Application Note
Stem Cell Biology

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Metabolic Phenotyping to Identify Cellular Transitions During Early Neuronal Differentiation from Neural Precursor Cells Using Seahorse XF Technology

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Abstract

In vitro stem cell differentiation systems enable the examination of the essential factors regulating cell differentiation and the generation of fully functional cells. Generation of functional cells from pluripotent cells such as induced pluripotent stem cells (iPSCs) involves dynamic changes in cellular metabolism. These metabolic changes occur continuously from the initial steps of cell commitment to differentiation and maturation, and thus time-dependent live cell analysis of cellular metabolism through the process is desired. Agilent Seahorse XF technology can provide a continuous and quantitative analysis platform to monitor the phenotypic changes of cell metabolism. This Application Note details a metabolic analysis during the early differentiation of nociceptor sensory neurons from committed neural precursor/stem cells (NPCs) to premature NPCs. It includes cell preparation protocols for XF analysis, Ara-C-mediated negative selection of highly proliferative cell types, and data normalization by genomic DNA. The data produced revealed a persistent downregulation of glycolysis in NPCs following the commitment stage in contrast to mitochondrial respiration, which showed more dynamic changes. This application model can be expanded for analysis over a prolonged differentiation period, through maturation and to the terminally differentiated neurons. It can also be adapted to studies on time-dependent metabolic changes of other stem cells, and more particularly in assessing cellular dynamics like metabolic oscillation.

Introduction

The development of reprogramming technologies that convert somatic cells into iPSCs has led to the creation of patient specific cell types and in vitro cell-based disease models. However, a major challenge is the lack of directed differentiation protocols for the generation of a pure population of the cell type of interest from PSCs[1]. As such, the differentiated cells that are produced are highly heterogeneous. This heterogeneity makes it difficult to evaluate the exact contribution of a specific cell type to disease development or to qualitatively analyze the molecular and metabolic trajectory of the differentiated cell types. Previous studies have identified a functional link between metabolism and stem cell development[2-3]. For example, a few recent studies have shown that NPCs have different metabolic profiles than their mature central nervous system (CNS) counterparts. This difference suggests that metabolic pathways have a regulatory role in stem cell fate decisions[4-5].

Numerous studies have used iPSCs to derive CNS neurons[6] and the subtypes of these neurons that are central for modeling neurodegenerative diseases, including Parkinson's disease and amyotrophic lateral sclerosis[7-10]. However, only a few recent studies report methods to differentiate PSCs into neurons of the peripheral nervous system (PNS)[11-12]. Therefore, there are limited protocols describing the derivation of pure populations of specific sensory neurons of the PNS and their application for modeling neurodegenerative diseases. Understanding the metabolic requirements during normal peripheral neural development and the metabolic defects that could drive aberrant neurogenesis would provide new metabolic checkpoints during NPC differentiation and peripheral nerve maturation. This understanding would also lead to the development of targeted treatment strategies for neuropathy.

Cellular metabolic changes in live NPCs and differentiated neurons can be studied using Seahorse XF technology. Mitochondrial function of those cells can be measured by using the XF Cell Mito Stress Test in which mitochondrial respiration activity is analyzed by monitoring real-time oxygen consumption rate (OCR) with serial administrations of mitochondrial complex inhibitors. Glycolytic activity of the cells can be assessed by using the XF Glycolytic Rate Assay, which measures extracellular acidification rate (ECAR) and calculates proton efflux rate (PER) out of it. More particularly, by applying HEPES buffered assay conditions, PER solely dependent on glycolysis can be assessed as glycolytic PER (glycoPER).

Results

Measurements of mitochondrial respiration and glycolysis in early differentiating NPCs

Initially, human iPSCs were differentiated towards an NPC phenotype, which were then banked and expanded as needed. The NPCs were then exposed to conditions that induce peripheral neural differentiation, through treatment with inhibitors and small molecules, such as CHIR, SU5402, and DAPT for the first four days. The differentiating cells were then dissociated and replated in the presence or absence of Ara-C and growth factors NGF and GDNF (Figure 1A; see methods for specific details). Ara-C treatment was performed to remove the proliferating cells, which tend to overtake the differentiating neural cultures, thus promoting a more homogeneous neural culture. Homogeneity in the differentiating cultures is required for accurate and representative measurement of molecular and functional parameters.

As shown in Figure 1, the metabolism of NPCs from day 0 to day 9 was compared after normalization. As differentiation of the NPCs progressed, cells became less energetic with the glycolytic rate, glycoPER decreasing more significantly than mitochondrial respiration rate, OCR. This decrease suggests that as stem/progenitor cells start differentiating they undergo a metabolic switch where glycolysis is downregulated.

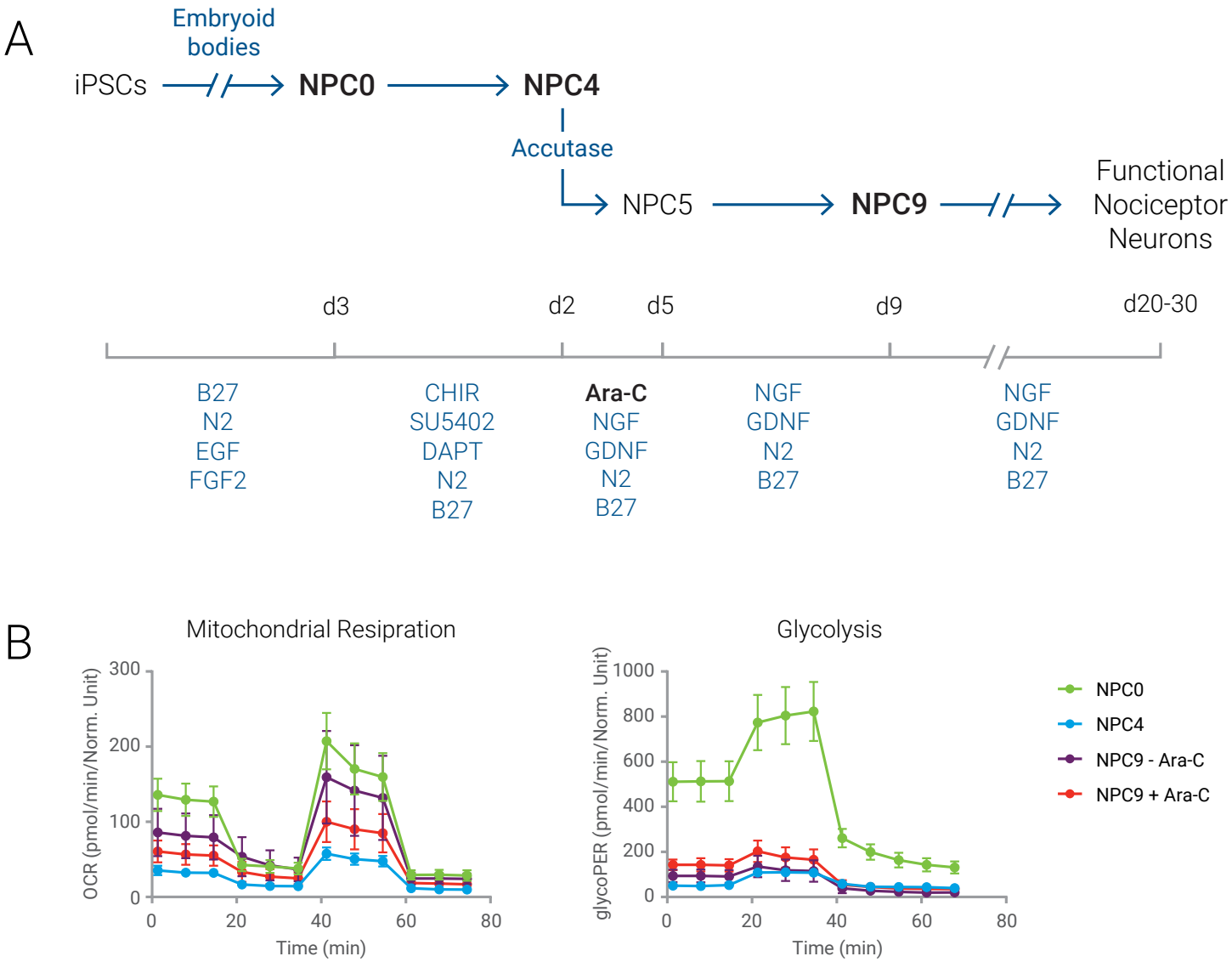

Figure 1. Sensory neuron differentiation timeline and cellular metabolism profile during early NPC differentiation. (**A**) Schematic showing the differentiation protocol from iPSCs to functional sensory neurons: Differentiation of iPSCs to NPCs was performed using the embryoid body procedure as mentioned in the materials and methods section. NPCs undergoing differentiation were dissociated at day 4 and replated on Matrigel coated cell culture dishes with and without Ara-C. (**B**) Metabolic changes during early NPC differentiation: mitochondrial respiration and glycolysis were measured by the XF Cell Mito Stress Test and the XF Glycolytic Rate Assay Kits respectively at specific time points during early NPC differentiation. Error bars reported as mean ± standard deviation (n=26).

Metabolic transition during the early differentiation stages from NPCs

As differentiation progressed from day 0 to day 9, a significant drop in basal and compensatory glycolysis was observed. This drop suggests that the cells dependence on glycolysis is downregulated within the first four days of differentiation (Figure 2A). In addition, during these first four days of NPC differentiation basal oxygen consumption rates and spare respiratory capacity also decreased. However, by day 9 of differentiation oxygen consumption levels and spare respiratory capacity recovered to similar levels to those levels observed for undifferentiated NPCs (Figure 2B). This recovery may be indicative of metabolic oscillations during the early differentiation. It is important to note that in the presence of Ara-C, which removes proliferating cells, the recovery of mitochondrial respiration was slower. This slower recovery was likely due to an effect of the Ara-C treatment on the cells resulting in slower recovery.

To demonstrate that NPCs followed the expected differentiation trajectory, gene expression of NPC markers (Nestin, Lin28B, Hes1, and NCAM2), mature general neuronal markers (Notch1, NeuN, NeuroD1), and peripheral neuronal markers (NF200) were examined (Figure 2C and D). As differentiation progressed over the nine-day timeline, the expression of NPC markers significantly decreased (Figure 2C) and neuronal marker expression gradually increased (Figure 2D).

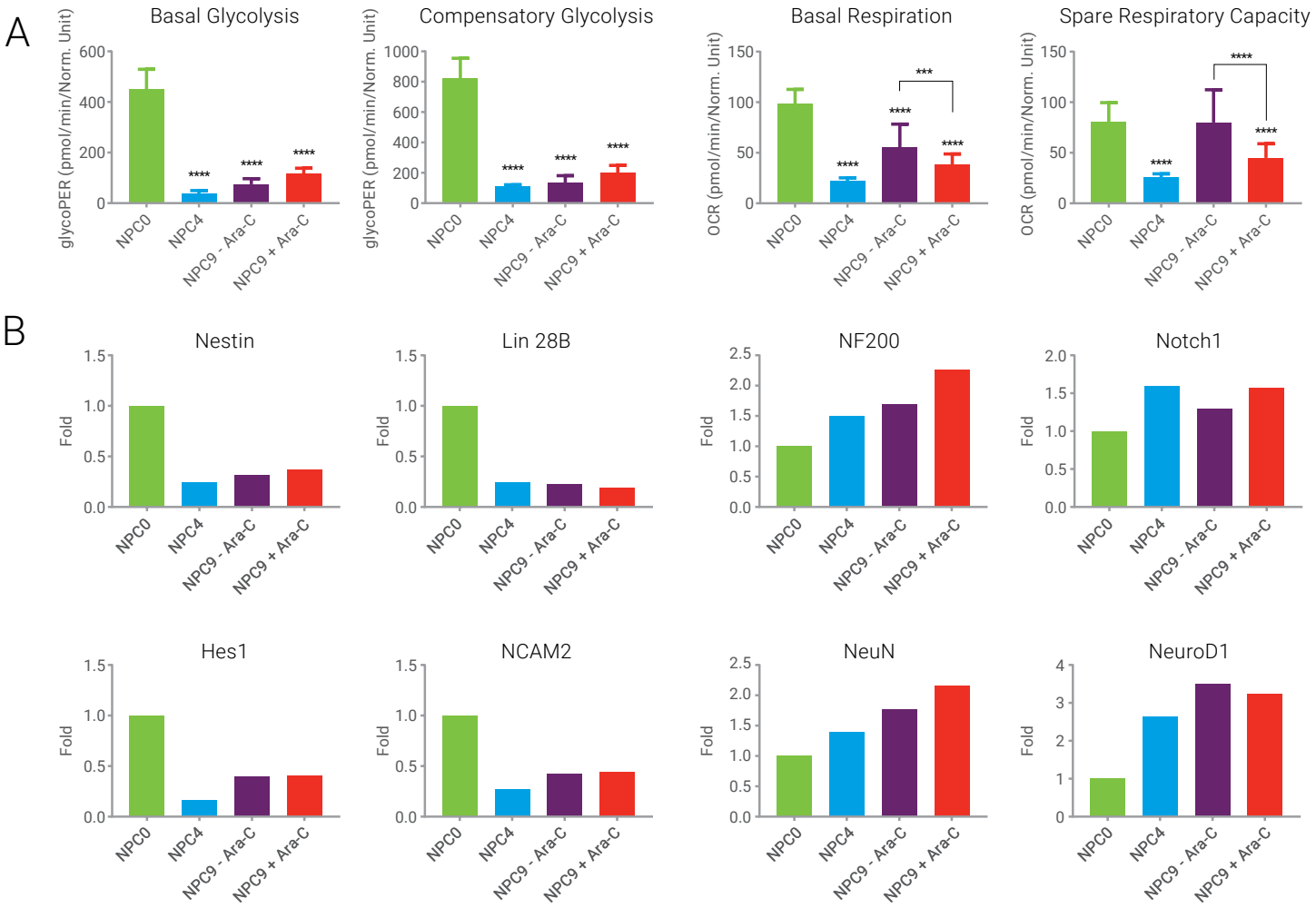

Figure 2. Cellular metabolism parameters correspond with neuronal gene expression during early NPC differentiation. (**A**) Glycolysis was measured at specific time points using the XF Glycolytic Rate Assay Kit. Basal and compensatory glycolysis was calculated using the report generator (Agilent) and the data was normalized to total cellular DNA content. (**B**) Mitochondrial respiration parameters, basal OCR, and spare respiratory capacity were calculated using the XF Cell Mito Stress Test Kit at and the report generator (Agilent). (**C**) Gene expression for NPC markers: Nestin, Lin28B, Hes1, and NCAM2 were measured at different time points during the NPC differentiation by qRT-PCR and fold change w.r.t. day 0 NPCs was calculated. (**D**) Gene expression data for neuronal markers: NF200, Notch1, NeuN, and NeuroD1. (****p<0.00001)

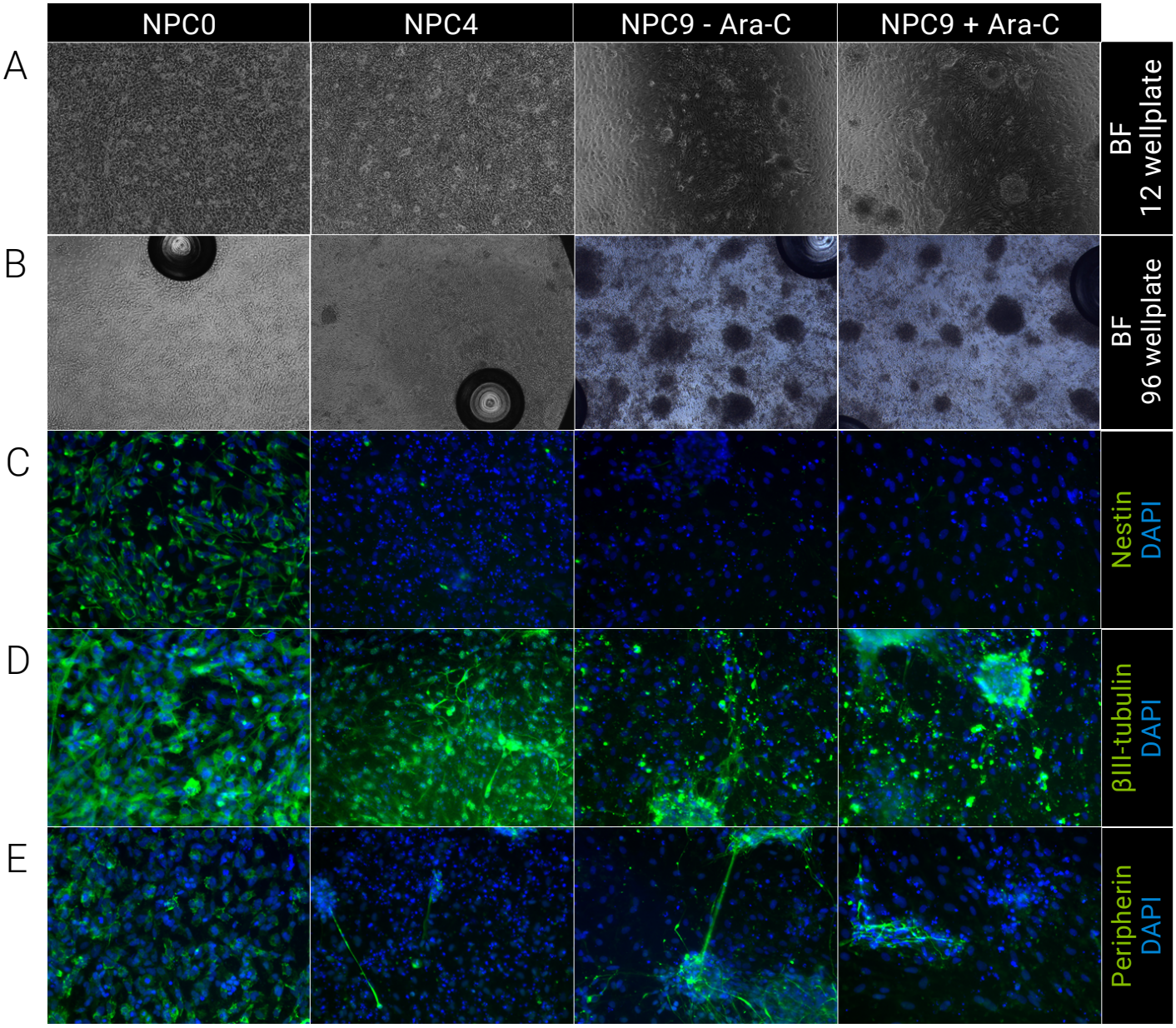

Figure 3. Phenotypic changes during early NPC differentiation. Representative 4X phase contrast images of the cells at specified time points during early NPC differentiation in 12-well plates (**A**) and in XF96 Seahorse plates (**B**). Cells were also stained with an NPC marker, Nestin (**C**), and the neuronal markers βIII-tubulin (**D**) and Peripherin (**E**). DAPI was used to stain the nuclei. (n=3)

The differentiation from NPCs to day 9 maturing neurons was evaluated by immunofluorescence analysis to demonstrate the presence of the neuronal markers at the protein level (Figure 3). As differentiation progresses the NPCs change morphology, beginning to form cell clumps by day 4 and axonal projections by day 9 (Figure 3A and B). As NPCs differentiate they lose expression of the NPC marker, Nestin (Figure 3C), show localization of the neuronal marker, beta-III tubulin (Figure 3D), and gain expression of the peripheral neuronal marker, Peripherin (Figure 3E).

Overall, comparison of mitochondrial respiration to mitochondrial independent glycolysis demonstrates that as differentiation progresses from day 0 to day 9 there is a clear shift in the metabolic dependence of the cells from glycolysis to mitochondrial respiration (Figure 4).

Discussion

Metabolic analysis and surrogate neuronal marker expression were performed on different days (0, 4, 5, and 9) during the initial peripheral neural differentiation timeline. It is critical that cell number per well is optimized, and a robust normalization method for the data points (i.e. protein or DNA content) is established. There should also be rigorous consideration given to the heterogeneity of the differentiating culture as numerous cell types are emerging alongside the peripheral neurons. At the early stage of neuronal differentiation, heterogeneity of the cultures is evidenced by morphological differences and well-to-well variation of the metabolic measurements. Therefore, it is important to monitor the cellular morphology of NPCs and differentiating cultures as an indicator of cellular health to minimize resources spent on metabolic analysis. If the cells are unhealthy, the metabolic profiles will reflect this by showing minimal OCR and glycoPER with substantial fluctuations between readings.

Another aspect of metabolic profiling that should be considered is the possibility that OCR and glycoPER measurements will oscillate during the differentiation timeline. The OCR showed oscillations between day 0 to day 9, which were similar to those oscillations observed in the numerous differentiation protocols examining gene expression profiles through the differentiation timeline. Thus, there may be multiple metabolic switches as the cells mature into peripheral neurons, which would be missed if metabolic parameters were only analyzed at the initial and final stage of differentiation. Establishing a robust protocol that improves purity of the differentiating neurons is also critical for the reliable and reproducible measurement of metabolic transitions during differentiation. A homogeneous population of neurons would also avoid the presence of other cell types masking any metabolic changes.

Conclusion

Metabolic profiling using the XF Cell Mito Stress Test and the XF Glycolytic Rate Assay to measure mitochondrial respiration and glycolysis in differentiating neurons demonstrated that this technology can be used to identify metabolic transitions that mirror cellular transitions during the neuronal differentiation timeline. These assays therefore provide a robust measurement, which is a step towards predicting the differentiation trajectory of peripheral neurons.

Materials and methods

NPCs culture and differentiation into peripheral sensory neurons

Human iPSC derived NPCs were cultured on Matrigel (Corning, 354234) coated cell culture dishes in DMEM/F12 media (Corning, 10-090-CV) supplemented with 1X N2 (Thermo, 17502048), 1X B27 (Thermo, 17504044), 10 ng/mL bFGF (PeproTech, 100-18B), and 50 ng/mL EGF (PeproTech, AF-100-15). For differentiation, cells were dissociated with Accutase (Corning, 25-058-CI), counted, and plated at a density of 5.6 x 106 cells/cm2 on cell culture plates coated twice with 1:15 Matrigel. The following day, neuronal differentiation was induced on a confluent NPCs monolayer with a Neurobasal media (Thermo, A3582901) containing 1X N2 (Thermo, 17502048), 1X B27 Plus (Thermo, A3582801), 10 µM DAPT (Abcam, Ab120633), 10 µM SU5402 (Cayman Chemicals, 13182), and 3 µM CHIR 99021 (Stem Cell Technologies, 72054). Differentiation was continued for five days with daily complete media changes using fresh differentiation media. At day 5 post induction, cells were dissociated with Accutase and replated on cell culture dishes coated twice with 1:15 Matrigel in Neurobasal media supplemented with 1X N2, 1X B27 Plus (Thermo, A3582801), 25 ng/mL NGF (PeproTech, 450-01), and 10 ng/mL GDNF (PeproTech, 450-10) with and without 10 µM Ara-C (Sigma, C1768) for 24 hours. Media was replaced next day and every second day thereafter.

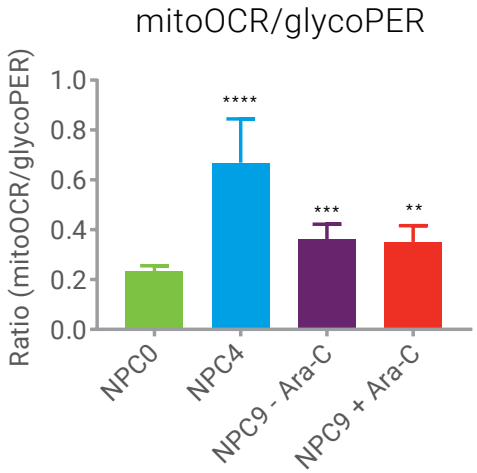

Figure 4. Glycolysis to mitochondrial respiration shift during early NPC differentiation. Ratios of mitochondrial OCR (mitoOCR) to glycolytic PER (glycoPER) were calculated from the XF Glycolytic Rate Assay data at the specific time points using the GRA assay report generator. Error bars reported as mean ± standard deviation (n=26; ****p<0.00001; *** p<0.0001; **p<0.001).

RNA isolation, cDNA synthesis, and quantitative real time PCR

NPCs were plated in 6-well plates and differentiated as described. RNA isolation was performed at specified time points along the differentiation course by Norgen RNA Isolation Plus kit (Norgen Biotek, 48400) following manufacturer's protocol. For each reverse transcription reaction, 1 µg of RNA was used, with the Bioline SensiFast cDNA synthesis kit (Bioline, BIO-65054) as per the kit protocol. 1 µL of cDNA was used as template for each qRT-PCR reaction using gene-specific primers for tracking downregulation of NPC markers and upregulation of neuronal markers along the differentiation timeline.

Cellular metabolic profiling

NPCs were plated and differentiated in XF96 microplates as described earlier. For day 0, XF Cell Mito Stress Test and XF Glycolytic Rate Assay, NPCs were plated at a density of 6 x 10^4 cells/well on Matrigel coated XF96 seahorse plates on day 1. In previous cell number optimization experiments, 6 x 10^4 cells/well was found to be the optimal cell density for the XF Cell Mito Stress Test and the XF Glycolytic Rate Assay. For day 4 measurements, NPCs were plated at 6 x 10^4 cells/well on day 1 and differentiated for four days as mentioned previously. For day 9 measurements, NPCs were plated at 4.8 x 10^6 cells/well on Matrigel coated 6-well plates and differentiated for five days as mentioned previously. On day 5, the cells were dissociated with Accutase and replated on Matrigel-coated XF96 seahorse plates, with and without Ara-C, and maintained until day 9 as mentioned previously. At specific time points, cells were rinsed twice with the complete XF assay media and XF Cell Mito Stress Test and the XF Glycolytic Rate Assay were performed as per the instructions provided with the respective kits. XF assay media for the XF Cell Mito Stress Test was XF DMEM base media without phenol red (Agilent, 103335-100) supplemented with 2.5 mM L-Glutamine, 1 mM sodium pyruvate, and 17.5 mM glucose. For the XF Glycolytic Rate Assay, the same media was further supplemented with 5 mM HEPES, pH 7.4 (Agilent, 103337-100). The pH of all media was adjusted to 7.4 at 37 °C. An optimized FCCP concentration (0.5 µM) was used for the XF Cell Mito Stress Test. OCR and glycoPER readouts were normalized to cellular DNA content after XF assays. Cellular DNA content was measured using CyQuant Cell Proliferation assay kit (Thermo, C7026) following the manufacturer's protocol.

Immunofluorescence staining

NPCs were plated in 12-well plates and differentiated as described. Cells were fixed at specified time points using BD Cytofix/CytoPerm kit (BD Bioscience, 554714). Upon fixation, cells were blocked in 10 % normal donkey serum for 1 hour at room temperature followed by overnight incubation with primary unconjugated antibodies against Nestin (Abcam, Ab22035), NF200 (Abcam, Ab78078), and Peripherin (Abcam, Ab123576) at 4 °C. The next day, cells were washed three times and stained with Alexa Fluor-488 conjugated secondary antibodies (Thermo, A11008, A11001) at room temperature for 1 hour. Cells were washed three times, stained with DAPI (Thermo, D3571), and then imaged.

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