasts beginning at day 2, and the schedule of medium changes outlined in the protocol was followed. On day 14, the maintenance medium was changed to mTeSR (Stem Cell Technologies) and colonies with PSC morphology were picked to wells of a 96-well plate beginning at about day 21. Viable colonies were expanded in mTeSR medium on Geltrex (Life Technologies)-coated plates at 37°C in a humidified CO₂ incubator with the oxygen level held at 5%. Growth medium supplemented with 10 μ M Rho-associated protein kinase (ROCK) inhibitor Y27632 (R&D Systems) was used for the first 24 h after colonies were split.

Neuralization of iPSCs was accomplished using PSC Neural Induction Medium (Life Technologies) according to the protocol with modifications. Briefly, iPSC colonies were maintained in PSC Neural Induction Medium beginning at day 1 after splitting. On day 5, colonies with good morphology were picked to fresh Geltrex-coated dishes and induction continued for another 5 days. On day 10, colonies were detached using Accutase (Life Technologies), passed through a 100- μ m strainer, centrifuged at 300 g for 4 min, and plated in neural expansion medium on Geltrex-coated dishes. Cultures were maintained at 37°C in a humidified CO₂ incubator with the oxygen level held at 5%. Growth medium supplemented

with 10 μ M ROCK inhibitor Y27632 (R&D Systems) was used for the first 24 h after the NSCs were split through passage 4.

Motor neuron differentiation

To remain in an undifferentiated state, GIBCO® Human Neural Stem Cells (H9 hESC derived; Invitrogen) were grown in Knockout Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 2 mM GlutaMAX-I supplement, 20 ng/mL human recombinant basic fibroblast growth factor, and epidermal growth factor and 2% StemPro neural supplement. When cells were ~90% confluent, they were dissociated using TrypLE™ and plated on culture vessels coated with CELLStart™ or Geltrex™. For differentiation into motor neurons, media were changed to neurobasal medium containing 2% B-27 serum-free supplement and 2 mM GlutaMAX-I supplement plus 0.1 μ M retinoic acid (RA; Sigma) for 7 days, followed by 7–21 days of 0.1 μ M RA plus 0.5 μ M purmorphamine (PM) [43,46–52].

After neural induction, iPSCs were differentiated as described previously, with some modifications [51]. Briefly, adherent cells were grown in neural induction media containing DMEM/F12 with 0.2 μ M LDN-193189 (LDN; Stemgent), 10 μ M SB431542 (SB; Stemgent), 10 ng/mL brain-derived neurotrophic factor (R&D Systems), 0.4 μ g/mL L-ascorbic acid (Sigma), 2 mM GlutaMAX-I supplement, 1% N-2 supplement, and 1% nonessential amino acids (NEAA). Two days later, 1 μ M RA was added. On day 4, LDN/SB was stopped and 1 μ M smoothed agonist (Calbiochem or Santa Cruz) and 0.5 μ M PM were added. On day 14, cells were switched to neurobasal media containing 2 mM GlutaMAX-I, 2% B-27, 1% NEAA, 0.4 μ g/mL AA, 10 ng/mL GDNF (R&D Systems), and 10 ng/mL CNTF (R&D Systems). Media were replaced every 2–3 days. Unless otherwise specified, all cell culture materials were purchased from Life Technologies. All cultures were grown at 37°C in 5% oxygen and 5% CO₂ conditions.

Lentiviral vectors

For generation of the HB9::GFP lentivirus, services and products generated by the VCU Massey Cancer Center Biological Macromolecule Shared Resource, supported, in part, with funding from NIH-NCI Cancer Center Support Grant P30 CA016059, were used. Addgene plasmid 37080 [50] was cotransfected with packaging and envelope plasmids, cCMVR8.74 and MD2G. Transfection and viral concentration were performed using standard published protocols. Virus titer was 5×10^7 TU/mL in phosphate-buffered saline (PBS). Cells were infected at a multiplicity of infection of 3–5 with 8 μ g/mL protamine sulfate 5 days before recording.

Electrophysiology

For whole-cell patch-clamp experiments, cells were differentiated for 28 days. Two days before recording, cells were moved to 12-mm-diameter circular glass coverslips (neuvitro). On the day of recording, coverslips were placed in a recording chamber on the stage of an inverted microscope (Olympus). An Axopatch 200B amplifier (Molecular Devices) and pClamp 10 software (Axon In-

struments) were used to record whole-cell currents. Patch pipettes were pulled from thick-walled borosilicate glass capillaries to resistances of 2–4 M Ω and filled with internal solution containing 120 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA, 10 mM HEPES, and 2 mM Na₂ATP (pH 7.2). Extracellular solution consisted of 135 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, and 10 mM glucose (pH 7.4). Two micromolar tetrodotoxin (TTX; R&D Systems) was added to block voltage-gated sodium channels.

Immunocytochemistry

For immunocytochemistry analysis on day 21 (D21), cells were fixed in 4% paraformaldehyde and 4% sucrose in PBS at room temperature for 15 min. Cells were incubated for 60 min in blocking buffer (5% goat serum, 1% bovine serum albumin, 0.1% Triton-X in PBS). Cells were incubated overnight at 4°C with primary antibody (1:100) diluted in 5% goat serum. Primary antibodies included mouse anti-nestin (Abcam), mouse anti-TOM20 (Abcam), rabbit anti-MAP2 (Millipore), and mouse anti-Isl1/2 and HB9 (MNR2; DSHB). Alexa Fluor-conjugated secondary antibodies (1:400; Life Technologies) were added for 60 min at room temperature. After washes, VECTASHIELD mounting media with DAPI were added for visualization of nuclei. Images were obtained with an Olympus FV1000 confocal microscope. For translocation of outer mitochondrial membranes 20 (TOM20) and percent positive quantification, 10 representative fields were taken and analyzed using MetaMorph image analysis software (Molecular Devices). For TOM20 analysis, pixel values were normalized to the number of cells in each image identified by DAPI nuclear staining.

Real-time quantitative PCR

For quantitative PCR (qPCR) analysis of hESC-derived cells, DNA and RNA were extracted at 7-day time points through day 28 using the AllPrep DNA/RNA Mini Kit (Qiagen). For iPSC-derived cells, RNA was extracted at day 0 (D0) and D21 with the RNeasy or RNeasy Plus Micro Kit (Qiagen), and DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Quantification of isolated DNA and RNA was performed using a Nanodrop 2000c spectrophotometer (Thermo Scientific). RNA was reverse transcribed into cDNA using the iScript or iScript Advanced cDNA Synthesis Kit (Bio-Rad). For qPCR, 25–50 ng cDNA or 0.1 ng DNA per well was loaded into a 96-well plate and analyzed with the CFX96 Real-Time PCR Detection System (Bio-Rad). All samples were analyzed in triplicate.

MtDNA copy number was determined using multiplex qPCR targeting human mtDNA-encoded genes around the mitochondrial genome (*12s rRNA*, *ND2*, *COX3*, *ND4*) as described in previous studies [16,53]. The mtDNA standards were prepared as described previously [16]. Six reference genes for both cDNA samples were analyzed and data were normalized to the geometric mean of two reference genes determined to have the greatest stability using the software qbasePLUS-GeNorm (14.3.3.Z and *CYC1*; BioGazelle). Primer sets are available upon request. Outliers were excluded at the $P < 0.05$ level by the Grubbs test. Statistics

were calculated using one-way ANOVA or unpaired *t*-test in Prism software (Prism; GraphPad). Error bars represent standard error of the mean.

Western blot

Twenty micrograms of total cell protein from each sample (day 0, 21, 28) was separated on a 4%–12% Bis-Tris Criterion precast gel and transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). ETC complex I–V subunits were detected by immunoblotting with the MitoProfile® Total OXPHOS Human WB Antibody cocktail (ab110411; Abcam). Anti-VDAC1 (Abcam) was assayed as an estimate of mitochondrial mass in each sample. Beta actin (β -actin; Abcam) was used as a loading control. Band intensity was quantified using the Odyssey infrared imaging system (LI-COR). Statistical analysis was performed with an unpaired *t*-test using Prism software.

XF24 extracellular flux analyzer

Cell metabolic rates were measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience) in unbuffered DMEM, pH 7.4 as described previously by our group [54]. Briefly, on day 16, hESC-derived cells were seeded onto an XF24 cell culture microplate (Seahorse Bioscience)

at 60,000 cells per well with 10 μ M Y-27632 dihydrochloride, a selective inhibitor of ROCK (Tocris), and incubated at 37°C in 5% oxygen. After 3 days, D0 hNSCs were seeded onto the same XF24 cell culture microplate at 40,000 cells per well with 10 μ M ROCK inhibitor and incubated at 37°C 5% oxygen for 24 h. iPSC-derived cells (D0 and D21) were plated at 60,000 cells per well the day before recording. Each group (D0 and D21) was tested at three to four concentrations to determine the most reliable plating density for each cell type. Inhibitors included oligomycin (1 μ M) and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (0.5–1 μ M). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were normalized to protein concentration (Micro BCA Kit; Pierce) for all experiments to account for differences in cell size. Statistical analysis was performed with an unpaired *t*-test using Prism software.

Results

hNSCs differentiate into cells with motor neuron characteristics

Peripheral blood MNCs were reprogrammed from one healthy male control patient, age 62 years, using an integration-free plasmid vector expression previously described [45]. Instead of starting from hESCs, we purchased hNSCs

FIG. 1. Generation of motor neurons (MN) from human neural stem cells (hNSCs). (A, B) Representative phase-contrast images of day 0 (D0) hNSCs and day 21 (D21) MNs from human embryonic stem cell (hESC)- (A) and induced pluripotent stem cell (iPSC)- (B) derived cultures. (C, D) Genes expressed by postmitotic spinal motor neurons, homeobox protein (HB9) and Islet 1 (ISL1), shown as normalized relative expression for hESC- ($n=4-6$) (C) and iPSC- ($n=3$) (D) derived cultures on D0 and D21. (E, F) Representative images of motor neuron markers, HB9 and ISL1/2 (green), and neuronal marker, microtubule-associated protein 2 (MAP2) (red), staining on D21 of differentiation from hESC- (E) and iPSC- (F) derived cultures. DAPI nuclear staining is in blue. HB9, homeobox protein; MAP2, microtubule-associated protein. Color images available online at www.liebertpub.com/scd

derived from the H9 (WA09) hESC line (Invitrogen). These cells have a normal karyotype and can remain in an undifferentiated state in the presence of growth factors or differentiate into neurons, oligodendrocytes, or astrocytes. To generate motor neurons, we followed protocols previously described for hESC-derived [46] and iPSC-derived [51] hNSCs.

Immunocytochemical analysis using confocal microscopy confirmed that on D0 >90% of hESC-derived and 67% of iPSC-derived cells stained positive for the NSC marker, nestin. Phase-contrast images of D0 and D21 cells showed morphological changes, including an extensive network of processes on D21 for both hESC-derived (Fig. 1A) and iPSC-derived (Fig. 1B) cultures. qPCR analysis revealed a significant increase in transcription of genes expressed by postmitotic spinal motor neurons after 21 days of differentiation for both hESC-derived (Fig. 1C) and iPSC-derived (Fig. 1D) cultures. Genes assessed include the motor neuron-specific transcription factor, homeobox gene (*HB9*) [55] and Islet 1 (*ISL1*; $n=4-6$ independent cultures for hESC-derived cells, and $n=3-4$ independent cultures for iPSC-derived cells). Both *HB9* and *ISL1* are essential for the formation of mature motor neurons [55,56]. After 21 days of differentiation, 46% of hESC-derived cells and 11% of iPSC-derived cells costained positive for microtubule-associated protein 2 (MAP2) and *ISL1* and 51% of hESC-derived cells and 16% of iPSC-derived cells costained positive for MAP2 and *HB9*. Representative images are shown in Fig. 1E for hESC-derived cells and Fig. 1F for iPSC-derived cells on D21. No glial fibrillary acidic protein staining was observed.

To identify motor neurons in live cultures, we infected cells with a lentivirus containing GFP under the control of the *HB9* promoter as previously described [50]. Membrane capacitance (C_m) was 14.88 ± 1.42 pF for hESC-derived cells ($n=5$) and 9.75 ± 0.45 pF for iPSC-derived cells ($n=7$; Fig. 2A). Resting membrane potential (V_m) was -39 ± 3.84 mV in hESC-derived cells ($n=6$) and -31.6 ± 4.82 mV in iPSC-derived cells ($n=10$; Fig. 2B). Membrane resistance (R_m) was 2 ± 0.3 G Ω in hESC-derived cells ($n=5$) and 3.77 ± 0.85 G Ω in iPSC-derived cells ($n=6$; Fig. 2C). Passive membrane properties were not statistically different between cell types.

Whole-cell patch-clamp recordings evoked currents by voltage steps from -80 to $+70$ mV in 10-mV increments from a holding potential of -60 mV. A representative current trace of a motor neuron on day 28 of differentiation in an iPSC-derived cell suggests the presence of voltage-gated sodium channels, accounting for the fast-activating, fast-inactivating inward current and voltage-gated potassium channels as suggested by the outward current (Fig. 2E-i). Addition of $2 \mu\text{M}$ TTX, a blocker of voltage-gated sodium channels, blocked the inward current (Fig. 2E-ii). Current densities normalized to cell size were determined by dividing peak current amplitudes by cell capacitance. Sodium current density was -29.10 ± 8.29 pA/pF for hESC-derived cells and -37.51 ± 5.39 pA/pF for iPSC-derived cells. Potassium current density was 29.55 ± 9.25 pA/pF for hESC-derived cells and 78.91 ± 8.04 pA/pF for iPSC-derived cells.

Action potentials were elicited by a series of current pulses (10 or 100 ms) of increasing current amplitude in the

FIG. 2. Functional properties of motor neurons identified by *HB9::GFP* on D28 of differentiation. (A) Membrane capacitance (C_m) ($n=5, 7$). (B) Resting membrane potential (V_m) ($n=6, 10$). (C) Membrane resistance (R_m) ($n=5, 6$) and (D) rheobase ($n=4, 4$) for hESC- and iPSC-derived cells, respectively. (E-i) A voltage-clamp trace showing voltage-activated sodium and potassium currents in an iPSC-derived motor neuron, (ii) the same cell after the addition of $2 \mu\text{M}$ tetrodotoxin (TTX). (F) Representative whole-cell current-clamp recording showing action potential formation in an hESC-derived motor neuron.

FIG. 3. Mitochondriogenesis is increased as cells differentiate into motor neurons. (A, B) Mitochondriogenesis gene expression on D0 and D21 for hESC- ($n=4-6$) (A) and iPSC- ($n=3$) (B) derived cultures. (C, D) Copy number of four mitochondrial DNA (mtDNA)-encoded genes in hESC- (C) and iPSC- (D) derived cultures. (E, F) mtDNA-encoded gene expression during differentiation. All data are presented as fold change from D0. * $P < 0.05$; ** $P < 0.01$.

current-clamp mode. Approximately, 40% of hESC-derived cells and 60% of iPSC-derived cells fired action potentials on D28. A representative trace from an hESC-derived cell is shown in Fig. 2F. Rheobase, the minimum amount of current necessary to generate an action potential, was also determined (Fig. 2D; 23.75 ± 9.44 pA hESC-derived cells; $n=4$, -10.75 ± 11.88 pA iPSC-derived cells; $n=4$). The fact that some iPSC-derived cells fire action potentials after hyperpolarizing current injection suggests the ability for spontaneous action potential formation.

Mitochondriogenesis increases with motor neuron differentiation

To investigate changes in mitochondriogenesis during the differentiation of hNSCs into motor neurons, we assessed gene expression of mitochondriogenesis signaling and transcription factors. qPCR analysis revealed increased expression of PGC-1 α and its downstream targets, *POLG*, *POLRMT*, *ERR α* , and *NRF1*, in hESC-derived cultures (Fig. 3A; $n=3-6$). Expression of all genes peaked at D21 of differentiation, coincident with motor neuron markers. *TFB2M* showed a slight increase in transcription during differentiation ($P=0.058$; Fig. 3A; $n=3-5$). Interestingly, *TFAM* and *NRF2* expression levels were not statistically different across the

time of differentiation. Expression of *ERR α* was increased in iPSC-derived cultures (Fig. 3B).

To measure the mtDNA copy number, we analyzed four mtDNA-encoded genes spatially distributed around the mitochondrial genome, *ND2*, *ND4*, *COX3*, and *12S*. On D21 and D28 of differentiation, we found a slight decrease in mtDNA copy number when compared with D0 for hESC motor neurons (Fig. 3C, $n=4-6$). This finding was statistically significant in the iPSC-derived motor neurons (Fig. 3D). The lack of variability in copy numbers of the four mtDNA-encoded genes assessed implies that there are no major mtDNA deletions in either cell type. Analysis of mtDNA-encoded gene expression revealed a statistically significant increase in *ND2* and *ND4* for hESC-derived cells (Fig. 3E), but iPSC-derived cells were too variable to determine a significant increase (Fig. 3F; $n=3-6$).

Motor neurons increase coupling to ATP synthesis and decrease glycolytic flux compared with hNSCs

Respiration analysis of hNSCs (D0) and motor neurons (D21) was performed in both hESC- (Fig. 4A, B) and iPSC- (Fig. 4C, D) derived cells with the XF24 Extracellular Flux Analyzer. The XF24 measures OCR and ECAR, an estimation of glycolysis from lactate production, in adherent

FIG. 4. Cells decrease glycolysis during differentiation into motor neurons. (A) Oxygen consumption rate (OCR) in hESC-derived cells. (B) Basal extracellular acidification rate (ECAR) values, a measure of glycolysis, in hESC-derived cells. (C) OCR in iPSC-derived cells. (D) ECAR in iPSC-derived cells. * $P < 0.05$.

cultures. Basal respiration was unchanged in D21 cells; however, hESC-derived cells showed a trend for increased coupling to ATP synthesis when treated with the ATP synthase inhibitor, oligomycin (Fig. 4A). There was a 57% decrease in D21 respiration with the addition of 1 μ M oligomycin compared with only a 48% decrease in D0 cells (Fig. 4A). Interestingly, the OCR did not increase above basal levels with the addition of 0.5 or 1 μ M FCCP in either cell type. This suggests that cells are already respiring at maximum capacity. There was a statistically significant decrease in ECAR on D21 in both hESC- (Fig. 4B) and iPSC- (Fig. 4D) derived cells, suggesting a decrease in glycolysis with motor neuron differentiation.

Respiratory proteins, but not mitochondrial mass, increase with motor neuron differentiation

To investigate if the decreased reliance on glycolysis and increased coupling to ATP synthesis was due to increased mitochondrial mass and/or respiratory proteins, we analyzed protein expression of ETC complexes and voltage-dependent anion channel 1 (VDAC1) for both cell types. VDAC1 is an abundant outer membrane protein and a marker of mitochondrial mass. Representative blots are shown for hESC- (Fig. 5A) and iPSC- (Fig. 5C) derived cell types. Western blot analysis revealed an increase in complex II (CII) protein expression for hESC-derived cells and complex III (CIII) and complex V (CV) for iPSC-derived cells on D21 when normalized to β -actin (Fig. 5A, C). Percent change from D0 of all respiratory proteins analyzed

revealed a statistically significant increase on D21 of motor neuron differentiation for both cell types (Fig. 5B, D).

Interestingly, VDAC1 expression remained unchanged in both cell types, suggesting no change in mitochondrial mass (Fig. 5A, C). To investigate this finding further, we stained D0 and D21 hESC-derived cultures with another marker of mitochondrial mass, TOM20. When normalized to the number of DAPI-stained nuclei per field, there was no change in the TOM20 area between hESC-derived D0 and D21 cultures (Fig. 5E, F). This implies that cells may be increasing the activity of the ETC, but not the total mitochondrial mass. Together, these results suggest that mitobiogenesis is upregulated and that cells switch from aerobic glycolysis to mitochondrial OXPHOS during motor neuron differentiation, perhaps by increasing protein levels of respiratory complexes.

Discussion

This study has three major findings. First, we have generated neurons expressing multiple markers of mature motor neurons from commercially available hNSCs and iPSCs derived from peripheral blood MNCs. To the best of our knowledge, this is the first report of MNC-derived iPSCs being differentiated into motor neurons and represents a novel finding. The use of peripheral blood MNCs for reprogramming may be more clinically favorable to fibroblasts because of their low exposure to environmental mutagens, less invasive sampling procedure, and favorable epigenetic profile [57]. Gene expression of postmitotic spinal motor neuron markers increased significantly during

FIG. 5. Individual electron transport chain (ETC) proteins, but not mitochondrial mass, increase during motor neuron differentiation. (A) Representative western blot and fold change for ETC complexes I–V and voltage-dependent anion channel 1 (VDAC1) normalized to beta actin (β -actin) for hESC ($n=3-4$) (A) and iPSC ($n=4$) (C) cultures. (B, F) Fold change in all respiratory proteins from D0 for hESC- (B) and iPSC- (D) derived cells. (E) Representative images of translocase of outer mitochondrial membranes 20 (TOM20) staining (red) on D0 and D21 of differentiation of hESC-derived cells. DAPI nuclear staining is in blue. (F) Quantification of the TOM20 area per cell in hESC-derived cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Color images available online at www.liebertpub.com/scd

differentiation, including a motor neuron-specific transcription factor, *HB9*, which peaked at D21. Interestingly, punctate nuclear staining of *HB9* and *ISL1/2* was observed in hESC-derived cells on D21. Previous studies have shown cytoplasmic and punctate nuclear staining of *HB9* in early stages of differentiation, similar to what is seen here [58,59]. Future studies using these cells may wish to explore later time courses to explore the change in cellular localization of these transcription factors.

Second, we showed that differentiation of hNSCs into motor neurons increased expression of the mitobiogenesis master regulator, *PGC-1 α* , and many of its downstream targets. These results are consistent with *PGC-1 α* acting through an *ERR α* -*NRF1*-mediated pathway to increase mitobiogenesis. The finding that not all mitobiogenesis genes were significantly increased in iPSC-derived cells after 21 days of differentiation could mean that these cells increase mitobiogenesis gene expression earlier or later in

the differentiation process. Our experimental design did not let us distinguish between these possibilities. By learning more about mitobiogenesis, a process impaired in ALS, we may be able to help identify targets for drug discovery.

Third, western blot and respiration analyses revealed that while mitochondrial mass, assessed by two independent measures, remained unchanged, representative members of ETC complexes increased with motor neuron differentiation. This is consistent with previous studies that found no increase in mitochondrial mass between hPSCs and differentiated cells [37,40]. We now extend these findings to a more specialized phenotype of clinical interest, motor neurons. However, we did not quantify TOM20 staining in iPSC-derived cells. Previous studies have shown a metabolic shift from glycolysis to OXPHOS with spontaneous differentiation. We show a switch from glycolysis to OXPHOS based on reduction of ECARs during motor neuron differentiation. Our findings build on the current literature and further understanding of mitobiogenesis

during the differentiation of hNSCs into motor neurons. However, our experiments do not provide insight into which upstream molecular signaling events are responsible for the stimulation of mitobiogenesis during hNSC differentiation.

In summary, we demonstrated in two different stem cell lines (one from hESC, one from iPSC) that increased mitobiogenesis signaling and functional increases in mitochondrial capacity accompany motor neuron differentiation. We demonstrated increased mitobiogenesis at multiple levels: expression of upstream regulatory genes (eg, PGC-1 α , ERR α); increased transcription of mtDNA-encoded genes; and increased levels of multiple representative mitochondrial OXPHOS proteins. We observed these mitobiogenesis changes without any detectable increase in mitochondrial mass, suggesting that altering the energy-producing capacity of exiting mitochondria can increase mitochondrial OXPHOS function. Increased mitobiogenesis during motor neuron differentiation suggests the possibility that motor neurons are both more dependent on mitochondrial energy production and more vulnerable to mitochondrial bioenergetic impairment.

Our studies of human cervical spinal cord and peripheral blood MNCs from sALS subjects found reduced mtDNA gene expression and impaired mitobiogenesis signaling [29]. We do not yet know the origin(s) of this deficit in mitobiogenesis in ALS, except that it does not appear to be derived primarily from the decreased mtDNA gene expression [30]. hNSC-derived motor neurons may (or may not) serve as reliable platforms for discovering the molecular defects leading to impaired mitobiogenesis signaling or testing survival-enhancing therapies in ALS. The production of biochemically differentiated and electrophysiologically active iPSC-derived motor neurons from peripheral blood MNCs shown in this work is both novel and implies that using these motor neurons from ALS and control subjects for therapy discovery is now approachable.

Acknowledgments

The authors would like to thank Amy Ladd for the thoughtful review, and Drs. Diomedes Logothetis, Carlos Villalba-Galea, and Jose Eltit for help with electrophysiological techniques, as well as the VCU Massey Cancer Center Biological Macromolecule Shared Resource Core. This research was supported by the Parkinson's and Movement Disorders Center at Virginia Commonwealth University and Harper's Hope ALS Research Fund, both administered through the Medical College of Virginia Foundation.

Author Disclosure Statement

No competing financial interests exist.

References

- Scarpulla RC. (2011). Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim Biophys Acta* 1813:1269–1278.
- Scarpulla RC, RB Vega and DP Kelly. (2012). Transcriptional integration of mitochondrial biogenesis. *Trends Endocrinol Metab* 23:459–466.
- Goffart S and RJ Wiesner. (2003). Regulation and coordination of nuclear gene expression during mitochondrial biogenesis. *Exp Physiol* 88:33–40.
- Schreiber SN, R Emter, MB Hock, D Knutti, J Cardenas, M Podvinez, EJ Oakeley and A Kralli. (2004). The estrogen-related receptor alpha (ERRalpha) functions in PPARgamma coactivator 1alpha (PGC-1alpha)-induced mitochondrial biogenesis. *Proc Natl Acad Sci U S A* 101:6472–6477.
- Lin J, C Handschin and BM Spiegelman. (2005). Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab* 1:361–370.
- Mootha VK, C Handschin, D Arlow, X Xie, J St Pierre, S Sihag, W Yang, D Altshuler, P Puigserver, et al. (2004). Erralpha and Gabpa/b specify PGC-1alpha-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. *Proc Natl Acad Sci U S A* 101:6570–6575.
- Falkenberg M, M Gaspari, A Rantanen, A Trifunovic, NG Larsson and CM Gustafsson. (2002). Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat Genet* 31:289–294.
- Posse V, E Hoberg, A Dierckx, S Shahzad, C Koolmeister, NG Larsson, LM Wilhelmsson, BM Hallberg and CM Gustafsson. (2014). The amino terminal extension of mammalian mitochondrial RNA polymerase ensures promoter specific transcription initiation. *Nucleic Acids Res* 42:3638–3647.
- Bonawitz ND, DA Clayton and GS Shadel. (2006). Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. *Mol Cell* 24:813–825.
- Wallace DC. (1999). Mitochondrial diseases in man and mouse. *Science* 283:1482–1488.
- Taanman JW. (1999). The mitochondrial genome: structure, transcription, translation and replication. *Biochim Biophys Acta* 1410:103–123.
- Byrne S, C Walsh, C Lynch, P Bede, M Elamin, K Kenna, R McLaughlin and O Hardiman. (2011). Rate of familial amyotrophic lateral sclerosis: a systematic review and meta-analysis. *J Neurol Neurosurg Psychiatry* 82:623–627.
- Thau N, S Knippenberg, S Korner, KJ Rath, R Dengler and S Petri. (2012). Decreased mRNA expression of PGC-1 alpha and PGC-1alpha-regulated factors in the SOD1G93A ALS mouse model and in human sporadic ALS. *J Neuropathol Exp Neurol* 71:1064–1074.
- Borthwick GM, MA Johnson, PG Ince, PJ Shaw and DM Turnbull. (1999). Mitochondrial enzyme activity in amyotrophic lateral sclerosis: implications for the role of mitochondria in neuronal cell death. *Ann Neurol* 46:787–790.
- Wiedemann FR, G Manfredi, C Mawrin, MF Beal and EA Schon. (2002). Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients. *J Neurochem* 80:616–625.
- Keeney PM and JP Bennett, Jr. (2010). ALS spinal neurons show varied and reduced mtDNA gene copy numbers and increased mtDNA gene deletions. *Mol Neurodegener* 5:21.
- Vielhaber S, D Kunz, K Winkler, FR Wiedemann, E Kirches, H Feistner, HJ Heinze, CE Elger, W Schubert and WS Kunz. (2000). Mitochondrial DNA abnormalities in skeletal muscle of patients with sporadic amyotrophic lateral sclerosis. *Brain* 123 (Pt 7):1339–1348.
- Dimos JT, KT Rodolfa, KK Niakan, LM Weisenthal, H Mitsumoto, W Chung, GF Croft, G Saphier, R Leibel, et al. (2008). Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* 321:1218–1221.
- Zhang Z, S Almeida, Y Lu, AL Nishimura, L Peng, D Sun, B Wu, AM Karydas, MC Tartaglia, et al. (2013). Down-

- regulation of microRNA-9 in iPSC-derived neurons of FTD/ALS patients with TDP-43 mutations. *PLoS One* 8:e76055.
20. Egawa N, S Kitaoka, K Tsukita, M Naitoh, K Takahashi, T Yamamoto, F Adachi, T Kondo, K Okita, et al. (2012). Drug screening for ALS using patient-specific induced pluripotent stem cells. *Sci Transl Med* 4:145ra104.
 21. Bilican B, A Serio, SJ Barmada, AL Nishimura, GJ Sullivan, M Carrasco, HP Phatnani, CA Puddifoot, D Story, et al. (2012). Mutant induced pluripotent stem cell lines recapitulate aspects of TDP-43 proteinopathies and reveal cell-specific vulnerability. *Proc Natl Acad Sci U S A* 109:5803–5808.
 22. Boulting GL, E Kiskinis, GF Croft, MW Amoroso, DH Oakley, BJ Wainger, DJ Williams, DJ Kahler, M Yamaki, et al. (2011). A functionally characterized test set of human induced pluripotent stem cells. *Nat Biotechnol* 29:279–286.
 23. Kiskinis E, J Sandoe, LA Williams, GL Boulting, R Moccia, BJ Wainger, S Han, T Peng, S Thams, et al. (2014). Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. *Cell Stem Cell* 14:781–795.
 24. Sareen D, JG O'Rourke, P Meera, AK Muhammad, S Grant, M Simpkinson, S Bell, S Carmona, L Ornelas, et al. (2013). Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion. *Sci Transl Med* 5:208ra149.
 25. Almeida S, E Gascon, H Tran, HJ Chou, TF Gendron, S Degroot, AR Tapper, C Sellier, N Charlet-Berguerand, et al. (2013). Modeling key pathological features of frontotemporal dementia with C9ORF72 repeat expansion in iPSC-derived human neurons. *Acta Neuropathol* 126:385–399.
 26. Donnelly CJ, PW Zhang, JT Pham, AR Haeusler, NA Mistry, S Vidsensky, EL Daley, EM Poth, B Hoover, et al. (2013). RNA toxicity from the ALS/FTD C9ORF72 expansion is mitigated by antisense intervention. *Neuron* 80:415–428.
 27. Mitne-Neto M, M Machado-Costa, MC Marchetto, MH Bengtson, CA Joazeiro, H Tsuda, HJ Bellen, HC Silva, AS Oliveira, et al. (2011). Downregulation of VAPB expression in motor neurons derived from induced pluripotent stem cells of ALS8 patients. *Hum Mol Genet* 20:3642–3652.
 28. Burkhardt MF, FJ Martinez, S Wright, C Ramos, D Volfson, M Mason, J Barnes, V Dang, J Lievers, et al. (2013). A cellular model for sporadic ALS using patient-derived induced pluripotent stem cells. *Mol Cell Neurosci* 56:355–364.
 29. Ladd AC, PM Keeney, MM Govind and JP Bennett, Jr. (2014). Mitochondrial oxidative phosphorylation transcriptome alterations in human amyotrophic lateral sclerosis spinal cord and blood. *Neuromolecular Med* 16:714–726.
 30. Zhang J, I Khvorostov, JS Hong, Y Oktay, L Vergnes, E Nuebel, PN Wahjudi, K Setoguchi, G Wang, et al. (2011). UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells. *EMBO J* 30:4860–4873.
 31. Xu X, S Duan, F Yi, A Ocampo, GH Liu and JC Izpisua Belmonte. (2013). Mitochondrial regulation in pluripotent stem cells. *Cell Metab* 18:325–332.
 32. Zhang J, E Nuebel, GQ Daley, CM Koehler and MA Teitell. (2012). Metabolic regulation in pluripotent stem cells during reprogramming and self-renewal. *Cell Stem Cell* 11:589–595.
 33. Folmes CD, PP Dzeja, TJ Nelson and A Terzic. (2012). Metabolic plasticity in stem cell homeostasis and differentiation. *Cell Stem Cell* 11:596–606.
 34. Prigione A, B Fauler, R Lurz, H Lehrach and J Adjaye. (2010). The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. *Stem Cells* 28:721–733.
 35. St John JC, A Amaral, E Bowles, JF Oliveira, R Lloyd, M Freitas, HL Gray, CS Navara, G Oliveira, et al. (2006). The analysis of mitochondria and mitochondrial DNA in human embryonic stem cells. *Methods Mol Biol* 331:347–374.
 36. Facucho-Oliveira JM, J Alderson, EC Spikings, S Egginton and JC St John. (2007). Mitochondrial DNA replication during differentiation of murine embryonic stem cells. *J Cell Sci* 120:4025–4034.
 37. Birket MJ, AL Orr, AA Gerencser, DT Madden, C Vitelli, A Swistowski, MD Brand and X Zeng. (2011). A reduction in ATP demand and mitochondrial activity with neural differentiation of human embryonic stem cells. *J Cell Sci* 124:348–358.
 38. Cho YM, S Kwon, YK Pak, HW Seol, YM Choi, J Park do, KS Park and HK Lee. (2006). Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochem Biophys Res Commun* 348:1472–1478.
 39. Saretzki G, T Walter, S Atkinson, JF Passos, B Bareth, WN Keith, R Stewart, S Hoare, M Stojkovic, et al. (2008). Downregulation of multiple stress defense mechanisms during differentiation of human embryonic stem cells. *Stem Cells* 26:455–464.
 40. Zhang J, I Khvorostov, JS Hong, Y Oktay, L Vergnes, E Nuebel, PN Wahjudi, K Setoguchi, G Wang, et al. (2011). UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells. *EMBO J* 30:4860–4873.
 41. Clarke L and D van der Kooy. (2009). Low oxygen enhances primitive and definitive neural stem cell colony formation by inhibiting distinct cell death pathways. *Stem Cells* 27:1879–1886.
 42. Studer L, M Csete, SH Lee, N Kabbani, J Walikonis, B Wold and R McKay. (2000). Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J Neurosci* 20:7377–7383.
 43. Stacpoole SR, B Bilican, DJ Webber, A Luzhynskaya, XL He, A Compston, R Karadottir, RJ Franklin and S Chandran. (2011). Efficient derivation of NPCs, spinal motor neurons and midbrain dopaminergic neurons from hESCs at 3% oxygen. *Nat Protoc* 6:1229–1240.
 44. Horie N, K So, T Moriya, N Kitagawa, K Tsutsumi, I Nagata and K Shinohara. (2008). Effects of oxygen concentration on the proliferation and differentiation of mouse neural stem cells in vitro. *Cell Mol Neurobiol* 28:833–845.
 45. Dowey SN, X Huang, BK Chou, Z Ye and L Cheng. (2012). Generation of integration-free human induced pluripotent stem cells from postnatal blood mononuclear cells by plasmid vector expression. *Nat Protoc* 7:2013–2021.
 46. Li XJ, ZW Du, ED Zarnowska, M Pankratz, LO Hansen, RA Pearce and SC Zhang. (2005). Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 23:215–221.
 47. Singh Roy N, T Nakano, L Xuing, J Kang, M Nedergaard and SA Goldman. (2005). Enhancer-specified GFP-based FACS purification of human spinal motor neurons from embryonic stem cells. *Exp Neurol* 196:224–234.

48. Lee H, GA Shamy, Y Elkabetz, CM Schofield, NL Harrison, G Panagiotakos, ND Socci, V Tabar and L Studer. (2007). Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. *Stem Cells* 25:1931–1939.
49. Zeng H, M Guo, K Martins-Taylor, X Wang, Z Zhang, JW Park, S Zhan, MS Kronenberg, A Lichtler, et al. (2010). Specification of region-specific neurons including forebrain glutamatergic neurons from human induced pluripotent stem cells. *PLoS One* 5:e11853.
50. Marchetto MC, AR Muotri, Y Mu, AM Smith, GG Cezar and FH Gage. (2008). Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. *Cell Stem Cell* 3:649–657.
51. Amoroso MW, GF Croft, DJ Williams, S O'Keeffe, MA Carrasco, AR Davis, L Roybon, DH Oakley, T Maniatis, CE Henderson and H Wichterle. (2013). Accelerated high-yield generation of limb-innervating motor neurons from human stem cells. *J Neurosci* 33:574–586.
52. Wang ZB, X Zhang and XJ Li. (2013). Recapitulation of spinal motor neuron-specific disease phenotypes in a human cell model of spinal muscular atrophy. *Cell Res* 23: 378–393.
53. Rice AC, PM Keeney, NK Algarzae, AC Ladd, RR Thomas and JP Bennett, Jr. (2014). Mitochondrial DNA copy numbers in pyramidal neurons are decreased and mitochondrial biogenesis transcriptome signaling is disrupted in Alzheimer's disease hippocampi. *J Alzheimers Dis* 40: 319–330.
54. Cronin-Furman EN, MK Borland, KE Bergquist, JP Bennett, Jr. and PA Trimmer. (2013). Mitochondrial quality, dynamics and functional capacity in Parkinson's disease cybrid cell lines selected for Lewy body expression. *Mol Neurodegener* 8:6.
55. Arber S, B Han, M Mendelsohn, M Smith, TM Jessell and S Sockanathan. (1999). Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* 23:659–674.
56. Qu Q, D Li, KR Louis, X Li, H Yang, Q Sun, SR Crandall, S Tsang, J Zhou, CL Cox, J Cheng and F Wang. (2014). High-efficiency motor neuron differentiation from human pluripotent stem cells and the function of Islet-1. *Nat Commun* 5:3449.
57. Chou BK, P Mali, X Huang, Z Ye, SN Dowey, LM Resar, C Zou, YA Zhang, J Tong and L Cheng. (2011). Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res* 21:518–529.
58. Kosaka Y, Y Akimoto, K Yokozawa, A Obinata and H Hirano. (2004). Localization of HB9 homeodomain protein and characterization of its nuclear localization signal during chick embryonic skin development. *Histochem Cell Biol* 122:237–247.
59. Leotta CG, C Federico, MV Brundo, S Tosi and S Saccone. (2014). HLXB9 gene expression, and nuclear location during in vitro neuronal differentiation in the SK-N-BE neuroblastoma cell line. *PLoS One* 9:e105481.

Address correspondence to:

Dr. James P. Bennett, Jr.
Neurodegeneration Therapeutics, Inc.
 3050A Berkmar Drive
 Charlottesville, VA 22902

E-mail: jpb8u@me.com

Dr. Laura C. O'Brien
Department of Physiology and Biophysics
Virginia Commonwealth University
 1101 East Marshall Street
 PO Box 980551
 Richmond, VA 23298-0678

E-mail: obrienl@vcu.edu

Received for publication February 24, 2015

Accepted after revision April 4, 2015

Prepublished on Liebert Instant Online April 19, 2015