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Nicotinamide Rescues Human Embryonic Stem Cell-Derived Neuroectoderm from Parthanatic Cell Death

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Abstract

Abundant cell death is observed when human embryonic stem cells (hESCs) undergo neuralization, a critical first step for future cell-based therapies addressing neurodegeneration. Using hESC neuralization as an *in vitro* model of human development, we demonstrated that the developing neuroepithelium acquires increased susceptibility to spontaneous cell death. We found that Poly(ADP-ribose) polymerase-1/Apoptosis Inducing Factor (PARP1/AIF)-mediated cell death (*parthanatos*) is a dominant mechanism responsible for cell loss during hESCs neuralization. The demise of neural progenitors cells (hNPCs), at least in part, is due to decreased endogenous antioxidant defenses and enhanced reactive oxygen species (ROS) leakage from mitochondria fuelled by non-physiological culture conditions. Under such conditions, PARP1 over-activation triggered cell death through the mitochondrial-nuclear translocation of AIF. Blocking PARP1 activity with shRNAi or nicotinamide dramatically enhanced hESC neuralization, providing optimal survival of the developing neuroepithelium. Because nicotinamide is a physiological metabolite, our results raise the possibility that neural stem/progenitor cell survival *in vivo* requires a metabolic niche. We argue that small natural metabolites provide a powerful physiological tool to optimize hESC differentiation compatible with requirements of regenerative medicine.

Keywords

Poly(ADP-ribose) polymerase-1; Nicotinamide; Human embryonic stem cells; Human neural progenitors; Parthanatos

INTRODUCTION

High hope is placed on potential uses of hNPCs as a tool for therapeutic treatment of neurodegenerative disorders like Alzheimer's Disease, Parkinson's Disease and spinal cord injury. NPCs, which have indeed proven to mitigate such disorders [1–3], can both be

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Author contribution

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derived from primary tissues and hESCs. While supplies of adult stem cells are limited, hESCs, with their virtually unlimited differentiation potential and high proliferation capacity, represent a rich source of hNPCs [4] as well as other cell lineages. The default model of neuralization proposes that during development the neuroectoderm develops from uncommitted ectoderm in the absence or by inhibition of bone morphogenetic protein (BMP) signalling [5]. It has also been demonstrated that, in the absence of BMP signalling, or, under most any extrinsic influence, undifferentiated mouse ESCs spontaneously undergo neuralization, although with a very low efficiency due to extensive cell death [6, 7].

In an attempt to exploit the default model paradigm to obtain a uniform population of hNPCs from hESCs, we modified a published protocol of neuralization [8] and used a feeder-free, serum-free chemically defined media (CDM) that abrogates the anti-neuralizing effect of the BMP pathway (*Bajpai et al., Cell Death and Differentiation 2009, in press*). Under these conditions hESCs uniformly assume a neural identity although with a very low efficiency due to extensive cell death. The molecular mechanisms of this phenomenon are poorly understood, although genetic interference with the apoptotic machinery seems to increase cell survival [6, 7].

Poly(ADP-ribose) polymerase-1 (PARP1) is a well characterized nuclear enzyme involved in the response to genotoxic damage [9, 10]. Following DNA damage, this enzyme modifies various proteins by catalyzing the attachment of long chains of poly (ADP-ribose) (PAR) to glutamate and aspartate residues in a NAD^+ -dependent fashion, thus mediating different physiological responses to the genotoxic stress [11]. Under mild stress conditions, PARP1 activates the DNA-repair machinery [12]. However, severe stress conditions can over-activate this enzyme and trigger cell death through the mitochondrial-to-nuclear translocation of Apoptosis Inducing Factor (AIF), a cell death executioner alternative to caspases [13–15]. The PARP1/AIF death cascade culminates in a highly orchestrated and caspase-independent programmed cell death (PCD) termed *parthanatos* [13], to distinguish it from classical caspase-dependent apoptosis.

Here we report that hESC neuralization is accompanied by increased mitochondrial respiration, oxidative stress and PARP1 activation, which ultimately translates into parthanatic cell death of developing neuroepithelial cells. Indeed, PARP1 inhibition *via* small molecule compounds or shRNAi-mediated genetic downregulation strongly improved the efficiency of our neuralization protocol, supporting survival of the developing neuroectoderm. Notably, nicotinamide (Nam), a natural PARP1 inhibitor and a physiological metabolite with neuro-protective properties *in vivo* [16, 17], proved very effective in rescuing hNPCs from PARP1-mediated cell death in our cultures. Furthermore, carrying out hESCs neuralization under more physiological oxygen tension such as 3% O_2 also effectively reduced PARP1 activity and PARP1/AIF-mediated cell death, suggesting that normoxic (20% O_2) conditions are genotoxic for early neural progenitors and thus not suitable for differentiation of hESCs into the neural lineage.

MATERIALS AND METHODS

Cell cultures

Maintenance of hESCs—the NIH approved hESC line H9, was used to generate homogeneous cultures of human neural progenitors. hESCs were maintained on MEF feeders and matrigel (BD Biosciences, final concentration = 1:30) coated plates in knockout DMEM (Gibco), 20% serum replacement (Gibco), 1X non essential aminoacids (Gibco), 2mM L-glutamine (Gibco), 0.1 mM β -mercaptoethanol (Gibco), 1X antibiotics/antimycotics (Omega) and 8 ng/ml bFGF (Sigma). hESCs were routinely passaged every 5–7 days using 1 mg/ml Collagenase IV (Gibco) diluted in knockout DMEM after manual removal of morphologically identifiable differentiated colonies. Medium was changed everyday.

Neuralization of hESCs—hNPCs were generated as previously described (*Bajpai et al., Cell Death and Differentiation 2009, in press*). In brief, hESCs were enzymatically separated from MEF feeders with collagenase IV and passaged as small clusters (Diameter = 80–200 μ m). Several washes with PBS followed by gravity-pelleting were used to completely remove contaminant MEF feeder cells, potentially seen at this stage as single cells. Neuralization was initiated by culturing hESC clusters in low attachment polypropylene dishes (Ted Pella) in neuralizing CDM (1:1 ratio of DMEM/F12 glutamax (Gibco): neurobasal medium, 0.05X B27 supplement without vitamin A (Gibco), 10% BIT 9500 (StemCell Technologies), 5 μ g/ml insulin (Sigma), 20ng/ml bFGF (Chemicon), 20ng/ml EGF (sigma), 1mM glutamine (Gibco)) at a density of $30 \cdot 10^3$ cells/cm² (unless otherwise stated, see supplementary figure S1). Medium was changed every day. The effect of Nam, 3ABA and Na (Sigma) on neuralization was tested by adding them to the CDM, unless otherwise stated, at a final concentration of 5 mM. For adherent cultures of neurospheres, spheres at different time points, as indicated in the figure legends, were either directly plated on poly-L-ornithine (10 μ g/ml, Sigma) coated plates (Corning) or after single cell dissociation with Accutase (Chemicon) (15 min at 37°C). For hypoxia experiments, a tri-gas incubator CB 150-UL (BINDER) was used.

Terminal differentiation of hNPCs—Terminal differentiation of hNPCs was obtained by growth factor withdrawal: day 5 neurospheres were plated on poly-L-ornithine (10 μ g/ml, 1hr at RT)+laminin (Invitrogen, 5 μ g/ml, 1hr at 37°C) -coated plates and maintained in CDM lacking bFGF, insulin and EGF. Cells were processed for immunostaining 7–10 days after growth factor withdrawal.

Immunocytochemistry

Cells were rinsed with PBS and fixed with 4% paraformaldehyde (diluted in PBS) for 15 min at room temperature. After blocking with PBSAT (3% BSA and 0.01% Triton X-100 diluted in PBS) for 1h at room temperature, cells were incubated overnight at 4°C with primary antibodies (see supplementary methods for a list of the antibodies) diluted in PBSAT. The appropriate fluorochrome-conjugated secondary antibody was used with every primary antibody at a dilution of 1:1000. Nuclear co-staining was performed by incubating cells with Hoechst nuclear dye (1:1000).

Western blots

Collected cells were washed with PBS and resuspended in Lysis Buffer (500 mM Tris-HCl pH 7.2, 1% Triton X-100, 0.1% SDS, 0.5% SDC, 500 mM NaCl₂, 1mM MgCl₂, 1:100 protease cocktail (Sigma)). 40 µg of cell lysates were resolved in 10% SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with the primary antibodies (see supplementary methods). Peroxidase-conjugated secondary antibodies were used, and proteins were visualized using ECL (Amersham Life Science).

Lentivirus-mediated shRNAi

Lentiviral-mediated small hairpin RNA interference (shRNAi) was used to derive H9 hES cell lines stably expressing shRNA against PARP1 or control scrambled (non-targeting) shRNA. In detail, the DNA sequence AAGTATCTGCTGAAACTGAACTCGAGTTTCAGTTTCAGCAGATACTT designed to target PARP1 mRNA from nucleotides 3142–3162 was cloned into the AgeI/EcoRI restriction sites of the lentiviral vector pLKO.1-puro. The derived PARP1 shRNA-plasmid and control plasmid pLKO.1-puro-non-target shRNA (scrambled) were used by the viral vectors facility at the Burnham Institute for Medical Research (10901 North Torrey Pines Rd, La Jolla, CA 92037) to obtain lentiviruses delivering, respectively, PARP1-shRNA and scrambled-shRNA. H9 hESCs were infected with the lentiviral particles and selected for 7 days with 2.5 µg/ml puromycin. During selection, hESCs were cultured in feeder-free conditions using MEF conditioned media as described by Xu and colleagues [18].

Flow-cytometric PCD/cell cycle assay

hESC clusters grown in the reported conditions were collected at different time points during the neuralization protocol by centrifugation at 300g for 3'. Cell clusters were washed with PBS and dissociated as single cells with Accutase (Chemicon, 10' at 37°C). Cells were spun again, resuspended in 0.1% Na-citrate, 0.1% Triton X-100 and 50 µg/ml propidium iodide (PI) and analysed by flow-cytometry. DNA content (PI red fluorescence) of each event (cell) was measured to determine the proportion of cells with less than 2n DNA (sub-G1/G0 population), consistent with cells undergoing PCD. To assess the number of cells in G1/G0, S and G2/M phases, data were gated to exclude dead cells from the count. Data were also gated in every case to exclude events with low complexity and size, consistent with cells lacking an integral membrane or contaminant small particles.

Microarray and quantitative-PCR (qPCR) analysis

For microarray-based gene expression analysis, RNA was isolated using Qiagen RNeasy columns. About 500ng total RNA per time point was used for cDNA synthesis followed by cRNA synthesis/amplification and labeling using the "Illumina RNA amplification" kit (Ambion). Labeled cRNA was hybridized to Human WG-6 sentrix BeadChip Arrays (Illumina) and scanned following the manufacturer's instructions. Analysis of results was done using algorithms implemented in the GeneSpring software. Because no data were available regarding catalase (CAT) transcript in our microarray analysis, we used qPCR to evaluate its expression. Total RNA was extracted using the RNeasy kit and 1 µg of total RNA reverse transcribed using the Quantitect kit (Qiagen), according to the manufacturer's

instructions. qPCR for CAT transcript was performed with SyberGreen master mix (Invitrogen) according to the manufacturer's recommendations using the following primers: CACTGAGGTCCACCCTGACT (forward) and GCCTCACAGATTTGCCTTCT (reverse). GAPDH was used for normalization and the data were analyzed using the $\Delta\Delta C_T$ method. GAPDH primers were: GAAGGTGAAGGTCGGAGTC (forward) and ATGGGATTTCCATTGATGAC (reverse).

Superoxide anion quantitation

Superoxide anions indicative of ROS were quantified using the specific probe dihydroethidium (HE, Sigma), which is freely permeable to cells and, once oxidized to ethidium, remains trapped inside cells by DNA intercalation. Cells were washed with PBS and incubated in PBS containing 10 μ M HE at 37°C for 45 min in the dark. Excess HE was removed by washing with PBS, and the oxidized product was detected using fluorescence (510–560 nm) microscopy. Fluorescence was quantified as described in the digital images analysis section.

Determination of cellular active mitochondria content

Intracellular levels of active mitochondria were determined using Mitotracker RED CMXRos (MTRED) (Lonza), which is retained only in active mitochondria. Cells were washed with PBS and incubated in fresh medium containing 250 nM MTRED for 17 min at 37°C. After several PBS washes, cells were co-stained with appropriate markers described in the immunocytochemistry section. Quantification of stained mitochondria was performed as described in the digital images analysis section.

Digital images analysis

Analyses of digital images were performed using the ImageJ software. For superoxide anion quantification, single cell fluorescence was determined after correcting for background intensity using cell-free fields of the same image. For quantification of the intracellular content of active mitochondria, the total area stained with MTRED was determined and normalized by dividing that computed surface by the total number of cells in the field. For sphere circularity determination, the following formula was used: $\text{Circularity} = 4\pi \cdot (\text{area} / \text{perimeter}^2)$. A value of 1 indicates a perfect sphere. At least three independent and randomly chosen fields were used for analysis, for a minimum total of 50 objects (cells or spheres).

Statistical analysis

Experiments were performed at least in triplicate. Statistical analyses were performed using Student's *t*-test with the exception of figure 2e, in which a paired Student's *t*-test was applied. *P* values < 0.05 were considered statistically significant.

RESULTS

Uniform neuralization of hESCs

Feeder-free neuralization of hESCs in a serum-free chemically defined media (CDM) was performed as described (Bajpai *et al.*, *Cell Death and Differentiation* 2009, *in press*). A

schematic of hESC neuralization is presented in supplementary figure S1. hESCs cultured for 5 days as floating clusters in our CDM were allowed to attach on poly-L-ornithine-coated plates. Non-viable spheres did not attach (fig. 1a), whereas viable spheres on day 6 generated a radial migratory cell population (fig. 1b). In line with the default model of neuralization, we found that several well known neuroepithelial markers, namely Nestin, Pax6 and Musashi1, are uniformly expressed in day 6 adherent neurospheres and radial cells, confirming the strong neurogenic character of this population of cells (fig. 1c–e). Recently, a primitive stage of hESC neuralization has been identified [19]. These primitive neuroepithelial cells, termed Rosette-neural stem cells, can be isolated from mouse embryonic neural plate tissue, have a broad differentiation potential, tend to form rosettes (neuroepithelial structures reminiscent of the neural tube) and express several markers not present in adult neural stem cells (termed neural stem cells^{FGF/EGF} due to their strong response to these two growth factors). When we undertook microarray-based gene expression analysis of hESC neuralization, we found that markers of rosette-neural stem cell were upregulated. At the same time markers of neural stem cells^{FGF/EGF} were not up-regulated (fig. 1f). Indeed, rosettes structures were often observed in our cultures (fig. 1g). hESC-derived hNPCs could be induced to undergo terminal differentiation into glia or neurons (fig. 1h) under appropriate conditions (see methods), serving as evidence of multipotency. Importantly, similar to what other investigators have observed [6, 7, 20, 21] our neuralization conditions resulted in substantial cell loss.

PARP1 inhibitors enhance hESC neuralization

Since PARP1 inhibition has proven to be strongly cytoprotective towards mature neurons under conditions such as excitotoxicity, oxidative stress and cerebral ischemia-reperfusion [22–24], we investigated the role of this enzyme in our *in vitro* model of early human neurogenesis. PARP1 was strongly expressed in hESC-derived hNPCs (fig. 2a) and its activity, which accounts for more than 90% of the total PARP activity [25], was readily detectable by probing hNPCs with an antibody against PAR polymers, the product of PARP-catalyzed reaction (fig. 2bII). PARP1 was also expressed in undifferentiated hESCs but at a significantly lower level compared to mature hNPCs (fig. 2a). Consistent with its upregulation during neuralization, PARP1 activity was also almost undetectable in undifferentiated hESCs (fig. 2bI). Considering that PAR polymers are cytotoxic [26] (*via* mitochondrial-nuclear AIF translocation [13]) we investigated the effect of PARP1 inhibition on hESC-derived hNPCs. Neurospheres differentiated in the presence of the PARP1 inhibitors nicotinamide (Nam) or 3-aminobenzamide (3ABA) showed clearly reduced PARP1 activity (fig. 2bIII–IV), consistent with a reduced global level of PAR. The addition of Nam or 3ABA to the differentiation cultures also induced evident morphological changes in neurospheres, namely the appearance of characteristic well-defined edges and a high degree of circularity (fig. 2c–d), properties lacking in hNPCs differentiated in the absence of PARP1 inhibitors. A comparison the total number of hNPCs obtained at day 8 (5 days of differentiation as floating spheres followed by 3 days of growth in adherent conditions) under different experimental conditions (fig. 2e) revealed that morphological changes induced by PARP1 inhibitors result in a significant increase in the efficiency of neuralization (a 3- and 4-fold increase in the total number of hNPCs with 3ABA and Nam, respectively). Notably, withdrawal of growth factors (bFGF and EGF) from the CDM

resulted in decreased neuralization efficiency by approximately 50%, demonstrating that bFGF and EGF enhance the default pathway of neuralization. Combining Nam with growth factors further potentiated hESCs neuralization, resulting in a 4-fold increase in hNPCs compared to the control (a net 8-fold increase compared to the “CDM-no growth factors” condition). Interestingly, addition of Nam to CDM lacking growth factors did not increase neuralization efficiency, suggesting that bFGF and EGF are required to support growth of neural progenitors [27, 28]. We further investigated the effect of Nam by assaying it at two concentrations and using different initial cell densities (supplementary figure S2). At 5 mM, Nam enhanced hESC neuralization under all assay conditions, and its efficiency decreased at low cell density, suggesting that PARP1 inhibition may synergize with the activity of endogenous factors. Without excluding potential pleiotropic effects associated with Nam and 3ABA treatment, our results overall suggest that chemical inhibition of PARP1 positively affects hESC neuralization.

PARP1 inhibition prevents cell death

We next investigated molecular mechanisms potentially underlying PARP1 effects on neuralization. We observed that cells arising from our differentiation protocol were uniformly positive for the neuroectodermal markers Nestin and Musashi1 in the presence or absence of PARP1 inhibitors (fig. 2f), ruling out the possibility that increased cell numbers seen with PARP1 inhibitors result from over-proliferation of non-neuroectodermal derivatives. We next asked whether PARP1 inhibitors promote either increased proliferation of neurospheres and/or a lower cell death rate during the differentiation process. To address this possibility, we performed cell cycle analysis and immunodetection of the mitotic marker Ki67. PARP1 inhibitors did not induce changes in the proportion of hNPCs in each phase of the cycle (supplementary figure S3) or changes in the percentage of Ki67⁺ proliferative cells (fig. 2g). Neurospheres and radial cells were in fact uniformly positive for Ki67⁺ in the presence or absence of PARP1 inhibitors and Ki67⁺ expression was independent of the degree of sphere maturation, underscoring that proliferation remains high throughout the entire neuralization process. A significant decrease in cell death induced by PARP1 inhibition was suggested by the increased number of viable adherent spheres observed in the presence of PARP1 inhibitors (fig. 3a). We followed the kinetics of cell death in our cultures by means of propidium iodide/flow cytometry and found that the propensity to PCD increases in parallel with hESC neuralization (fig. 3b), highlighting enhanced vulnerability of the developing neuroectoderm compared to undifferentiated hESCs. The addition of PARP1 inhibitors significantly rescued hNPCs from cell death (fig. 3b–c), suggesting a primary role played by PARP1 in neuroectodermal cell death in our cultures.

Molecular mechanism of PARP1-mediated cell death

In PARP1-mediated PCD, AIF substitutes for caspases as a cell death executioner [15]. We therefore investigated AIF involvement in hNPC cell death. PCD was readily detectable in day 6 hNPCs as with evidenced by condensed chromatin and fragmented nuclei revealed by Hoechst staining (fig. 4a,4c). Hoechst and AIF co-staining (fig. 4aI) revealed AIF nuclear translocation in a large portion of pyknotic cells, suggesting a role for this cell death executioner in neurosphere PCD. The addition of Nam or 3ABA to the CDM decreased the number of pyknotic cells showing nuclear translocation of AIF (fig. 4a–c), underscoring the

significant cyto-protective effect exerted by PARP1 inhibitors. To validate the role played by PARP1 in hNPC *parthanatos* we used an hESC line stably expressing shRNAi targeting PARP1. PARP1 knockdown (fig. 5a) resulted in a reduced Poly(ADP-ribosyl)ation (fig. 5b) and AIF nuclear translocation in mature hNPCs (fig. 5c–d), confirming PARP1 involvement in hNPC cell death. PARP1 consumes the redox cofactor NAD⁺ to produce PAR: in addition to a direct role of PAR in the pro-parthanatic activation of AIF, another PARP1-mediated trigger for AIF nuclear translocation is reportedly the energetic crisis caused by the high NAD⁺ consumption following PARP1 over-activation [29]. Since nicotinic acid (Na), along with Nam, is the major precursor in the anabolic pathway to NAD⁺ [30], we reasoned that, if NAD⁺ depletion was an important cell death-mediator in our system, addition of Na to neuralizing media would have had a protective effect on hNPCs and enhance neuralization. However, Na treatment did not induce morphological changes in neurospheres observed with PARP inhibitors (fig 2c–d), nor did it enhance neuralization efficiency, (fig. 2e) suggesting that PARP1-mediated cell death during hESC neuralization is independent of energetic failure.

Parthanatos in hNPCs is fuelled by oxidative stress

A major stimulus for PARP1 activity is oxidative stress and DNA damage [24, 31, 32]. Since mitochondria are among the major sources of intracellular genotoxic ROS, and considering that our microarray data suggested increased expression of mitochondrial biogenesis-related genes during the hESCs-hNPCs transition (supplementary figure S4), we used the mitochondrial probe MTRED, which accumulates only in active mitochondria, and the ROS probe dihydroethidium (HE). We observed an increase in active mitochondria content (fig. 6a–b) and oxidative stress (fig. 6c–d) (the latter despite of the presence of antioxidants in our CDM in the form of the B-27 supplement) in parallel with hESC neuralization. Catalase (CAT) and the glutathione peroxidase (GPX) family of enzymes are primarily responsible for intracellular detoxification of ROS. We observed an increase in ROS coupled to a decrease in these endogenous antioxidant systems, as evidenced by downregulation of CAT, GPX2 and GPX3 transcripts (fig. 6e). Other GPX isozymes showed constant levels, indicating comparable expression levels in hESCs and hNPCs (data not shown). Finally, we found that carrying out hESC neuralization at a more physiological [33] oxygen tension (3% O₂) reduced PARP1 activity (fig. 7a) and blocked AIF nuclear translocation (fig. 7b–c). Our results suggest that mitochondrial ROS leakage increases to genotoxic, pro-parthanatic levels during neuralization due to increased oxidative mitochondrial metabolism and its excessive activation fuelled by non-physiological exposure to the normoxic culture environment (20% O₂). Overall, increased intracellular ROS content, PARP1 expression and decreased levels of ROS-detoxifying enzymes during neuralization account for sustained, cytotoxic PARP1 activity (fig. 7d).

DISCUSSION

We identified PARP1/AIF-dependent *parthanatos* as an important molecular mechanism of cell death in undifferentiated neuroepithelial cells during early phases of hESC neuralization that precede development of young neurons. Consistent with our results, substantial cell death has been previously reported during neural differentiation of ES cells [6, 7, 20, 21]. In

particular, Bieberich and colleagues observed apoptosis dependent on endogenous biosynthesis of the sphingolipid ceramide [20], a known activator of AIF nuclear translocation [34, 35]. We observed that blocking PARP1 activity resulted in a robust 4-fold increase in the number of hNPCs by the end of the differentiation protocol. Since PARP1 inhibition did not alter proliferation in our hands, we estimate that overall 75% of hNPCs die by parthanatos by the end of neuralization as a result of progressive susceptibility to PARP1 activation. We therefore propose PARP1 inhibition as a valid strategy to improve hES-neuralizing protocols. Such treatment could facilitate large-scale production of hNPCs needed for the therapeutic exploitation of this promising cell type. In this regard, we note significant advantages of Nam. Unlike other xenobiotic, anti-apoptotic compounds (such as zVAD-fmk), Nam is a vitamin and therefore fully suitable for use as a PARP1 inhibitor in protocols aimed at obtaining therapeutic grade hNPCs. This metabolite, along with Na, is one of the principal NAD⁺ precursors [30] and it is, along with PAR, the principal product of the poly(ADP-ribosyl)ation reaction. It acts on PARP1 through a product-inhibition mechanism, which is also shared with a number of other enzymes (e.g. SIRT1) implicated in self-renewal and differentiation of neural progenitors [36]. It is tempting to speculate that Nam plays a physiological role in the regulation of PARP1-mediated cell death *in vivo* in the neural stem/progenitor cell niche.

We demonstrated that administration of a single metabolite resulted in a very robust and uniform population of hNPCs under serum-free conditions. We speculate that these results are, at least in part, due to the restoration of the “metabolic niche” allowing stem/progenitor cell survival. It is also possible that metabolic niches are a vital part of stem cell microenvironment, which, as we demonstrated here, is amenable to manipulation using physiological small molecule metabolites. Giving that oxygen is essential for aerobic respiration, it can be considered a critical metabolite. It has been estimated that the oxygen tension in mammalian brain ranges between 0.55% and 8% [33]. Indeed we observed a profound effect of oxygen tension in regulating PARP1 activity and *parthanatos* in hNPCs. Carrying out hESC neuralization under a more physiologically relevant oxygen tension such as 3% O₂ could in fact decrease PARP1/AIF mediated cell death, further supporting the concept of a metabolic hNPC niche able to provide optimal survival. Indeed, the cytoprotective effect of culturing hNPCs in low oxygen has been reported by several groups [37, 38]. Mechanistically, our data suggest that the low oxygen tension effect on hNPCs involves blockade of the PARP1/AIF death cascade. Even though we found that Nam and low oxygen converge to inhibit PARP1, we cannot exclude the possibility that other pro-survival mechanisms are associated with both Nam and low oxygen pathways. Metabolites can in fact exert a very heterogeneous effect on cell physiology and affect simultaneously several pathways depending on their concentration. Therefore, additional experiments are required to confirm the presence of the postulated “metabolic NPC niche” and to elucidate its properties.

Finally, we found that in our uniform model of early human neurogenesis, the developing neuroepithelium acquires a progressive susceptibility to oxidative stress and cell death paralleling maturation of hESCs toward the neuroectodermal lineage.

Programmed cell death was previously reported *in vivo* during vertebrate neurogenesis where it is required to properly sculpt the developing nervous system [39]. Indeed, in mouse more than half of initially generated neurons undergo apoptosis at later stages of development [39]. Here we provide evidence that during hESC neuralization the cell death machinery is activated, likely due to non-physiological oxygen levels that could mimic sustained pathological oxidative stress. We speculate that during normal development early neuroepithelial cells might be selectively susceptible to pathological conditions and prone to *parthanatos*. PARP1 was previously identified as a key player in pathological PCD *in vivo* in differentiated cells and shown to be activated in response to stressors such as ischemia/reperfusion, excitotoxicity, and oxidative stress [22–24], supporting the hypothesis that it could be involved in pathological PCD at early stages of human neural development. In fact, oxidative stress is suggested to play a key role in development of brain lesions that during early human neurogenesis ultimately result in cerebral palsy, the most common childhood neuromotor disability [40]. Indeed, in clinical studies, increased lipid peroxidation and decreased antioxidant capacity have been found in cerebral palsy patients compared to healthy individuals [41]. Furthermore, autopsy of periventricular leukomalacia brains (a major cause of cerebral palsy) show increased lipid peroxidation in oligodendrocyte precursors compared to non-periventricular leukomalacia brains [42]. Considering these studies, our results suggest possible involvement of a parthanatic demise of the neural progenitor pool in early developmental neurological lesions and suggest that the neuroectodermal cell lineage might be a major target of oxidative stress. We further speculate that PARP1 inhibition during pregnancy might be a valid strategy to prevent cerebral palsy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of hESC-derived hNPCs

(a) Inviable spheres do not adhere to poly-L-ornithine-coated plates. (b) An isolated adherent viable sphere (*) generating radial neuroectoderm (**). (c–e) Immunostainings for the neuroepithelial markers Nestin (c), Pax6 (d) and Musashi1 (e) in day 6 hNPCs. Each figure shows results obtained in spheres (bottom row) and radial cells (top row). Nuclei were counterstained with Hoechst dye. (f) Microarray data show increased expression of rosette-neural stem cell markers LIX1 and LEF1 (right graph, red bars) and no up-regulation of the neural stem cell^{FGF/EGF} markers OLIG1 and AQP4 (left graph, blue bars) in day 6 hNPCs. Data are expressed as fold increase compared to undifferentiated hESCs. (g) Hoechst staining of hESC-derived hNPCs shows their tendency to form rosettes (arrows). (h) hESC-derived hNPCs show multipotency by their capacity to terminally differentiate into astrocytes and neurons (GFAP and Map2ab staining, respectively). Scale bars: a–b = 400 μ m, c–e and g–h = 50 μ m.

Figure 2. PARP1 inhibition enhances hESC neuralization without affecting proliferation or differentiation

(a) PARP1 is detectable by immunocytochemistry in both hNPCs (right) and, at a lower level, in hESCs (left); scale bars = 100 μ m. (b) Immunodetection of PAR in undifferentiated hESCs (I) and in mature neurospheres derived under reported conditions. PARP1 activity is readily detectable in hNPCs maturing in CDM (II) and ostensibly decreases in the presence of PARP1 inhibitors (III and IV). In agreement with lower PARP1 expression seen in hESCs, its activity was almost undetectable before neural induction (I). Insets are western

blots showing increased levels of high-molecular weight poly(ADP)-ribosylated proteins, confirming immunocytochemical analyses. Loading control (bottom panel in each inset) is γ -Tubulin. Bottom edges of upper insets correspond to 150 kDa, whereas γ -Tubulin is 48 kDa. Scale bars in figures a–b = 100 μ m. (c) Phase contrast images of day 6 neurospheres maturing under reported conditions. Note major morphological changes in hNPCs derived in the presence of PARP1 inhibitors (Nam and 3ABA). Addition of Na does not alter neurosphere morphology. Scale bars = 400 μ m. (d) Quantification of morphological changes induced by PARP1 inhibitors in terms of sphere circularity (see methods for details). (e) Neuralization efficiency, evaluated as fold increase in the total number of derived hNPCs compared to the standard protocol, increases in the presence of PARP1 inhibitors Nam and 3ABA. Note that withdrawal of growth factors (NoGFs) halves the efficiency of neuralization, and Nam treatment cannot increase the number of derived hNPCs in the absence of growth factors (NoGF-Nam), even following addition of Na. In figures d–e * P <0.001 and ** P <0.05 compared to control. (f) Immunodetection of the neuroectodermal markers Nestin and Musashi1 in day 6 mature hNPCs in the presence (right panel) or absence (left panel) of the PARP1 inhibitor Nam indicates no alteration in the differentiation fate of hESCs. Scale bar = 50 μ m. (g) Cell proliferation during hESCs neuralization evaluated by immunocytochemical detection of the mitotic marker Ki67 in immature (day 1, upper panel) and mature (day 6, bottom panel) neurospheres in the presence (left panel) or absence (right panel) of Nam shows highly proliferative spheres independent of their degree of maturation and addition of PARP1 inhibitors. Scale bars = 100 μ m. For (f) and (g) identical results were obtained using 3ABA instead of Nam (data not shown).

Figure 3. Cell death in neuralizing hESCs parallels the maturation process and is blocked by PARP1 inhibitors

(a) The PARP1 inhibitor Nam increases the number of viable spheres able to adhere on poly-ornithine-coated plates (right) compared to untreated controls (left). Scale bar = 400 μm . The graph at right shows quantification of adherent spheres under reported conditions. (b) Kinetics of PCD during hESC neuralization evaluated by PI staining and flow-cytometric analysis of intracellular DNA content. Note that cell death increases in parallel with maturation of hESCs toward the neural lineage, and PARP1 inhibitors effectively block it. (c) Representative cell cycle/PCD flow-cytometric profiles of day 3 and day 6 neurospheres showing the cytoprotective effect of PARP1 inhibitors. The sub-G1 hypodiploid (apoptotic/parthanatic) population of cells is marked with a blue asterisk (*).

Figure 4. Nuclear translocation of AIF and cell death in hESC-derived hNPCs

(a) Immunodetection of AIF (red) in dissociated, mature (day 6) neurospheres grown in the reported conditions shows its nuclear translocation in pyknotic cells (bright condensed or fragmented nuclei, Hoechst blue nuclear counterstaining). White and yellow arrows indicate respective examples of mitochondrial perinuclear (red color) and nuclear (pink color, overlapping red and blue channels) AIF staining. Red arrows point to apoptotic cells lacking nuclear AIF. Note that addition of PARP1 inhibitors strongly reduces the number of pyknotic cells showing nuclear AIF. Scale bars = 100 μ m. (b) Quantitation of AIF nuclear translocation in reported conditions. * $P < 0.001$ and ** $P < 0.05$ compared to control. (c) High magnification images of different cellular subtypes according to PCD and AIF localization: (I) a non-apoptotic/parthanatic cell, (II and III) parthanatic cells (condensed and fragmented nuclei, respectively) whose PCD is PARP1/AIF-dependent as evidenced by AIF nuclear localization, and (IV and V) apoptotic cells (condensed and fragmented nuclei, respectively) whose PCD is PARP1/AIF-independent. Scale bar = 5 μ m.

Figure 5. PARP1 genetic knockdown decreases AIF-dependent cell death

(a) shRNAi strongly reduces PARP1 expression (right) compared to shRNAi off-target control (scrambled, left image). Scale bars = 100 μ m (b) Western blot against PAR in hNPCs showing that shRNA-mediated PARP1 knockdown almost completely abolishes PARP1 activity. (c) AIF immunodetection (red) showing that shRNAi against PARP1 decreases the number of cells showing nuclear AIF (pink color, overlapping red and blue channels) compared to scrambled control. Scale bars = 100 μ m. (d) Quantification of AIF nuclear translocation in the reported conditions. * P <0.001.

Figure 6. Oxidative stress and mitochondrial respiration increases with hESC neuralization
 (a) Mitotracker RED CMXRos (MTRED)-based staining for active mitochondria in hESCs and hNPCs. Immunolabelling of Oct4 (hESC nuclear marker) and Pax6 (hNPC nuclear marker) counterstains nuclei in hESCs and hNPCs, respectively. Scale bar = 100 μ m. (b) Quantification of data shown in figure a. * $P < 0.001$ (c) Representative fluorescent photomicrographs of hESCs probed for superoxide anions ($O_2^{\bullet-}$) with dihydroethidium (HE) at different time points during the neuralization process. Scale bar = 100 μ m. (d) HE-based $O_2^{\bullet-}$ quantitation in hESCs during neuralization. Note increased oxidative stress during the hESCs-hNPCs transition. (e) Gene expression analysis shows downregulation of ROS-detoxifying enzymes CAT, GPX2 and GPX3 during hESC neuralization.

Figure 7. Hypoxia reduces PARP1/AIF-mediated cell death

(a) Western blot showing reduced levels of PAR in hESCs neuralized in 3% O_2 compared to standard normoxic (20 % O_2) conditions. (b) AIF immunodetection (red) showing that low oxygen tension (3% O_2) decreases the number of cells showing nuclear AIF (pink color, overlapping red and blue channels) compared to 20% O_2 . Scale bar = 100 μm . (c) Quantification of AIF nuclear translocation in reported conditions. * $P < 0.05$. (d) Model of PARP1-mediated cell death during hESCs neuralization. Early phases of hESC neuralization are characterized by increased mitochondrial respiration, ROS production and PARP1 expression, which, coupled to lower expression of ROS-detoxifying enzymes, results in a sustained, cytotoxic PARP1/AIF activation.