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Derivation of neural precursor cells from human ES cells at 3% O₂ is efficient, enhances survival and presents no barrier to regional specification and functional differentiation

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In vitro stem cell systems traditionally employ oxygen levels that are far removed from the *in vivo* situation. This study investigates whether an ambient environment containing a physiological oxygen level of 3% (normoxia) enables the generation of neural precursor cells (NPCs) from human embryonic stem cells (hESCs) and whether the resultant NPCs can undergo regional specification and functional maturation. We report robust and efficient neural conversion at 3% O₂, demonstration of tri-lineage potential of resultant NPCs and the subsequent electrophysiological maturation of neurons. We also show that NPCs derived under 3% O₂ can be differentiated long term in the absence of neurotrophins and can be readily specified into both spinal motor neurons and midbrain dopaminergic neurons. Finally, modelling the oxygen stress that occurs during transplantation, we demonstrate that *in vitro* transfer of NPCs from a 20 to 3% O₂ environment results in significant cell death, while maintenance in 3% O₂ is protective. Together these findings support 3% O₂ as a physiologically relevant system to study stem cell-derived neuronal differentiation and function as well as to model neuronal injury.
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The capacity of human embryonic stem cells (hESCs) to generate defined neuronal and glial lineages offers a major opportunity to study neurodevelopment and model neurological disease *in vitro*, as well as having potential direct therapeutic applications in the field of regenerative neurology. Notwithstanding major advances in hES neural specification and differentiation over the last decade, there remain significant challenges to overcome before the promise of hESCs for neurological diseases can be fully realised.[1–4] These include the need to optimise survival, fate and function of neural derivatives upon both neural conversion and long-term differentiation *in vitro* and *in vivo*.

Neural stem cells (NSCs) can be readily generated from ESCs by culture in defined conditions in the absence of extrinsic signals; the so-called default model of neuralisation.[5] However, significant cell death is observed under such serum-free, defined conditions.[6] The mechanism through which the cells die involves both apoptotic and parthanatic pathways,[6–10] accompanied by the generation of reactive oxygen species (ROS).[10] Consequently, neuralisation protocols often contain antioxidants, which may increase the propensity to accumulate genetic mutations, or involve co-culture with stromal feeder layers.[2,6,7,10–12] In addition, antagonism of the TGF-β signalling pathway has been shown to augment the efficiency of neural conversion and thereby increase survival; however, this can also influence the default identity of neural progenitors and potentially limit their ability to be directed towards defined cell types.[13,14]

The importance of ROS in mediating cell death during neural conversion under routine culture at oxygen (O₂) levels of 20%, which is far removed from than that found under physiological conditions in the central nervous system (CNS), suggests higher oxygen tension may be deleterious to neural specification and differentiation.[7,10] In the CNS, oxygen levels vary from 8% at the pia to 0.55% in the midbrain, with measurements from the human brain recording a mean level of 3.2% at 22–27 mm below the dura and 4.4% at 7–12 mm.[15,16] Overall, the mean tissue level of oxygen in adult organs is about 3%, although it is considerably less in the developing embryo where stem cells abound.[17] There is a growing literature around the critical influence of oxygen levels on stem cell fate, proliferation and survival.[7–10,12,17–27] Furthermore, oxygen has been proposed to act as a developmental morphogen;[24] low oxygen promotes tyrosine hydroxylase positive dopaminergic neurons from midbrain neural precursor cells (NPCs) and oligodendrocyte differentiation from human fetal NPCs.[9,18,23] In addition, oxygen tension is thought to be tightly regulated in the stem cell niche and it is thought that changes in the partial pressure of oxygen

(pO_2) contribute to the mobilisation of stem cells in an injury response.[25–27]

In view of the importance of low pO_2 in maintenance of pluripotency, mediated in part through Notch signalling and upregulation of Oct-4, it remains unclear as to whether low O_2 interferes with both neural conversion of hESCs and subsequent neuronal differentiation of hESC-derived NPCs.[21,22] Mouse ES studies suggest that culture at 4% O_2 does not limit neural conversion or terminal differentiation.[7] Furthermore, our understanding of the effect of low, physiological levels of oxygen on hESC-derived neuronal sub-type specification, as well as long-term differentiation and function, is incomplete. One prediction from rodent and human fetal literature is that low oxygen could enable longer-term culture of differentiated progeny.[28] A benefit of longer-term culture under physiological oxygen levels is that this would allow more accurate disease modelling paradigms, particularly for neurodegenerative diseases in which ROS and oxidative stress have been widely postulated to have a role in cell death.[29,30] Moreover, for both disease modelling and pre-clinical assessments, a key functional assay of neuronal derivatives requires transplantation. Given that routine transplantation studies cause, in effect, a stress challenge consequent on an oxygen switch from 20% to ~3–4% upon transplantation, it would be of considerable value to model the effect of such a 'switch' *in vitro*.

Against this background, we sought to investigate whether 3% O_2 can allow the generation of NPCs from hESCs, the timescale involved and whether the resultant NPCs can undergo regional specification and functional maturation.

Finally, we examine the survival of these NPCs following transfer from a 20 to 3% O_2 environment, providing an *in vitro* model of the oxygen challenge that occurs during transplantation.

Results

NPCs can be reliably and efficiently derived from hESCs in a 3% O_2 environment.
To address whether hESC-NPCs could be efficiently derived in low oxygen conditions, feeder-free hESCs, grown in a chemically defined medium (CDM)[31–33] at 20% O_2, were enzymatically detached and transferred to suspension culture at 3% O_2, along with removal of activin and FGF-2. A pimonidazole-binding assay was used to biochemically confirm growth of cells at low oxygen; pimonidazole adducts on the surface of hypoxic cells, binding most efficiently at a pO_2 <10 mm Hg (Figure 1d).[34] Over 14 days, efficient neural conversion was confirmed by quantitative immunolabelling that revealed loss of expression of the pluripotent marker OCT4 ($1.1 \pm 0.7\%$ positive) with concomitant upregulation of the neuroepithelial markers SOX1 ($98.7 \pm 0.5\%$) and NESTIN ($97.4 \pm 0.3\%$), and maintenance of the stem cell marker SOX2 (Figures 1a–c). There was no significant difference between the efficiency of neural conversion at 3 and 20% O_2, with a neural identity acquired by D14 in both instances (Figure 1c), consistent with previous reports of a 2-week timescale for neural conversion of hESCs at 20% O_2.[14,33,35,36] Neural conversion at 3% O_2 was robust, highly reliable and reproducible across two independent hESC
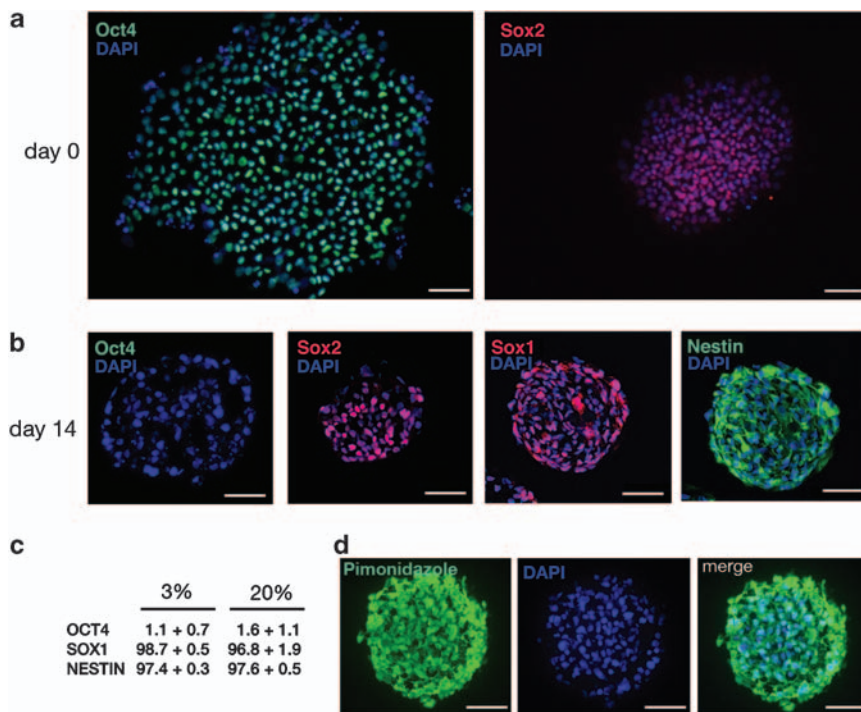
Figure 1 hESCs can generate NPCs in 3% O_2. (**a**) hES cells were uniformly positive for the embryonic stem cell markers OCT4 and SOX2 at day 0. (**b**) OCT4 is lost at the protein level by day 14 ($1.1 \pm 0.7\%$ positive), and cells were positive for the stem cell marker SOX2 and the neural stem cell markers SOX1 ($98.7 \pm 0.5\%$) and NESTIN ($97.4 \pm 0.3\%$). (**c**) Comparative quantification between 3 and 20% O_2 demonstrated equivalence in the efficiency of neural conversion at D14. (**d**) Pimonidazole staining confirmed the low O_2 environment. Scale bar $= 50\,\mu m$

lines, irrespective of whether feeder-dependent (H9, $n=5$; HUES9, $n=10$) or feeder-free (H9, $n=10$).

Improved survival with neuralisation of hESCs at 3% O_2 compared with 20% O_2. Having established that hES-NPCs can be reliably generated at 3% O_2, this system was compared with the standard of 20% O_2. Cultures appeared healthier at 3% O_2, with more rounded, brighter spheres and fewer dead cells (Supplementary Figure 1A). Growth curves confirmed a significant increase in numbers of NPCs generated at 3% compared with 20% O_2, under basal culture conditions (Figure 2a; day 21, $P=0.00115$). Furthermore, at D14, a greater proportion of cells grown at 3% O_2 (82.9%) were viable compared with 20% O_2 (66.7%), demonstrated though propidium iodide (PI) and annexin V exclusion (Figure 2b). The protective effect of 3% O_2 could be partly reproduced by the addition of the antioxidant *N*-acetyl-L-cysteine (NAC) (1 μM) to 20% O_2 cultures, resulting in an increase of viable cells to 75.5% at D14 (Supplementary Figure 1B).

The intracellular response to low oxygen is coordinated by the hypoxia-inducible factors (HIFs), with low oxygen stabilising the α-subunit, allowing for a rapid response to changes in oxygen that does not rely upon alterations in mRNA expression.[37] A time course analysis revealed stabilisation of HIF-1α protein was transient; it appeared within 6 h, was maximal at 24 h and became undetectable by 6 days (Figure 2c). By contrast, HIF-2α stabilisation demonstrated a delayed kinetic compared with HIF-1α, protein levels being upregulated as HIF-1α is downregulated at day 3, with HIF-2α expression persistent at D14 (Figure 2c).

hESC-NPCs derived at 3% O_2 display tri-lineage phenotypic potential and such cultures can be maintained for up to 3 months in the absence of exogenous growth factors. To address whether low oxygen levels would prevent differentiation of hESC-derived NPCs,[21] cells were plated on poly-D-lysine-laminin-coated coverslips in the absence of FGF-2 at day 30 and maintained long term at 3% O_2. The neuronal marker β-III tubulin was observed by 24 h, synapsin at 48 h and the more mature neuronal marker MAP2a + b was expressed by 5 days post plating (Figure 3a). To confirm functional maturation, electrophysiological studies were undertaken that revealed MAP2 + β-III tubulin + neurons generated action potentials, as early as 10 days post plating ($n=6/6$, Figure 3d). Recordings performed 30 days after plating demonstrated more mature-looking action potentials ($n=25/27$), which were inhibited by the addition of the voltage-gated sodium channel blocker tetrodotoxin (TTX) ($n=16$, Figure 3b). The frequency of these action potentials depended on the amount of current injected (Figure 3c), as expected for functional neurons.[38] There was also evidence of spontaneous activity in neurons after 30 days of differentiation at 3% O_2 (Figure 3e). These findings were remarkably similar to the electrophysiological maturation of neurons observed at 20% O_2 (Supplementary Figure 2), clearly demonstrating that there was no maturation block at low oxygen.

In addition to neurons, long-term differentiated cultures at 3% O_2 consistently contained GFAP-positive astrocytes and O4/MBP expressing oligodendrocytes (Figure 3f). Furthermore, and in contrast to this and previous studies at 20% O_2, we found that healthy differentiated cultures could be maintained for at least 3 months in basal B27-supplemented medium in the absence of exogenous neurotrophins.[2,39]

Directed differentiation of 3% O_2-derived NPCs into midbrain dopaminergic and spinal motor neurons. A key aspect of hES-NPCs is their competence to respond to developmental cues that direct differentiation into defined cell
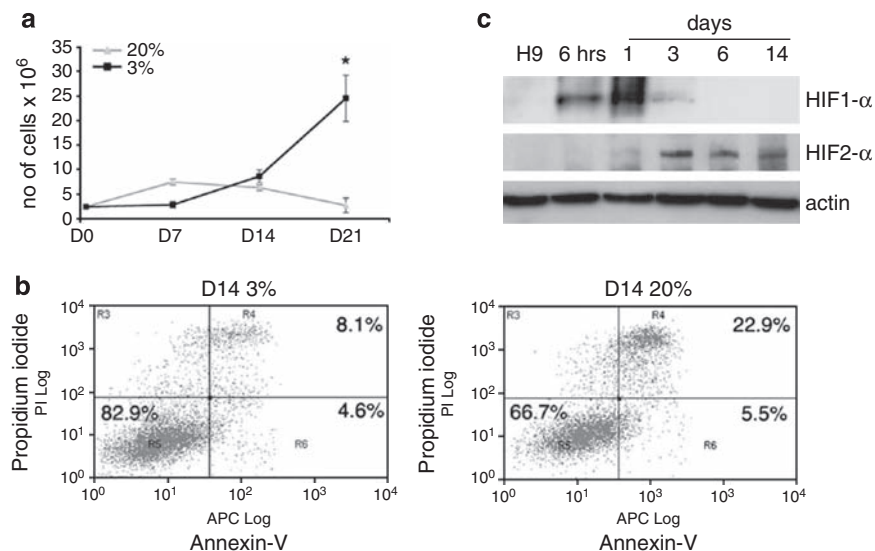
Figure 2 The 3% O_2 system carries a significant survival advantage when compared with traditional methods at 20% O_2. (**a**) Growth curves at 3 and 20% O_2 show a lag phase in growth at 3% between D0 and D7, but a highly significant increase in cell numbers in 3 *versus* 20% by D21 ($P=0.00115$). (**b**) Enhanced viability of the 3% NPCs compared with those derived at 20% was observed by flow cytometry analysis of PI and annexin V exclusion. (**c**) Western blot analysis of cells neuralised at 3% O_2 showed that the HIF-1α response is transient, demonstrating stabilisation of protein level by 6 h, which is maximal at 24 h before disappearing completely by 6 days. By contrast, HIF-2α stabilisation is detectable by 3 days and is maintained at 14 days
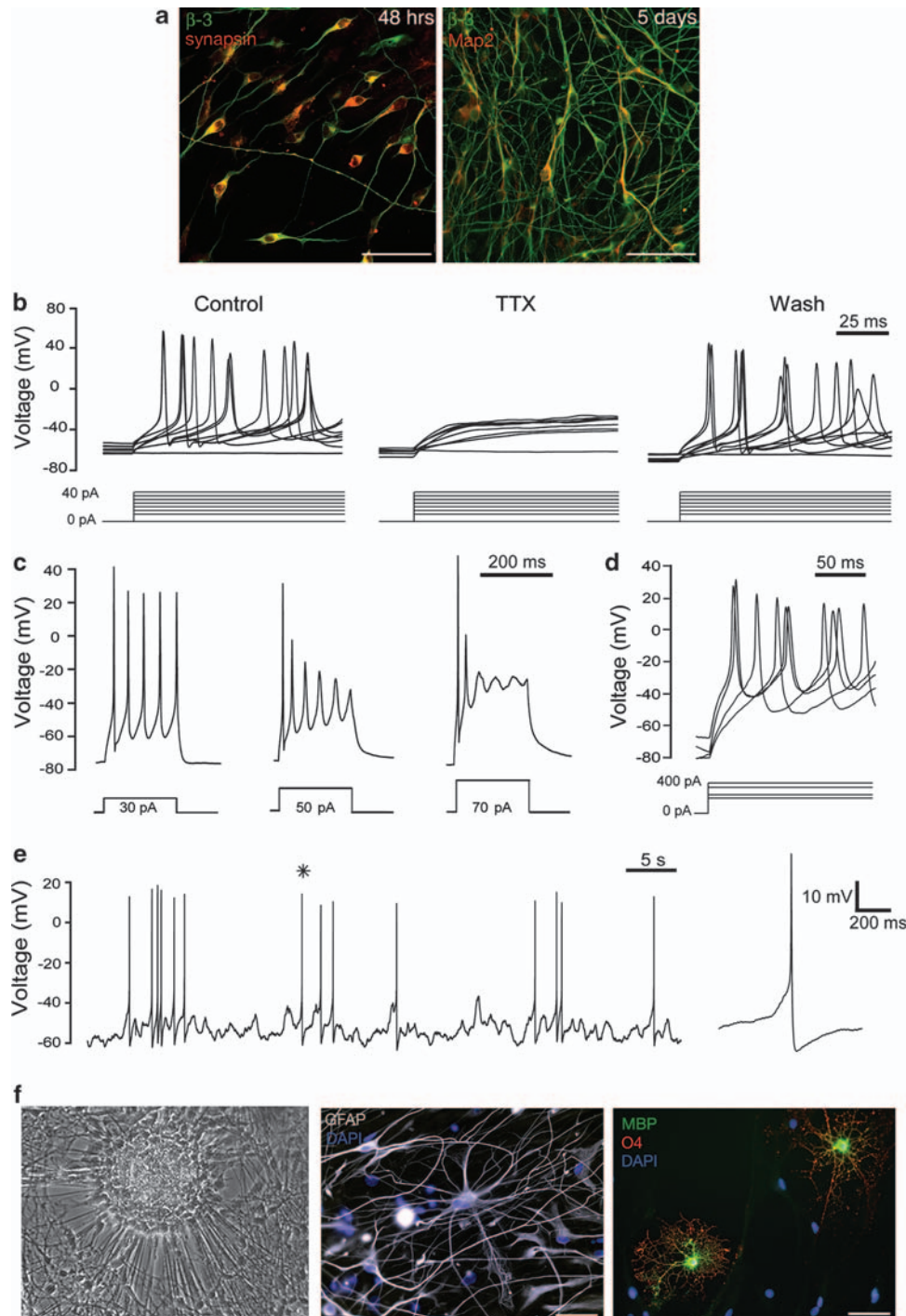
Figure 3 NPCs generated at 3% O_2 differentiate and mature into glia and electrically functional neurons. (**a**) Removal of FGF-2 led to the differentiation of NPCs generated at 3% O_2 into β-III tubulin-positive neurons, with expression of synapsin by 48 h and MAP2a + b by 5 days post plating. (**b**) Action potentials were reversibly blocked by the sodium channel blocker TTX (30 days post plating, $n = 16$). (**c**) Representative response of a neuron to increasing current injection at 30 days post plating. (**d**) These neurons fired action potentials as early as 10 days post plating ($n = 6/6$). (**e**) Spontaneous action potentials were detected in neurons after 30 days of differentiation. Asterisk denotes the action potential, which is magnified on the right. (**f**) NPCs were also able to generate GFAP + ve astrocytes and MBP and O4 + ve oligodendrocytes, shown after 8 weeks of plating. Scale bar = 50 μm

types. To determine whether 3% O_2-derived NPCs retain this competence, we next applied existing protocols for directed differentiation of hES-NPCs into spinal motor neurons and midbrain dopaminergic neurons.[11,35,36] Motor neuron specification was achieved through sequential application of 0.1 μM retinoic acid (RA) for 1 week to specify a caudal, neuronal identity, followed by a further week of RA with 1 μM purmorphamine to ventralise the cells. RT-PCR confirmed

Figure 4 NPCs derived at 3% O_2 can be differentiated into midbrain dopaminergic and spinal motor neurons. (**a**) Spinal motor neurons were generated through the application of 0.1 μM RA (D14–D21) to caudalise the cells (i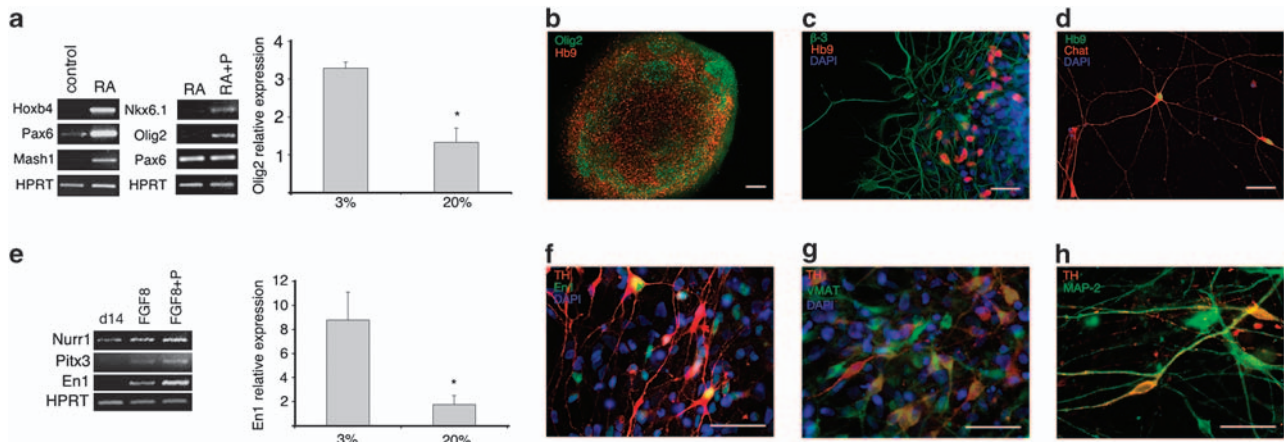nduction of *HOXB4*, *PAX6* and *MASH1*), followed by RA and 1 μM purmorphamine (D21–D28) to ventralise them (induction of *NKX6.1* and *OLIG2*). Quantitative RT-PCR demonstrated a twofold increase in expression of *OLIG2* at 3 *versus* 20% O_2. (**b** and **c**) HB9 and β-III positive motor neurons emerged following plating in the absence of growth factors for 2–10 days. (**d**) These HB9 positive motor neurons were also positive for ChAT at 10 days. (**e**) RT-PCR after sequential addition of 100 ng/ml FGF-8 alone for 1 week and FGF-8 with 1 μM purmorphamine for 1–2 weeks demonstrated induction of the midbrain marker *EN1* and *NURR1* and *PITX3*, which are required for the development of substantia nigra dopaminergic neurons. Quantitative RT-PCR showed a fivefold greater induction of EN-1 at 3% O_2. (**f**) Co-expression of EN-1 and tyrosine hydroxylase. (**g**) Resultant tyrosine hydroxylase positive neurons also stained for VMAT and (**h**) MAP2a+b. Asterisk denotes a significant difference $P < 0.05$. Scale bar = 50 μm apart from panel **b** (100 μm)

induction of *HOXB4* and *MASH1* with upregulation of *PAX6*, followed by induction of *OLIG2* and *NKX6.1* (Figure 4a). Immunolabelling demonstrated expression of HB9, a transcription factor specifically expressed by post-mitotic motor neurons (Figures 4b and c) and, importantly, ChAT expression was also observed by 10 days (Figure 4d).

Similarly, midbrain dopaminergic specification was achieved following sequential application of 100 ng/ml FGF-8 for 1 week and FGF-8 with 1 μM purmorphamine for 1–2 weeks. PCR characterisation showed expression of the midbrain marker *EN1*, along with *PITX3* and *NURR1*, which are required for the development of substantia nigra dopaminergic neurons (Figure 4e). Immunolabelling also revealed EN-1 and tyrosine-hydroxylase (TH)-positive neurons (Figure 4f). A proportion of TH + neurons also stained for MAP2a + b, and co-expressed vesicular monoamine transporter (VMAT2), which is required for packaging dopamine into sub-cellular compartments in monoamine neurons (Figures 4g and h). As with non-patterned neurons, specified dopaminergic and motor neurons were readily cultured for at least 30 days without the requirement for exogenous BDNF, GDNF, IGF, ascorbic acid or cAMP at 3% O_2.

The relative efficiency of induction of midbrain dopaminergic and spinal motor neurons at 3 *versus* 20% O_2 was addressed by quantitative RT-PCR analysis of expression of *OLIG2* and *EN-1*, which are specific progenitor markers of motor neurons and midbrain dopaminergic neurons, respectively. This revealed a twofold increase in the expression of *OLIG2* at 3% O_2, and furthermore, a fivefold increase in *EN-1* induction at day 28 (Figures 4a and e).

In vitro switch simulation of transplantation from 20 to 3% O_2 leads to NPC death, while maintenance at 3% O_2 is protective. To model the oxygen challenge that occurs with transplantation, *in vitro* 'switch' experiments were

performed. Single cell dissociates from day 30 NPCs derived both at 3 and 20% O_2 were shown to be comparable by FACS analysis, that demonstrated uniform co-expression of MUSASHI and SOX2: 99.6% (3%) and 99.8% (20% O_2) (Figure 5a) with absence of pluripotency and other germ layer markers (Supplementary Figure 3). PI exclusion without annexin V binding also demonstrated comparable levels of viability at day 30 of 3 and 20% O_2-derived NPCs (Figure 5b). This finding is consistent with the view that it is the neuralisation process itself that imposes maximal stress,[10] with subsequent longer-term maintenance of NPCs being more readily achieved under either 3% or 20% O_2.

Having confirmed equivalence in terms of neural identity and viability, NPCs were then plated as dissociated single cells in the absence of growth factors and maintained at either 20 or 3% O_2, or switched from 20 to 3% O_2. Ethidium bromide (dead cell) and calcein (live cell) staining at 48 h showed that the survival of differentiating cells swapped from 20 to 3% O_2 was much worse than cells maintained at 3% O_2 (57 $\pm$ 2.4 % *versus* 79.9 $\pm$ 2.4%, $P = 0.0001$), with cells remaining at 20% O_2 intermediate between the two groups (67 $\pm$ 0.7% *versus* 79.9 $\pm$ 2.4%, $P = 0.0033$) (Figure 5c). Together, these findings strongly suggest that switching cells from high to low oxygen levels results in significant cell death that can be prevented by maintaining cells throughout at physiological levels of oxygen.

Discussion

We report that a physiological, 3% O_2 environment does not present a barrier to the generation of tri-potential NPCs from human ESCs, or to their specification into midbrain dopaminergic and spinal motor neurons, the efficiency of which is markedly enhanced at low oxygen. Furthermore, compared with basal conditions at 20% O_2, the application of a defined,
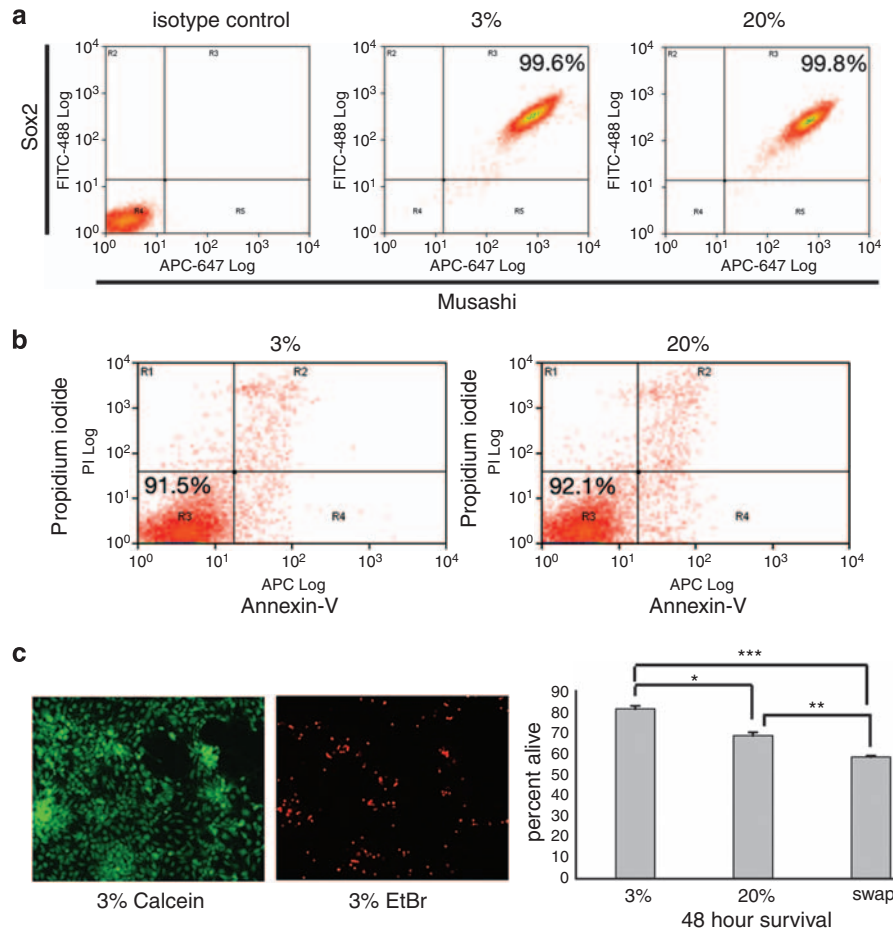
Figure 5 Switching cells from 20 to 3% O$_2$ leads to cell death. (**a**) Flow cytometry characterisation of D30 NPCs showed that cells derived at both 3 and 20% O$_2$ uniformly expressed the neural precursor cell markers MUSASHI and SOX2. (**b**) Annexin V and PI flow cytometry analysis at D30 showed that NPCs propagated at 3 and 20% O$_2$ were equally viable, with 92% of cells negative for both markers. (**c**) Representative ethidium bromide and calcein staining of NPCs plated for differentiation for 48 h at 3% O$_2$ in the absence of growth factors; cells from the same field of view are shown. Cell counts showed that these differentiating cultures survived better at 3% than at 20% O$_2$, with those that were swapped from 20 to 3% faring worst of all. *$P = 0.0033$, **$P = 0.0024$ and ***$P = 0.0001$

feeder-free neuralising system to this low oxygen environment results in the generation of greater numbers of NPCs, and, upon differentiation, allows the establishment of mixed cultures of neurons and glia that can be maintained for at least 3 months, without the requirement for exogenous growth factors. Significant cell death was observed on switching differentiating NPCs from a high to low oxygen environment, modelling the oxygen challenge presented by transplantation.

The cellular response to low oxygen is co-ordinated by the three HIF isoforms, with HIF-1α believed to be the global regulator of the hypoxic response. HIFs are heterodimeric DNA-binding proteins with α- and β-subunits. At 20% O$_2$, the α-subunits are degraded, whereas at low O$_2$, HIF-α is stabilised, allowing binding to the HIF-β/ARNT subunit and activation of target genes, which are involved in a myriad of diverse functions, including metabolism, angiogenesis, survival and migration.[37] Previous reports that HIF-1α interacts with Notch,[21] and that HIF-2α regulates *Oct4*,[22] might suggest that low O$_2$ would maintain pluripotency in ESCs and present a barrier to efficient neural induction and terminal differentiation. However, we observed that at 3% O$_2$, efficient neuralisation was completed by 14 days, a timescale

comparable to that at 20% O$_2$,[14,33] indicating that a low O$_2$ environment does not adversely affect acquisition of a neural fate. Additionally, under basal conditions, significantly more NPCs were generated at 3% than at 20% O$_2$, with a greater proportion of viable cells; a finding consistent with a previous study based on feeder and matrigel-maintained human ES cells, reporting a decrease in parthanatic cell death in neurectoderm derived at 3% O$_2$.[10] In agreement with the studies on mouse ES cells[7] and cortical NSCs,[8,9] the addition of NAC to neuralising conditions at 20% O$_2$ could partly reproduce the beneficial effect of low O$_2$, suggesting that ROS contribute to cell death during neural conversion. Furthermore, 3% O$_2$ did not prevent or delay neuronal or glial differentiation of hESC-NPCs, and in particular, the speed of electrophysiological maturation of neurons was remarkably similar in both the low and high oxygen environments.

The finding of an interaction between HIF-1α and Notch, promoting the stem cell state and inhibiting differentiation (including into β-III tubulin-positive neurons), was based on observations on the embryonic carcinoma line P19, myogenic C2C12 cells and embryonic rat NSCs cultured at 1% oxygen, for a matter of hours.[21] This is a rather more extreme pO$_2$ than

that found physiologically, and the time course examined does not exclude a later downregulation of HIF-1α. Indeed, we found that HIF-1α was only transiently stabilised, appearing within 6 h and disappearing completely by 6 days, correlating to the lag phase in the growth curve at 3% O_2. In the majority of previous reports, the time course of the HIF response has not been fully examined, simply demonstrating the stabilisation of HIF-1α upon transfer to low O_2 conditions at up to 3 days,[1,21,23] but the later disappearance of HIF-1α has been commented upon twice before, in studies based on hESCs.[19,40] We propose that this transient HIF-1α stabilisation represents an adaptive response to the low O_2, whereas HIF-2α maintains a physiological response.[19] This later appearance and persistence of HIF-2α may also contribute to the increased numbers of NPCs generated at 3% O_2: HIF-2α inhibits the p53 pathway and also regulates *SOD1*, *SOD2*, *GPX1* and *CATALASE* expression,[41] so is well placed to modulate the survival of NPCs. Moreover, HIF-2α appears to have a critical role in the proliferation of neuroblastoma cells,[41] and the ability of our system to isolate the downstream effects of HIF-2α from HIF-1α could provide further insights into this observation, as well as into the mechanisms of maintenance of endogenous NSCs.

In addition to our finding of efficient neural conversion and tri-lineage differentiation we also observed that 3% O_2 allowed long-term maintenance of healthy, mixed differentiated cultures for over 3 months, in the absence of any exogenous neurotrophins that are typically required in cultures differentiated at 20% O_2 for considerably shorter periods such as to 28 days.[2,39] This observation is supported by a report that mouse cortical neurons thrive at 1% O_2, with enhanced survival at 7–14 days in comparison to those at 20% O_2.[28] The establishment of viable long-term cultures provides a unique opportunity to study the development of human neurons and glia over a much greater time course than previously possible. Taken alongside our report of successful neuronal sub-type specification at 3% O_2 to both midbrain dopaminergic and spinal motor neurons, this system will provide a more physiologically relevant model to investigate disease processes *in vitro*, a major avenue of research for both ES and induced pluripotent stem cell (iPS) neural cell derivatives. This is of particular importance to both motor neuron and Parkinson's disease, where oxidative stress has been implicated in neuronal injury.[29,30]

The finding that an *in vitro* simulation of the oxygen challenge that occurs in cell transplantation studies resulted in significant cell death, in contrast to maintenance at 3% O_2, is of considerable interest, and is reflected in the emerging concept of 'hypoxic' pre-conditioning before transplanting stem cells or their derivatives.[42] While neuronal and glial populations cultured at 20% O_2 do survive and achieve functional improvements after transplantation,[3] our findings warrant comparison of survival and phenotypic potential of transplanted NPCs derived from high and low oxygen environments.

Conclusion

A gradual shift in the long held view that low oxygen equates to hypoxia has led to the realisation that in fact it often represents

in situ normoxia.[43] The 3% O_2 system described in this study affords a novel approach for the generation of functional, defined cell types for *in vitro* and *in vivo* disease modelling and provides a platform for future studies exploring the therapeutic effects of cell-based therapies for neurological disorders.

Materials and Methods

Cell culture. H9 hESCs (Madison, WI, USA) were maintained in feeder-free conditions in CDM ((50% IMDM (Invitrogen, Carlsbad, CA, USA), 50% F12 (Invitrogen), insulin 7 μg/ml (Roche Diagnostics, UK), transferrin 30 μg/ml (Roche), bovine serum albumin 5 mg/ml (Sigma, St Louis, MO, USA), 1% lipid 100 $\times$ (Invitrogen) and monthioglycerol 450 μM (Sigma)), supplemented with 12 ng/ml FGF-2 (R&D Systems, Minneapolis, MN, USA) and 10 ng/ml activin, between passages 82 and 96. All cultures were supplemented with penicillin and streptomycin (Invitrogen). The six-well plates (Nunc, Rochester, NY, USA) were coated overnight with MEF-medium and colonies were passaged with collagenase 1 mg/ml (Invitrogen) every 3–5 days. HUES-9 cells (hES facility, Harvard University, Cambridge, MA, USA), between passages 30 and 40, were grown on irradiated mouse embryonic fibroblast feeders, supplemented with 10 ng/ml FGF-2, 10 ng/ml activin and 10 ng/ml insulin.

For neural conversion, the colonies were lifted off with liberase 125 μg/ml (Roche), incubated for 15–20 min, allowed to settle in 15 ml tubes and rinsed with CDM before chopping with a McIlwain Tissue Chopper (Mickle Engineering, Gomshall, UK) at 120 μm distances in two directions perpendicular to each other. Resulting cellular aggregates were then grown in repellent tissue culture flasks (Nunc) at a density of $\sim$200 000 cells/ml of CDM ($\pm$1 μM *N*-acetyl-cysteine (Sigma) at 20% O_2), in the absence of growth factors. At this stage, cells were cultured either in a standard 20% O_2 and 5% CO_2 incubator or in a 3% O_2 and 5% CO_2 incubator, with oxygen displaced by nitrogen. The resultant spheres were fed every other day (50% media change) and chopped again at day 10 before transfer to an orbital shaker, to prevent aggregation. From D12 onward 20 ng/ml FGF-2 plus heparin 5 mg/ml (Sigma) was added to the basal CDM, with media changes every 2–3 days and mechanical passaging approximately every 10–14 days. For regional specification, FGF-2 was added between days 12 and 15, and morphogens applied as described in the text. For terminal differentiation, NPCs were plated onto poly-D-lysine/laminin 10 μg/ml (Sigma)-coated coverslips and cultured in DMEM/2% B27 (Invitrogen)/1% penicillin–streptomycin for up to 3 months, with a 50% medium change after every 2–3 days.

Oxygen switch experiments. NPCs were dissociated with Accutase (Sigma) and plated at 40 000 cells per coverslip in 30 μl plating medium, to allow adherence. In all, 500 μl plating medium was added after 30 min. For live–dead staining, cells plated for 48 h were incubated for 10 min on ice with 4 μM of calcein and ethidium bromide (Invitrogen) in dPBS (Invitrogen). Four random fields from each of three coverslips in each group were counted (on an inverted microscope), on three occasions.

RNA isolation, RT-PCR and immunoblotting. These were carried out according to the standard procedures (detailed in Supplementary Information). Primer sequences are contained in Supplementary Table 1.

Immunocytochemistry and flow cytometry. Immunocytochemistry and flow cytometry were performed using standard protocols (see Supplementary Information). Details of primary antibodies used are contained in Supplementary Table 2.

Electrophysiology. Whole-cell current-clamping of neurons was performed at room temperature, using glass micro-electrodes of 3–6 MΩ resistance containing an internal solution consisting of 130 mM potassium gluconate, 4 mM NaCl, 10 mM HEPES, 10 mM BAPTA, 4 mM MgATP, 0.5 mM Na_2GTP, 0.5 mM $CaCl_2$ and 2 mM K-Lucifer yellow (pH adjusted to 7.3 with KOH). Series resistance was 6–14 MΩ. Cultures were superfused with HEPES-buffered external solution containing 144 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 10 mM glucose, 2 mM $MgCl_2$, pH set to 7.35 with NaOH, bubbled with medical oxygen. Traces were corrected for -14 mV junction potential. Tetrodotoxin (Tocris, Ellisville, MO, USA) was applied as indicated in the text; all other reagents come from Sigma.

Quantification and statistical analysis. All experiments were performed at least three times, unless otherwise stated. A Student's unpaired *t*-test was used

for statistical analysis. *P*-values of < 0.05 were considered significant; data are presented as mean ± standard error of the mean (S.E.M.).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)