Published in final edited form as:

J Proteome Res. 2008 January ; 7(1): 328–338. doi:10.1021/pr070300g.

A quantitative proteomic analysis of mitochondrial participation in P19 cell neuronal differentiation

Jermel Watkins, Siddhartha Basu, and Daniel F. Bogenhagen*

Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, NY 11794-8651

Abstract

A quantitative proteomic analysis of changes in protein expression accompanying the differentiation of P19 mouse embryonal carcinoma cells into neuron-like cells using isobaric tag technology coupled with LC-MS/MS revealed protein changes reflecting withdrawal from the cell cycle accompanied by a dynamic reorganization of the cytoskeleton and an up-regulation of mitochondrial biogenesis. Further study of quantitative changes in abundance of individual proteins in a purified mitochondrial fraction showed that most mitochondrial proteins increased significantly in abundance. A set of chaperone proteins did not participate in this increase, suggesting that neuron-like cells are relatively deficient in mitochondrial chaperones. We developed a procedure to account for differences in recovery of mitochondrial proteins during purification of organelles from distinct cell or tissue sources. Proteomic data supported by RT-PCR analysis suggests that enhanced mitochondrial biogenesis during neuronal differentiation may reflect a large increase in expression of PGC-1 α combined with down-regulation of its negative regulator, p160 Mybbp1a.

Keywords

embryonal carcinoma; mitochondrial biogenesis; quantitative proteomics; cell cycle; neurogenesis

Introduction

The P19 embryonal carcinoma cell model has been adopted by many laboratories studying neuronal differentiation^{1,2}. P19 cells are undifferentiated, divide rapidly, and, like stem cells, are multi-potential. In the presence of varied inducers, P19 cells can differentiate in vitro into derivatives of all three germ layers, endoderm, mesoderm, and ectoderm². When these cells are injected into mouse blastocysts, they differentiate into a broad range of cell types³. P19 cells can be induced to withdraw from the cell cycle and to differentiate along the neuronal pathway with the addition of a vitamin A derivative, all-trans-retinoic acid (ATRA), combined with altered culture conditions. The cells differentiate into neurons, glia and fibroblast-like cells in much the same way as neural stem cells are thought to develop through radial glia as common progenitors of both neurons and glia^{1,4,5}. P19 neuron-like cells (NLCs) in culture have small cell bodies with long processes similar to the axons and dendrites of cultured brain cells.

Previous studies in other labs have investigated the global proteomic changes of neuronal differentiation. A study of the in vitro differentiation of adult rat hippocampal neural stem cells

*To whom correspondence should be addressed: Daniel F. Bogenhagen, Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, NY 11794-8651. Telephone: 631-444-3068. Fax: 631-444-3218. email: dan@pharm.sunysb.edu. AUTHOR EMAIL ADDRESS dan@pharm.stonybrook.edu

by two-dimensional electrophoresis (2DE), resulted in the identification of 367 regulated spots, of which 128 could be identified, although many of these proteins were isoforms⁶. A similar 2DE analysis of protein abundance in P19 cells undergoing neuronal development revealed 500 abundant polypeptides, 17 of which were regulated⁷. However, this early study did not identify many of these proteins. A subsequent study of P19 cell neural differentiation identified only 28 differentially expressed proteins⁸. A distinct set of proteomic changes is observed when P19 cells are induced to differentiate along the cardiomyocyte lineage⁹. Approximately 1200 proteins were studied via 2DE analysis of E14 cells during differentiation into dopaminergic neurons, but only 23 spots were expressed differentially in a consistent pattern¹⁰. A recent 2DE-proteome analysis of proliferating and differentiating human neuronal ReNcell VM stem cells identified 402 spots with differential expression of 49 protein spots¹¹.

We compared the protein profile of dividing P19 cells with that of NLCs using a relatively new isobaric tagged proteomic approach that incorporates multidimensional chromatography coupled with MS/MS, as an alternative to conventional 2DE. The use of this isobaric tag method, which has been stimulated by the commercialization of iTRAQ[®] reagents (Applied Biosystems), enables complex sets of proteins to be compared quantitatively using mass spectrometry without 2D gels¹².

The pattern of protein expression in our study shows dynamic changes that accompany differentiation into NLCs. We identified multiple polypeptides for over 500 proteins, 182 of which were observed consistently in replicate experiments using total cell lysates. Statistical analysis of the data revealed 119 (~65%) of these 182 proteins significantly increased or decreased in abundance during neuronal differentiation. Mitochondrial purification allowed for a deeper coverage of mitochondrial proteins in response to NLC differentiation. A total of 59 mitochondrial proteins were observed consistently in replicate experiments. This work provides the most extensive quantitative analysis to date of proteomic changes accompanying neuronal differentiation along with a novel approach to monitor changes in protein abundance in a subcellular fraction.

Experimental Section

Cell culture

P19 cells were maintained in complete medium (alpha modified MEM, 2.5% fetal bovine serum (FBS), 7.5% bovine calf serum (BCS), and 1% penicillin-streptomycin) at 37°C in a 5% CO₂ atmosphere. The cultures were passaged every 2–3 days at a 1:20 to 1:30 dilution. P19 cells in exponential growth were trypsinized and transferred to siliconized glass dishes at a density of 10⁵ cells per ml of ATRA induction medium (alpha modified MEM, 2.5% fetal bovine serum (FBS) and penicillin-streptomycin containing 500 nM ATRA), where the cells aggregated for 4 days with a medium change on day 2. On day 4, the aggregates were trypsinized and cells were plated at a density of 2×10⁷ per 10 cm poly-L-lysine treated tissue culture dish in complete medium (alpha modified MEM, 2.5% fetal bovine serum (FBS), 7.5% bovine calf serum (BCS), and penicillin-streptomycin). From day 5 to 7, the complete medium was supplemented with cytosine arabinoside (AraC) to kill fibroblast-like cells that did not respond to ATRA induction. Cells were maintained in NB/B27 (Neurobasal-A medium with the B27 supplement) from days 7 to 11 when neuron-like cells were harvested.

Total cell protein preparation

P19 and ATRA-induced neuron-like cells (NLC) were lysed in an extraction buffer containing 9 M urea, 2.5 M thiourea, 5% CHAPS, 0.2% ampholytes, 2 mM DTT. The proteins samples

were diluted 1 to 5 in distilled water before protein was determined by Bradford assay, with diluted lysis buffer serving as the blank and BSA as the quantitative protein standard.

Mitochondrial purification

All handling was at 4°C, except for the nuclease treatment step. All buffers contained the protease inhibitors PMSF, Pepstatin A, Leupeptin and E64. 30 plates of P19 day 0 or 60 plates of NLC day 11 cells were washed off dishes and pelleted at $900 \times g$ for 5 min, resuspended in TD buffer (135 mM NaCl, 5 mM KCl, 25 mM Tris-HCl, pH 7.6, 0.7 mM Na₂HPO₄), and repelleted by spinning at $900 \times g$ for 5 min. The cells were resuspended in 9 ml of hypotonic buffer (20 mM Hepes pH 8, 5 mM KCl, 1.5 mM MgCl₂, 2 mM DTT) and homogenized with 10–15 strokes of a glass dounce homogenizer. 6 ml of 2.5X MSH (525 mM mannitol, 175 mM sucrose, 50 mM Hepes, pH 8, 5 mM EDTA), were added per 9 ml. Nuclei were removed by two successive centrifugation steps for 5 min at 1500 g. The post nuclear supernatant (PNS) containing the mitochondria was centrifuged for 15 min at 13,000 rpm to pellet the crude mitochondria (CM).

Mitochondria were resuspended in 3 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 20 mM Hepes, pH 8, 2 mM EDTA) containing 60 mM KCl, 1 mM K₂HPO₄, 10 mM MgCl₂, 1 mM ADP, 5 mM Na-glutamate, 5 mM Na-malate, 60 µl of 2 mg/ml RNase-free DNase I (Sigma Type II; 2000 U/mg) and of 0.05 U/ml Benzonase (adapted from ¹³). The crude mitochondria were incubated at 37 °C for 15 min. EDTA was added after the incubation to chelate Mg. The crude mitochondria were then layered above 10 ml of 0.8 M sucrose, 20 mM Hepes, pH 8, 1 mM EDTA pelleted at 4 °C at 13,000 rpm for 15 min, and resuspended in 0.5 ml 1X MSH containing 50 µg/ml BSA.

Crude mitochondria were resuspended in 0.5 ml 1X MSH and layered on a 0.8 M/1.5 M preformed sucrose step gradient in a Beckman SW41 tube. Gradients were centrifuged at 23,000 rpm at 4 °C for 30 min. Mitochondria, which sediment to the 0.8 M/1.5 M sucrose interface, were collected with a sterile Pasteur pipette. The mitochondria were diluted with 4 volumes of 1X MSH containing 50 µg/ml BSA, repelleted at 13000 rpm and resuspended in 0.5 ml 1X MSH. Protein content was determined using the Bradford dye binding assay as above. 50 to 100 µg aliquots of mitochondria were pelleted and frozen at –80°C.

Proteolysis and iTRAQ® reagent labeling

50 µg samples of protein from undifferentiated P19 cells and day 11 NLC were precipitated with acetone for 1 h at –20 °C and collected by centrifugation at 14,000 rpm. The pellet of protein from each sample was then resuspended in 30 µl of dissolution buffer (iTRAQ® reagent, Applied Biosystems) containing 0.05% SDS final concentration. Each 50 µg sample of protein was reduced, alkylated, digested with trypsin and labeled with the isobaric reagents according to the manufacturer's protocol. Differentially labeled peptide samples were pooled and subjected to strong cation exchange chromatography fractionation on a Waters 625 HPLC, equipped with a 200 × 4.6 mm, 5 µm PolyLC Polysulfoethyl A column (The Nest Group, Inc.). Peptides were eluted with a gradient of 0 to 0.4 M KCl in 5 mM KH₂PO₄, 25% acetonitrile, pH 3.0. The resulting peptide fractions were lyophilized under vacuum to 50 µl volume.

Protein mass spectrometric analysis

The HPLC-separated peptides were run on a nano-LC/ESI/MSMS API Qstar Pulsar i quadrupole-time of flight (Q-TOF) tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) interfaced with an LC Packings Nano LC System which included an Ultimate micro pump, a FAMOS micro auto-sampler and a Switchos micro column switch module (LC Packings, Sunnyvale, CA). The peptide separation was carried out on a Waters NanoEase Symmetry column (3.5 µm C18, 75 µm ID × 15 cm) with a 60 min linear gradient

from 2% to 30% solvent B. Solvent A was 2% acetonitrile (ACN) with 0.1% formic acid and solvent B was 88% ACN, 10% isopropanol with 0.1% formic acid. The flow rate was 300 nL/min. A 300 μ m id $\times$ 5 mm μ -Pre-column Cartridge (LC Packings) was used for sample desalting and pre-concentration.

Immunoblot Analysis

Total proteins (3 μ g) were separated by SDS-PAGE and electrophoretically transferred to PVDF membrane. The membranes were blocked with 5% non-fat dried milk in Tween-PBS (1x PBS containing 0.5% Tween 20) for 1 hr at room temperature. Following blocking, membranes were incubated with the primary antibodies as follows (source and dilution factor in parentheses): monoclonal antibodies Oct 4 (Santa Cruz, 1:1000), β 3 tubulin (R&D Systems, 1:1000), GAPDH (RDI, 1:5000), complex I, II and III (MitoSciences MSC101 vs NDUFA9 1:1000, MSC 204 vs SDHA 1:10,000 and MSC303 vs UQCRC1 1:2000, respectively), Complex IV (Calbiochem, Cox I 1:1000), Porin (Calbiochem, 1:1000), AIF (Santa Cruz, 1:200), prohibitin (Neomarkers, 1:500); polyclonal antibodies, goat anti-NRF1 (gift from R. Scarpulla, 1:1000) or rabbit anti-HSP60 (Santa Cruz, 1:500), MnSOD (RDI, 1:1000), PGC-1 α (Chemicon, 1:500) and GFAP (DAKO, 1:1000). Primary antibodies were incubated overnight at room temperature. Secondary anti-mouse or anti-rabbit antibodies were either fluorescein-modified (Amersham Life Science, 1:2500) or phosphatase-labeled (KPL, 1:5000) and were incubated for 1 hr at room temperature. Between the primary and secondary antibodies or before detection, membranes were washed three times for 5 min each in Tween-PBS.

Flow Cytometry

β 3-tubulin and glial fibrillary acidic protein (GFAP) expression in P19 and NLCs were measured using a Becton Dickinson FACSCAN analyzer. 1×10^6 cells in suspension were dispersed and centrifuged at 200 g for 5 min. The supernatant was removed and the cells were washed with PBS twice then centrifuged again. The PBS was removed and the cells were fixed in 4% PFA in PBS for 30 min at room temperature. The cells were centrifuged at 200 g for 5 min to remove excess PFA and washed twice with PBS. Permeabilization buffer (0.25 % Triton X-100 in PBS) was added and the cells incubated at room temperature for 30 min. After washing twice with PBS, the cells were blocked for 30 min at room temperature in 5% normal swine serum, 0.25 % Triton X-100 in PBS. The cells were washed with PBS twice then centrifuged again and incubated in primary antibody (β 3-tubulin (R&D Systems, 1:1000) or GFAP (DAKO, 1:500)) at room temperature for 2h. The cells were washed with PBS twice then centrifuged again and incubated in secondary antibody (Alexa fluor 488 goat anti-mouse IgG or Alexa fluor 488 goat anti-rabbit IgG; both Molecular Probes reagents) at 1:10,000 dilution at room temperature for 1h. The cells were washed with PBS twice then centrifuged again and resuspended in 500 μ l of PBS for analysis.

Nucleic Acid Extraction and Analysis

Total DNA was purified from cells at days 0, 4 and 11 of the differentiation program using a standard Proteinase K/phenol extraction method¹⁴. DNA was quantified by UV absorbance and digested with EcoRI. Fragments were separated by agarose gel electrophoresis and Southern blotted to Hybond N+ membrane (Waters). Specific fragments were detected by simultaneous hybridization with random-primer labeled ³²P probes for mouse mtDNA residues 1751–3804 and mouse rDNA (BK000964) residues 10198–10743, which hybridize to fragments of 2053 and 6606 base pairs, respectively. Total RNA from P19 day 0 and day 11 NLCs was extracted by homogenization in TRIzol® reagent (Invitrogen). After mixing with chloroform the lysate was centrifuged at 12,000 $\times$ g for 15 min at 4° C. RNA was precipitated from the aqueous phase with isopropyl alcohol and dissolved in ribonuclease-free water. RNA

was quantified and first strand cDNA was generated from 5 µg RNA template using SuperscriptTM III Reverse Transcriptase (Invitrogen). The cDNA was stored at -20°C.

Quantitative RTPCR

Quantitative PCR was carried out using the Qiagen QuantiTect SyBr Green PCR Kit and a MJ Research DNA Engine Opticon 2 PCR machine. The gene expression of 3-phosphoglycerate dehydrogenase (Phgdh) was used as an internal control to normalize the results obtained for genes of interest with respect to mitochondrial biogenesis at neuronal differentiation time points days 0, 4 and 11. Equal amounts of starting cDNA template for each time point were added to PCR reactions with the gene of interest in neuronal differentiation as follows:

Phgdh (CCTCACCAGTGCCTTCTCTC and TCTCTCAGAAGGCCGACAAT),

NRF1 (GCACCTTTGGAGAATGTGGT and CTGAGCCTGGGTCATTTTGT),

PGC-1 α (AAGGTCCCCAGGCAGTAGAT and GCGGTATTCATCCCTCTTGA),

PGC1 β (TTCCCAGAACTGGATGAAGG and TCTGGAAGTGAAGGCTGGTCT),

PRC-1 (TTCAGTGGTCAGATGCTTGC and ACCAGCTCACTCAAGGAGGA),

Mybbp1a (GGAGATCTTGCTGTCTTGC and TCATCAGTGACCACCACGTT), and

TFAM (CTTCAACCACCACCACTG and TCTCTCCTCCATGCGTTCTT). The cDNA was heated at 95 °C for 15 min before PCR consisting of 40 cycles of 30 s at 94 °C, 30s at 60 °C and 1 minute at 72. Quantitative PCR products were analyzed using Opticon Monitor Analysis Software Version 2.02.

Protein identification and quantification

Four experiments were performed comparing undifferentiated P19 and day 11 ATRA-treated NLC. Mass spectrometer data files in wiff format were processed with Pro-Quant software (Applied Biosystems) for quantification and identification of iTRAQ[®] labeled peptides by searching against an interrogator database constructed from GenBank NR release 19. Carboxamidomethylcysteine was selected as a fixed modification and methionine oxidation was permitted. One missed trypsin cleavage was allowed. Mass tolerance was set to 0.15 Da in MS mode and 0.1 Da in MS/MS analysis. To attempt to assess the frequency of false positives, we repeated data analysis with a mock database containing reversed-sequences. We saw occasional apparent peptide hits, as might be expected by chance, but never observed a reversed protein with more than one peptide hit. Pro-Group software (Applied Biosystems) was utilized to search results from Pro-Quant to determine protein ID confidence, description of expression differences and detection of protein isoforms. This software combines peptide hits from several fractions that together comprise a complete experiment to determine the number of peptides identified for a particular protein. Results were then imported into an Excel file and truncated to a list of peptides identified with confidence > 90%. Each individual peptide was linked to an independent mass tag ratio. The resulting peptides that identified a single protein were used to calculate a new averaged mass tag ratio for that protein. These averages characteristically had a standard deviation of less than $\pm 17.6\%$. Complete data sets from independent experiments were compared to one another and a final average ratio for each protein identified in the data set was calculated. Only those proteins identified in at least two independent experiments by peptides with > 90% confidence were interpreted in this analysis. All of the identified proteins are then submitted to the MiGenes database (<http://www.pharm.stonybrook.edu/migenes/>) using the batch search mode. MiGenes is a

relational database of mitochondrially-related gene products that collates information on all proteins with GO annotations identifying them as mitochondrial¹⁵.

Statistical analysis

Each protein was characterized by a ratio of mass tag signal intensity reflecting the relative abundance of the protein in neuronal-like cells relative to undifferentiated cells. These abundance ratios were converted to a $\log_{10}$ scale to overcome the lack of symmetry in raw protein abundance ratios and to calculate a mean, μ , and standard deviation, σ , for the log protein abundance. The observed mean of the relative protein abundance was $\mu = 1.10$ and the log of this value was 0.04. The standard deviation of the log relative protein abundance was $\sigma = 0.29$. In order to determine the biological significance of relative protein expression data, we formulated the null hypothesis H_0 that all protein abundance data fit a normal distribution characterized by the population mean $= \mu$. The hypothesis of interest is whether the relative $\log_{10}$ abundance ratio of a protein $X = \mu$, or if the alternative hypothesis is correct, that $X \neq \mu$. Using a two-tailed t test, we defined criteria to determine whether the abundance of a protein was significantly distinct from the normal distribution characterized by the population mean, μ . We consider a protein to be significantly up or down-regulated if the p-value of its abundance ratio relative to the mean was ≤ 0.05 .

Results

Homogeneous differentiation of P19 cells to NLC

We chose the P19 cell line for our studies as a system expected to be capable of homogeneous differentiation. The non-induced, replicating P19 cells express the pluripotent stem cell marker Oct4, which is quickly lost as cells differentiate (Figure 1A)¹⁶. Upon ATRA induction, cell aggregation, and a total of 11 days of culture, including growth in supplemented neurobasal medium from days 7–11, P19 cells differentiate to NLC that express the mature neuronal marker β 3-tubulin (Figure 1A)¹⁷. Since neurons and glia share a common neuroectodermal progenitor, we sought to investigate whether our differentiated cultures might contain a mix of neurons and glia. Parallel immunoblot experiments failed to detect glial fibrillary acidic protein (GFAP) as a glial marker under conditions that readily detected this protein in a mouse brain homogenate (Figure 1B)¹⁸. Similarly, flow cytometry of NLC detected homogeneous staining with β 3-tubulin and no detectable sub-population expressing GFAP (Figure 1C). We conclude that the ATRA induction protocol results in a fairly uniform population of cells.

Proteomic analysis of total cell lysates

Total cell protein was prepared from undifferentiated P19 cells and differentiated NLC for quantitative multidimensional LC-MS/MS analysis of isobaric tagged peptides as described in Materials and Methods. In four replicate experiments, we obtained quantitative data on multiple peptides for over 500 proteins. 182 proteins were identified in at least two experiments by peptides with >90% confidence (Supplementary Table 1). In over 90% of the cases, multiple qualifying peptides were observed in each experiment. Since the isobaric tag approach to quantitative proteomics using iTRAQ[®] reagents is a new technology, we examined the data to determine their reliability. First, we observed that multiple peptides in replicate experiments converged to an average characterized by a standard deviation of less than $\pm 17\%$. Second, in several instances, we obtained quantitative data on proteins that are known to be components of larger complexes. These proteins generally increased or decreased in parallel with their binding partners. For example, two subunits of Na⁺/K⁺ ATPase were increased 3.26 and 2.64-fold while two subunits of mitochondrial F1 ATPase were increased 1.86 and 1.77-fold (Supplementary Table 1). Finally, in total cell lysates, 19 ribosomal proteins all decreased to 70 $\pm$ 14% of control abundance during differentiation (Supplementary Table 1). These results

provide additional confidence in the precision of protein abundance measurements using the isobaric tag method.

119 of the 182 proteins identified, approximately 65%, were differentially expressed at the $p=0.05$ confidence level, with 53 of these up-regulated and 66 down-regulated (Figure 2). This result indicates that neuronal differentiation entails a major re-organization of cell structure and function. We grouped the proteins listed in Supplementary Table 1 according to gene ontology classifications to reveal a number of categories that showed significant disparities in the numbers of up- and down-regulated proteins, as shown in Table 1. The most consistent patterns of alteration in protein abundance reflect withdrawal of cells from the cell cycle, reorganization of the cytoskeleton to adopt a neuronal morphology and a shift in metabolism involving enhanced mitochondrial biogenesis.

The withdrawal of P19 cells from the cell cycle may be expected to entail remarkable changes, since the undifferentiated cells grow very rapidly with a generation time of approximately 13 hours. However, we observed relatively few proteins with GO annotations involved in roles in cell cycle regulation, no doubt reflecting the fact that most regulatory proteins are of low abundance and difficult to capture in a quantitative proteomic screen of total cell proteins. The two cell cycle-related proteins we observed were among the most significantly down-regulated proteins in the data set: SET translocation (SET), an oncoprotein involved in cell cycle regulation and chromatin remodeling^{19–23}, and prothymosin alpha, a highly abundant oncoprotein necessary for cell cycle progression and proliferation²⁴.

The non-proliferative state is characterized by a decrease in protein synthesis⁸. While neuronal differentiation requires new synthesis of specific proteins, as discussed below, the overall quantity of new protein required is evidently less than that required for the continued rapid doubling of the undifferentiated cell population. Thus, Table 1 shows a significant decrease in abundance of several translation initiation factors as well as ribosomal proteins. Overall, 27 of 31 proteins annotated as having a role in protein biosynthesis showed a significant decrease in abundance, while only one, ribophorin I, increased. Ribophorin I is not a core component of the protein synthesis machinery, but is an endoplasmic reticulum protein involved in protein glycosylation. The modest up-regulation of ribophorin I may reflect the increased synthesis and ER processing of membrane proteins in NLCs.

We quantified significant increases in abundance of ten proteins involved in neurogenesis during the differentiation of P19 cells to NLC including Basp1, brain abundant membrane attached signal protein 1 (also known as NAP22 and CAP23), a downstream effector of neuregulins²⁵ and a functionally related protein, GAP-43²⁶. These proteins function with MARCKS, another protein we found to be significantly up-regulated, in the remodeling of the actin cytoskeleton²⁷. Several members of a family of dihydropyrimidinase-like proteins were also up-regulated during neuronal differentiation. These proteins, which are also known as collapsing response mediator proteins, are thought to be involved in axonal growth, neuronal differentiation, and one of them, dihydropyrimidinase-like 5, is reported to co-localize with mitochondria^{28, 29}. These examples illustrate the intimate co-regulation of markers of neurogenesis with the remodeling of the cytoskeleton in NLC (Table 1). We documented significant increases in the abundance of 16 other cytoskeletal proteins in this study. These proteins play clear functional and/or regulatory roles in axonal outgrowth in developing neurons, extending other recent work by Inberg et al.³⁰.

Enhanced mitochondrial biogenesis in NLC

The results in Table 1 and Supplementary Table 1 also document increased levels of a number of proteins involved in mitochondrial energy metabolism, including TCA cycle proteins and components of the mitochondrial respiratory complexes. To obtain a deeper coverage of

mitochondrial proteins, we repeated the iTRAQ[®] analysis with mitochondria purified from undifferentiated cells and NLC. Western blot analyses to detect representative markers for mitochondrial and cytosolic fractions confirmed that the mitochondria were highly enriched (Figure 3). We performed three experiments using isobaric tags to compare proteins in independent preparations of mitochondria from undifferentiated P19 cells and NLC obtained using the 11-day in vitro differentiation protocol. These experiments provided quantitative data on 129 mitochondrial proteins, but our analysis was confined to 59 proteins for which we obtained multiple peptides in repeat experiments (Supplementary Table 2).

The raw data on peptide ratios obtained using purified mitochondrial fractions consistently showed a lower degree of up-regulation than we observed in total cell extracts prepared by homogenizing the cell pellets directly in CHAPS/urea buffer. This discrepancy is illustrated by analyzing the behavior of 14 proteins that we observed in both total cell homogenates and purified mitochondrial preparations. These 14 proteins showed a range of 33 to 89% greater relative abundance in total homogenates than in purified mitochondria (Table 2). What is the source of this disparity? Since cell fractionation involves increased handling, we consider that the total cell data most likely provides the best estimate of the absolute change in relative abundance of these proteins. Apparently, there is either a greater loss of mitochondria during purification from NLCs or that there may be a greater representation of non-mitochondrial proteins in the mitochondrial fraction from NLC than from undifferentiated P19 cells. To evaluate the latter possibility, we analyzed the iTRAQ[®] abundance data on all non-mitochondrial proteins identified in the mitochondrial fraction that were also observed in the total cell lysates. 38 proteins fitting these criteria are listed in Supplementary Table 3. These were increased in abundance in the mitochondrial fraction by an average of 2.06-fold. This trend is led by a set of 15 proteins annotated as cytoskeletal or neurogenesis proteins that were increased in abundance in the mitochondrial fraction by an average factor of 3.3-fold. These proteins could simply represent contaminants, but their selective nature is consistent with a remodeling of the contacts of the outer mitochondrial membrane with other cellular components during neurogenesis. For example, it appears that neurogenesis involves increased association of mitochondria with such proteins as tubulin, MARCKS, GAP43 and collapsing response mediator (dihydropyrimidinase-like) protein family members (Supplementary Table 3) possibly to aid in transport of mitochondria to neuronal processes. As a second example, we note that although the abundance of proteins in the glycolysis pathway is decreased during neurogenesis, this trend is reversed for two proteins, aldolase 1 and pyruvate kinase 3, that showed a slightly increased abundance in the mitochondrial fraction in NLCs. The docking of proteins on the outer mitochondrial membrane is of increasing interest in cell signaling and will be an important subject for future research.

For our present purpose of understanding changes in the abundance of known mitochondrial proteins, we sought to develop a strategy to normalize the data in Supplementary Figure 2 to account for the discrepancy in relative protein abundance in total lysates and purified mitochondria. We considered that one way to accomplish this would be to develop an internal standard. However, as an alternative to a single standard, we decided to use a set of internal standards, the group of 14 mitochondrial proteins observed in both total cell lysates and the mitochondrial fraction. The data on relative abundance of these 14 proteins in both the total cell lysate and the mitochondrial fraction is shown in Table 2. These are all relatively abundant proteins representing chaperones, membrane carriers, TCA cycle enzymes and members respiratory complexes. They are contained in outer membrane, inner membrane and matrix fractions. It is evident that the total cell data show a greater increase in abundance with differentiation than the corresponding data from the mitochondrial fraction, with an average increase of 61 $\pm$ 15%. Thus, we normalized the raw data on relative abundance of each of the 59 mitochondrial proteins by multiplying by a factor of 1.61 to reflect the apparent selective loss or dilution (with other proteins) of these 14 internal standard proteins (Supplementary

Table 2). This normalization procedure brings the relative abundance of the internal standard proteins into line with their abundance in the total cell lysate and provides a basis to consider the behavior of the less abundant mitochondrial proteins that were not observed in the total cell lysate. This is a generally applicable method to deal with systematic changes in recovery of subcellular organelles in proteomic research.

Our detailed analysis of the normalized data on abundance of mitochondrial proteins is shown in Figure 4, which illustrates the relative abundance of most of the mitochondrial proteins listed in Supplementary Table 2, organized according to the functional classification of proteins as components of the electron transport chain, ATP synthetase, the TCA cycle, transporters and chaperones. This analysis shows that neuronal differentiation is accompanied by a broadly-based up-regulation of mitochondrial proteins. However, some individual classes, such as the chaperone proteins and components of the mitochondrial protein import machinery did not increase in parallel with other mitochondrial proteins. In order to validate the quantitative changes in mitochondrial proteins observed using LC-MS/MS, immunoblots were performed to monitor individual proteins in respiratory complexes as well as transporters, chaperones and a scavenger of reactive oxygen species (Figure 5). Components of the electron transport chain and cytochrome C were all up-regulated, although only a relatively minor increase was observed for complex II, SDHA. This is consistent with the increased reliance of neurons on glycolysis, which provides reducing equivalents to the electron transport chain at the level of NADH through complex I. Relatively little change was observed for ETFB (Supplementary Table 2), which participates in the delivery of electrons at the level of FADH₂ to respiratory complex II, and for the SDHA subunit of complex II itself (Figure 5). We did not observe peptides derived from complex II in our proteomic analysis. The marked increase observed for the superoxide scavenger MnSOD in Figure 5 may reflect the participation of PGC-1 α in the neuronal development program, as discussed below.

Two results obtained in this study suggested a mechanism for the enhanced mitochondrial biogenesis that we observed. First, differentiation resulted in increased abundance of a diverse collection of mitochondrial proteins, suggesting a broadly-based regulatory switch. This is also indicated by a 2.1-fold increase in mtDNA copy number measured relative to nuclear ribosomal DNA (rDNA) during the 11-day developmental program (Figure 6). Second, one of the most significantly down-regulated cellular proteins we identified was the protein Mybbp1a, also known as p160 myb binding protein 1. Fan et al.³¹ showed that binding of Mybbp1a to PGC-1 α can down-regulate mitochondrial respiration. We reasoned that down-regulation of Mybbp1a during neuronal differentiation might relieve repression of PGC-1 α . Since PGC-1 α stimulates NRF-1, which up-regulates expression of numerous nuclear genes encoding mitochondrial proteins³², decreased Mybbp1a results in enhanced mitochondrial biogenesis. Unfortunately, protein sequencing did not identify PGC-1 α or NRF-1. Therefore, we monitored expression of these factors at either the RNA level using quantitative RT-PCR or at the protein level using immunoblots (Table 3 and Figure 7). In addition to studying the mRNA abundance of PGC-1 α we monitored two other potential regulators of mitochondrial respiration, PGC-1 β and PRC-1 from the same family. Undifferentiated cells contain an abundance of PRC-1 mRNA, along with a lower quantity of PGC-1 β , while PGC-1 α is, rather surprisingly, very rare. In contrast, in NLCs the abundance of PGC-1 α mRNA is increased approximately 150-fold while PGC-1 β mRNA increases slightly and PRC1 mRNA levels are similar to those of undifferentiated cells. The increased PGC-1 α mRNA level is also reflected at the protein level (Figure 7). Interestingly, these changes in levels of mRNAs encoding PGC1-family co-activators do not result in a marked increase in mRNA for either NRF-1 or TFAM (data not shown). We suggest that this increase in PGC-1 α , coupled with relief of inhibition by Mybbp1a, promotes overall mitochondrial biogenesis, with selectively greater effects on some proteins, such as MnSOD, than on others.

Discussion

Quantitative proteomic analysis of a neuronal differentiation pathway

The P19 embryonal carcinoma cell system has proven to be a very valuable model for studying neuronal differentiation^{1, 33, 34}. This process provides a clear illustration of the concept that proliferation and differentiation are mutually incompatible cell states. Upon exposure to ATRA and altered cell culture conditions, P19 cells engage in two to three rounds of cell division, then withdraw from the cell cycle and undergo a fairly uniform transition to NLCs through a differentiation program characterized by decreased expression of Oct4 and other markers of proliferative cells coincident with the acquisition of neuronal markers, such as β 3-tubulin (Figure 1). The P19-derived NLCs express an array of other neuronal markers and exhibit synaptic structures and electrophysiological characteristics of neurons¹.

The P19 cell neuronal program has been studied previously at both the RNA^{35, 36} and protein levels⁷. The previous 2DE study by Ray and Gottlieb⁷ did not reveal any down-regulated proteins, nor did they indicate the identities of the 17 up-regulated proteins observed. Advances in 2DE technology and peptide mass spectrometry made after the publication of this early study, including the use of DIGE as an internally standardized quantitative method³⁷, would presumably permit a more complete analysis of proteomic changes. However, we elected to use an isobaric tag proteomic method to quantify changes in protein abundance. Our study documenting the relative abundance of over 200 proteins represents a major advance in understanding this neuronal differentiation pathway, although this still represents only a sampling of the full proteome. We found that a substantial fraction of cellular proteins vary in abundance during neuronal differentiation. Significant decreases were observed for proteins involved in the cell cycle, nuclear DNA synthesis and translation, while marked increases were observed for proteins involved in remodeling the cytoskeleton and related activities required for neurogenesis, as noted above.

Modulation of bioenergetic pathways and mitochondrial function during neuronal differentiation

It is well known that neurons use glucose as their major energy source. The enhanced mitochondrial oxidative phosphorylation in NLCs involves induction of the aspartate-glutamate carrier aralar (Slc25a12; Supplementary Table 2) to assist in transfer of NADH reducing equivalents to the mitochondrial matrix through the malate-aspartate shuttle³⁸. The primary structure of aralar differs from that of the majority of mitochondrial carriers in that it contains a large EF-hand calcium binding domain that presumably functions to couple calcium signaling to neuronal metabolism^{39, 40}. We were surprised to find that differentiation of P19 cells to NLCs entailed a modest but consistent depression in the levels of enzymes involved in glycolysis, including aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, enolase and pyruvate kinase (Supplementary Table 1). A simultaneous decrease in lactate dehydrogenase coupled with a broad up-regulation of proteins involved in the citric acid cycle and oxidative phosphorylation indicate that the differentiating cells produce less lactic acid, but transfer acetate more efficiently to mitochondrial metabolism. This transition makes more efficient use of carbohydrate energy, providing more ATP to satisfy the demands of the developing neuron. The mitochondrially-generated ATP is made available to the cell through up-regulation of the adenine nucleotide transporter (principally ANT1; Supplementary Table 2) and creatine kinase (Supplementary Table 1). It would be of great interest to conduct a comparative study of these shifts in metabolic proteins using normal stem cells induced to undergo neuronal differentiation. P19 cells are highly transformed tumor-derived cells that may be even more dependent on glycolysis than neurons.

The changes in mitochondrial protein composition indicated in Figure 4 and Supplementary Table 2 reflect a generalized increase in mitochondrial biogenesis. Singh and Veltri⁴¹ previously documented an increase in the fraction of cytoplasm occupied by mitochondria in P19-derived NLC. However, their study also reported the paradoxical result that the quantity of mtDNA appeared to decrease during differentiation. This result would be difficult to reconcile with the requirement of mtDNA transcription and translation to provide the 13 mtDNA-encoded subunits of mitochondrial respiratory complexes. We reinvestigated the quantity of mtDNA in P19 and NLC cells using an internally-controlled Southern blotting procedure and found that the mtDNA content relative to nuclear rDNA increased 2.1-fold (Figure 6), in parallel with the increases observed for most components of the electron transport chain. While most components of the mitochondrial respiratory complexes observed in our work increased in abundance approximately 1.6 to 2-fold, Western blotting suggests that the COX1 subunit of cytochrome oxidase increased more dramatically (Figure 5). It has long been known that neurons are distinguished by an unusually high content of cytochrome oxidase⁴², and that this content responds to neuronal activity. However, the basis for the disproportionate increase in cytochrome oxidase subunit 1 remains uncertain.

Neuronal differentiation does not involve a coordinated increase in abundance of all mitochondrial proteins. Some proteins, such as chaperones and three components of the protein import apparatus, showed little change (Supplementary Table 2). It is interesting to note that the accumulation of proteins involved in importation and refolding of mitochondrial proteins does not keep pace with the general increase in abundance of cargo proteins. This is illustrated by the behavior of HSP60, which does not increase perceptibly during neuronal differentiation according to our mass spectrometry (Figure 4) and immunoblot results (Figure 5). HSP60 is a known disease gene that is mutated in some patients with spastic hereditary paraplegia⁴³. It will be interesting to determine whether a reduced relative level of this or other chaperones is observed in neurons in other settings.

Overall control of the differentiation program

Much of the developmental transition during neurogenesis is likely to result from changes at the level of transcription. Since the developmental program is primed by exposure of cells to all-trans retinoic acid, early transcriptional modulation may involve RAR and RXR factors⁴⁴, or may reflect the down-regulation of cyclin D1 as cells exit the cell cycle. However, much of the increase in mitochondrial proteins documented in Figure 5 occurs later in the differentiation program as primitive NLCs begin to extend axonal processes.

The list of nuclear transcription factors most commonly implicated in control of mitochondrial proliferation includes nuclear respiratory factors (NRF1 and 2), and peroxisome proliferator-activated receptor α/γ (PPAR α/γ), all of which can employ PGC-1 α as a coactivator. PGC-1 α also collaborates with estrogen related receptor- α (ERR α)⁴⁵ to influence expression of nuclear genes encoding mitochondrial proteins. While expression of PGC-1 α has been shown previously to respond to RXR receptor activation, our data in Table 3 and Figure 7 provide the first evidence, to our knowledge, that PGC-1 α is significantly up-regulated during neuronal differentiation. Additionally these data show that there is a consistently high abundance of PRC-1 mRNA from day 0 to 11, with a dramatic increase in abundance of PGC-1 α . PGC-1 α is known to induce mitochondrial biogenesis and oxidative metabolism in muscle cells, adipocytes and cardiomyocytes^{46, 47}. Recently, St. Pierre et al.⁴⁸ showed that PGC-1 α stimulates MnSOD expression, as we observed (Figure 5). The phenotype of PGC-1 α knockout mice provided the first clue to the importance of this factor in neuronal function⁴⁹. These KO mice contain spongiform lesions in the striatum leading to hyperactivity that is phenocopied to some extent by the action of mutant Huntingtin protein which has recently been shown to

antagonize expression of PGC-1 α ⁵⁰. These results will stimulate additional study of the complex role of PGC-1 α in neurogenesis.

Our proteomic data suggest that down-regulation of Mybbp1a may also impact the regulation of mitochondrial biogenesis by PGC-1 α during neuronal differentiation. Mybbp1a can inhibit the ability of PGC-1 α to stimulate transcription³¹. Thus, enhanced mitochondrial biogenesis during neuronal differentiation may reflect a major effect of increased PGC-1 α and secondary effect of decreased levels of its antagonist, Mybbp1a. The potential interaction of these mechanisms is illustrated in Figure 8, which depicts the manner in which these two mechanisms together may contribute to increased mitochondrial biogenesis. Despite this apparent complexity, Figure 8 clearly oversimplifies the situation since it does not account for the possible contribution of either PGC-1 β or PRC1 to this pathway. Another regulatory pathway that might exert an influence during neuronal differentiation is the anticipated decrease in cyclin D1, which is reported to control expression of a cohort of genes involved in mitochondrial biogenesis⁵¹. We suggest that the P19 neuronal differentiation pathway provides a fertile opportunity to explore these regulatory circuits in greater detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Margaret Romaine and Stephanie Burke for technical assistance and Dr. Weiping Xie of the USB Proteomics Facility for LC-MS/MS services. We thank Chun Zhou and Erich Bremer for bioinformatics assistance, Dr. John Chen for statistical advice and Dr. Stella Tsirka for critical review of the manuscript. This research was supported by NIEHS grant R01-ES012039.

References

1. Bain G, Ray WJ, Yao M, Gottlieb DI. From Embryonal Carcinoma Cells to Neurons: The P19 Pathway. *BioEssays* 1994;16:343–348. [PubMed: 8024542]
2. McBurney MW, Rogers BJ. Isolation of male embryonic carcinoma cells and their chromosome replication patterns. *Dev Biol* 1982;89:503–508. [PubMed: 7056443]
3. Rossant J, McBurney MW. The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. *J Embryol Exp Morph* 1982;70:99–112. [PubMed: 7142904]
4. Meeks JP, Mennerick S. Feeding hungry neurons: astrocytes deliver food for thought. *Neuron* 2003;37(2):187–9. [PubMed: 12546812]
5. Voutsinos-Porche B, Bonvento G, Tanaka K, Steiner P, Welker E, Chatton JY, Magistretti PJ, Pellerin L. Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. *Neuron* 2003;37(2):275–86. [PubMed: 12546822]
6. Maurer MH, Feldmann RE Jr, Futterer CD, Butlin J, Kuschinsky W. Comprehensive Proteome Expression Profiling of Undifferentiated versus Differentiated Neural Stem Cells from Adult Rat Hippocampus. *Neurochemical Research* 2004;29(6):1129–1144. [PubMed: 15176470]
7. Ray WJ, Gottlieb DI. Regulation of Protein Abundance in Pluripotent Cells Undergoing Commitment to the Neural Lineage. *J Cell Physiology* 1996;168:264–275.
8. An J, Yuan Q, Wang C, Liu L, Tang K, Tian HY, Jing NH, Zhao FK. Differential display of proteins involved in the neural differentiation of mouse embryonic carcinoma P19 cells by comparative proteomic analysis. *Proteomics* 2005;5(6):1656–68. [PubMed: 15789344]
9. Wen J, Xia Q, Lu C, Yin L, Hu J, Gong Y, Yin B, Monzen K, Yuan J, Qiang B, Zhang X, Peng X. Proteomic analysis of cardiomyocytes differentiation in mouse embryonic carcinoma P19CL6 cells. *J Cell Biochem* 2007;102:149–160. [PubMed: 17520663]
10. Wang D, Gao L. Proteomic analysis of neural differentiation of mouse embryonic stem cells. *Proteomics* 2005;5(17):4414–26. [PubMed: 16222718]

11. Hoffrogge R, Mikkat S, Scharf C, Beyer S, Christoph H, Pahnke J, Mix E, Berth M, Uhrmacher A, Zubrzycki IZ, Miljan E, Volker U, Rolfs A. 2-DE proteome analysis of a proliferating and differentiating human neuronal stem cell line (ReNcell VM). *Proteomics* 2006;6(6):1833–47. [PubMed: 16475233]
12. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Mol Cell Proteomics* 2004;3(12):1154–1169. [PubMed: 15385600]
13. Enriquez J, Fernandez-Silva P, Perez-Martos A, Lopez-Perez M, Montoya J. The synthesis of mRNA in isolated mitochondria can be maintained for several hours and is inhibited by high levels of ATP. *Eur J Biochem* 1996;237:601–610. [PubMed: 8647103]
14. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular cloning: a laboratory manual*. 2. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1989.
15. Basu S, Bremer E, Zhou C, Bogenhagen DF. MiGenes: a searchable interspecies database of mitochondrial proteins curated using gene ontology annotation. *Bioinformatics* 2006;22(4):485–492. [PubMed: 16368773]
16. Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK, Trosko JE. Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. *Carcinogenesis* 2005;26(2):495–502. [PubMed: 15513931]
17. Braun H, Schafer K, Holtt V. BetaIII tubulin-expressing neurons reveal enhanced neurogenesis in hippocampal and cortical structures after a contusion trauma in rats. *J Neurotrauma* 2002;19(8):975–83. [PubMed: 12225657]
18. Lu QR, Park JK, Noll E, Chan JA, Alberta J, Yuk D, Alzamora MG, Louis DN, Stiles CD, Rowitch DH, Black PM. Oligodendrocyte lineage genes (OLIG) as molecular markers for human glial brain tumors. *Proc Natl Acad Sci U S A* 2001;98(19):10851–6. [PubMed: 11526205]
19. Li M, Makkinje A, Damuni Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem* 1996;271:11059–11062. [PubMed: 8626647]
20. Karetsoy Z, Martic G, Sflomos G, Papamarcaki T. The histone chaperone SET/TAF-I beta interacts functionally with the CREB-binding protein. *Biochem Biophys Res Comm* 2005;335:322–327. [PubMed: 16061203]
21. Adler HT, Nallaseth FS, Walter G, Tkachuk DC. HRX leukemic fusion proteins form a heterocomplex with the leukemia-associated protein SET and protein phosphatase 2A. *J Biol Chem* 1997;272:28407–28414. [PubMed: 9353299]
22. Estanyol JM, Jaumot M, Casanovas O, Rodriguez-Vilarrupla A, Agell N, Bachs O. The protein SET regulates the inhibitory effect of p21(Cip1) on cyclin E-cyclin-dependent kinase 2 activity. *J Biol Chem* 1999;274:33161–33165. [PubMed: 10551888]
23. Canela N, Rodriguez-Vilarrupla A, Estanyol JM, Diaz C, Pujol MJ, Agell N, Bachs O. The SET protein regulates G2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. *J Biol Chem* 2003;278:1158–1164. [PubMed: 12407107]
24. Letsas KP, Frangou-Lazaridis M. Surfing on prothymosin alpha proliferation and anti-apoptotic properties. *Neoplasia* 2006;53(2):92–96. [PubMed: 16575463]
25. Mattar P, Britz O, Johannes C, Nieto M, Ma L, Rebeyka A, Klenin N, Polleux F, Guillemot F, Schuurmans C. A screen for downstream effectors of Neurogenin2 in the embryonic neocortex. *Dev Biol* 2004;273(2):373–389. [PubMed: 15328020]
26. Frey T, Mannella C. The internal structure of mitochondria. *Trends Biochem Sci* 2000;25:319–325. [PubMed: 10871882]
27. Laux T, Fukami K, Thelen M, Golub T, Frey D, Caroni P. GAP43, MARCKS, and CAP23 Modulate PI(4,5)P2 at Plasmalemmal Rafts, and Regulate Cell Cortex Actin Dynamics through a Common Mechanism. *J Cell Biol* 2000;149(7):1455–1472. [PubMed: 10871285]
28. Fukada M, Watakabe I, Yuasa-Kawada J, Kawachi H, Kuroiwa A, Matsuda Y, Noda M. Molecular Characterization of CRMP5, a Novel Member of the Collapsin Response Mediator Protein Family. *J Biol Chem* 2000;275(48):37957–37965. [PubMed: 10956643]

29. Takahashi S, Inatome R, Yamamura H, Yanagi S. Isolation and expression of a novel mitochondrial septin that interacts with CRMP/CRAM in the developing neurones. *Genes Cells* 2003;8(2):81–93. [PubMed: 12581152]
30. Inberg A, Bogoch Y, Bledi Y, Linial M. Cellular processes underlying maturation of P19 neurons: Changes in protein folding regimen and cytoskeleton organization. *Proteomics* 2007;7(6):910–920. [PubMed: 17370269]
31. Fan M, Rhee J, St-Pierre J, Handschin C, Puigserver P, Lin J, Jaeger S, Erdjument-Bromage H, Tempst P, Spiegelman BM. Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1{alpha}: modulation by p38 MAPK. *Genes Dev* 2004;18(3):278–289. [PubMed: 14744933]
32. Cam H, Balciunaite E, Blais A, Spektor A, Scarpulla RC, Young R, Kluger Y, Dynlacht BD. A Common Set of Gene Regulatory Networks Links Metabolism and Growth Inhibition. *Mol Cell* 2004;16(3):399–411. [PubMed: 15525513]
33. Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI. Retinoic Acid Induces Embryonal Carcinoma Cells to Differentiate into Neurons and Glial Cells. *J Cell Biol* 1982;94:253–262. [PubMed: 7107698]
34. Jones-Villeneuve E, Rudnicki M, Harris J, McBurney M. Retinoic acid-induced neural differentiation of embryonal carcinoma cells. *Mol Cell Bio* 1983;3:2271–2279. [PubMed: 6656766]
35. Bain G, Mansergh F, Wride M, Hance J, Isogawa A, Rancourt S, Ray W, Yoshimura Y, Tsuzuki T, Gottlieb D, Rancourt D. ES cell Neural Differentiation Reveals a Substantial Number of Novel ESTs. *Funct Integr Genomics* 2000;1:127–139. [PubMed: 11793228]
36. Wei Y, Harris T, Childs G. Global gene expression patterns during neural differentiation of P19 embryonic carcinoma cells. *Differentiation* 2002;70:204–219. [PubMed: 12147139]
37. Unlu M, Morgan M, Minden J. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. *Electrophoresis* 1997;18:2071–2077. [PubMed: 9420172]
38. Nicholls, D.; Ferguson, S. *Bioenergetics*. 3. Academic Press; Amsterdam: 2002.
39. Ramos M, del Arco A, Pardo B, Martinez-Serrano A, Martinez-Morales JR, Kobayashi K, Yasuda T, Bogonez E, Bovolenta P, Saheki T, Satrustegui J. Developmental changes in the Ca2+-regulated mitochondrial aspartate-glutamate carrier aralar1 in brain and prominent expression in the spinal cord. *Developmental Brain Research* 2003;143(1):33–46. [PubMed: 12763579]
40. Pardo B, Contreras L, Serrano A, Ramos M, Kobayashi K, Iijima M, Saheki T, Satrustegui J. Essential role of aralar in the transduction of small Ca2+ signals to neuronal mitochondria. *J Biol Chem* 2006;281(2):1039–47. [PubMed: 16269409]
41. Singh G, Veltri K. Effects of Differentiation of Embryonal Carcinoma cells (P19) on Mitochondrial DNA Content in vitro. *In Vitro Cell Dev Biol* 1991;27A:557–561. [PubMed: 1890073]
42. Wong-Riley MTT. Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends in Neurosciences* 1989;12(3):94–101. [PubMed: 2469224]
43. Hansen JJ, Durr A, Courmu-Rebeix I, Georgopoulos C, Ang D, Nielsen MN, Davoine CS, Brice A, Fontaine B, Gregersen N, Bross P. Hereditary spastic paraplegia SPG13 is associated with a mutation in the gene encoding the mitochondrial chaperonin Hsp60. *Am J Hum Genet* 2002;70:1328–1332. [PubMed: 11898127]
44. Leid M, Kastner P, Chambon P. Multiplicity generates diversity in the retinoic acid signalling pathways. *Trends Biochem Sci* 1992;17:427–433. [PubMed: 1333659]
45. Mootha VK, Handschin C, Arlow D, Xie X, StPierre J, Sihag S, Yang W, Altshuler D, Puigserver P, Patterson N, Willy PJ, Schulman IG, Heyman RA, Lander ES, Spiegelman BM. Err{alpha} and Gabpa/b specify PGC-1{alpha}-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle. *Proc Natl Acad Sci U S A* 2004;101(17):6570–6575. [PubMed: 15100410]
46. Schreiber S, Emter R, Hock MB, Knutti D, Cardenas J, Podvinec M, Oakeley EJ, Kralli A. The estrogen-related receptor α (ERR α) functions in PPAR γ coactivator 1 α (PGC-1 α)-induced mitochondrial biogenesis. *Proc Natl Acad Sci U S A* 2004;101(17):6472–6477. [PubMed: 15087503]
47. Scarpulla R. Transcriptional activators and coactivators in the nuclear control of mitochondrial function in mammalian cells. *Gene* 2002;286:81–89. [PubMed: 11943463]

48. St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, Handschin C, Zheng K, Lin J, Yang W. Suppression of Reactive Oxygen Species and Neurodegeneration by the PGC-1 Transcriptional Coactivators. *Cell* 2006;127(2):397–408. [PubMed: 17055439]
49. Lin J, Wu P-H, Tarr PT, Lindenberg KS, St-Pierre J, Zhang C-Y, Mootha VK, Jager S, Vianna CR, Reznick RM. Defects in Adaptive Energy Metabolism with CNS-Linked Hyperactivity in PGC-1 [alpha] Null Mice. *Cell* 2004;119(1):121–135. [PubMed: 15454086]
50. Cui L, Jeong H, Borovecki F, Parkhurst CN, Tanese N, Krainc D. Transcriptional Repression of PGC-1[alpha] by Mutant Huntingtin Leads to Mitochondrial Dysfunction and Neurodegeneration. *Cell* 2006;127(1):59–69. [PubMed: 17018277]
51. Sakamaki T, Casimiro MC, Ju X, Quong AA, Katiyar S, Liu M, Jiao X, Li A, Zhang X, Lu Y, Wang C, Byers S, Nicholson R, Link T, Shemluck M, Yang J, Fricke ST, Novikoff PM, Papanikolaou A, Arnold A, Albanese C, Pestell R. Cyclin D1 Determines Mitochondrial Function In Vivo. *Mol Cell Biol* 2006;26(14):5449–5469. [PubMed: 16809779]
52. Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metabolism* 2005;1(6):361–370. [PubMed: 16054085]

Figure 1.

Differentiation of P19 embryonal carcinoma cells to neuron-like cells. A. In vitro differentiated P19 cells express markers of mature neurons. 3 μ g samples of total protein from days 0, 2, 4, 7, 9 and 11 of the developmental program were fractionated by SDS-PAGE, transferred to a PVDF membrane and probed with various antisera: $\beta 3$ -tubulin, marker for mature neurons; Oct4, a marker for pluripotent stem cells; GAPDH, a loading control. B. 3 μ g samples of total P19 cell protein from days 0 or 11 or from a mouse brain homogenate (Br) were fractionated by SDS-PAGE and probed by immunoblotting with antibodies directed against $\beta 3$ tubulin and GFAP (glial fibrillary acidic protein). C. Undifferentiated P19 cells (day 0) and day 11 NLCs were subjected to flow cytometry to analyze the distribution of cells responding to a glial marker GFAP, and a neuronal marker $\beta 3$ -tubulin as indicated.

Figure 2. Changes in abundance of total cell proteins during neuronal differentiation. The distribution of the number of proteins with log iTRAQ[®] ratios in the indicated ranges is shown.

Figure 3.

Purity of the mitochondrial preparation. 3 μ g of protein from each fraction during purification was subjected to electrophoresis. Proteins were transferred to PVDF membrane and probing with antibodies directed against tubulin as a cytoskeletal marker and prohibitin as a mitochondrial marker. Lanes contained the following fractions: 1. Total homogenate; 2. Post-nuclear supernatant; 3. Nuclear pellet; 4. Post-mitochondrial supernatant; 5. Crude mitochondrial pellet; 6. Microsomal fraction; and 7. Purified mitochondria.

Figure 4.

Quantitative changes in abundance of mitochondrial proteins during differentiation. The changes in abundance during neuronal differentiation are shown for several classes of mitochondrial proteins. Panel A includes proteins belonging to respiratory complexes I, III and IV. Panel B shows subunits of F1, F0 ATPase (Complex V). Panel C shows changes in abundance of proteins in the TCA cycle. Panels D and E depict data for membrane transporters and chaperones, respectively. Note the differences in scale between different panels.

Figure 5. Immunoblots validate changes in expression of mitochondrial proteins during differentiation. 3 μ g samples of total cell proteins from cultures at different stages in the induction program from day 0 through 11 were subjected to SDS-PAGE, blotted and probed for a variety of mitochondrial proteins. Antibodies used were specific for individual polypeptides of the respiratory complexes I–IV, GAPDH (loading control), Porin (VDAC), Cyt. C (cytochrome C), AIF (apoptosis inducing factor), PHB (prohibitin 1), MnSOD (manganese superoxide dismutase), and HSP 60 (heat shock protein 60).

Figure 6.

Increase of mtDNA during neuronal differentiation. A. Total cellular DNA was prepared from undifferentiated cells and from cells at days 4 and 11 of the differentiation program. 10 μ g samples of DNA were digested with EcoRI, fractionated by agarose gel electrophoresis, transferred to a Hybond N+ membrane and probed with a mixture of 32 P-labeled probes to detect both nuclear rDNA and mtDNA. Hybridization of probes to DNA was detected and quantified with a phosphorimager. B. Quantification of mitochondrial DNA to nuclear ribosomal DNA.

Figure 7. Differential expression of protein regulated upon NLC differentiation. Immunoblot analysis of NRF1, PGC-1α and GAPDH at days 0, 4 and 11 of the differentiation time course.

Figure 8.

PGC-1 α and NRF1 modulate expression of nuclear genes encoding mitochondrial proteins. Neuronal differentiation involves a dramatic up-regulation of expression of PGC-1 α . Upon phosphorylation it can actively bind its regulatory partner NRF1 to activate expression of nuclear genes encoding mitochondrial proteins such as cytochrome C (Cyt C)⁵². A second regulatory mechanism is illustrated when PGC-1 α is bound by Mybbp1a (p160), cannot be phosphorylated and cannot activate NRF1³¹.

Table 1**Analysis of quantitative changes in abundance of total cell proteins by GO category**

Proteins identified by LC-MS/MS were classified according to their GO annotations. The numbers of proteins in each class that were increased, decreased or unchanged (NC) in abundance are indicated along with the average fold-change for proteins in each class.

GO Category	Up	NC	Down	Avg
Cytoskeleton	16	6	3	3.10
Transcription	0	7	4	0.86
Translation/Protein Biosynthesis	1	3	27	0.68
Metabolism	1	6	3	1.03
Cell Cycle/DNA metabolism	0	1	7	0.54
mRNA Processing	2	12	6	0.98
Protein Modification	0	2	0	1.03
Protein Catabolism	0	2	1	0.83
Neurogenesis	10	0	0	3.77
Nucleotide Biosynthesis	0	3	0	0.95
Protein Folding/Chaperone	4	8	5	0.98
Signal Transduction	5	3	0	1.92
Hydrolase Activity	1	0	2	1.00
Transport	3	7	1	1.42
Response to Stress/Oxidative stress	0	0	2	0.69
Mitochondrial categories:				
TCA cycle	3	0	0	2.00
Transport	4	1	0	1.66
ATP Synthetase	2	0	0	1.82
Electron Transport	1	2	0	1.45
Oxidative Stress	0	1	0	1.22
Protein Folding/Chaperones	0	4	0	0.97

Comparison of abundance changes for 14 mitochondrial proteins observed in both the total cell protein and the mitochondrial fraction

The relative abundance of 14 mitochondrial proteins for NLC relative to undifferentiated cells is shown for total cell lysates and for the purified mitochondrial fraction. The right-hand column shows the ratio of total cell to mitochondrial protein data.

Accession	Protein Name	Gene	iTRAQ® Cell	Abundance Mito	Data Cell/Mito
NP_031531	F1 ATPase alpha	Atp5a1	1.861	1.137	1.637
NP_058054	F1 ATPase beta	Atp5b	1.770	0.951	1.861
NP_035824	VDAC 1	Vdac1	2.034	1.193	1.705
NP_079843	cytochrome c-1	Cyc1	1.313	0.695	1.888
NP_079986	Complex III, Rieske Fe-S 1	Uqcrls1	1.786	1.050	1.701
NP_034607	HSP 60	Hsp60	0.845	0.636	1.328
NP_034611	HSP 70	GRP75	1.016	0.654	1.554
NP_032329	HSP 10	Hspe1	0.846	0.506	1.673
NP_031557	Prohibitin 2	BAP	1.170	0.686	1.706
NP_032643	MDH 2	MDH	1.751	1.154	1.518
NP_080720	Citrate synthase	Cis	1.911	1.275	1.499
NP_542364	aconitase 2	Aco2	2.332	1.533	1.521
NP_031477	Ant 2	Slc25a5	1.242	0.844	1.472
NP_598429	Phosphate carrier	Phc	1.523	0.986	1.543
	Average				1.615

Table 3
Differential expression of mRNAs regulated upon NLC differentiation
Quantitative mRNA analysis of cycle threshold (Ct) changes of Phgdh (internal control), PGC1 α , PRC1, and mybbp1a for undifferentiated P19 cells day 0, 4, and 11. (S.D. = standard deviation).

Gene	Relative Ct Values				Relative to Day 0					
	Day 0	S.D. +/-	Day 4	S.D. +/-	Day 11	S.D. +/-	Δ Ct Day 4	Δ Ct Day 11	Fold Δ Day 4	Fold Δ Day 11
PHGDH	19.83	0.30	18.67	0.27	22.54	0.10	-1.16	2.71	2.24	0.15
PGC1 α	30.21	0.33	30.95	0.23	22.93	0.09	0.74	-7.28	0.60	155.88
PGC1 β	30.07	0.24	28.06	0.20	26.31	0.11	-2.01	-3.75	4.03	13.49
PRC1	23.77	0.32	22.26	0.51	23.52	0.22	-1.51	-0.25	2.85	1.19
Mybbp1a	20.10	0.19	20.78	0.41	21.99	0.49	0.68	1.89	0.63	0.27