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Non-hypoxic Stabilization of Hypoxia-Inducible Factor Alpha (HIF-α): Relevance in Neural Progenitor/Stem Cells

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Abstract Hypoxia-inducible factor-1 (HIF-1) plays an important role in neural progenitor cell (NPC) propagation and dopaminergic differentiation. In the presence of oxygen and iron, hypoxia-inducible factor 1 alpha (HIF-1α) is rapidly degraded via the prolyl hydroxylase (PHD)/VHL pathway. In addition to hypoxia, various non-hypoxic stimuli can stabilize HIF-1α in NPCs and influence the transcription of HIF-regulated genes. Here, we investigate various hypoxia mimetics: deferoxamine (DFO), ciclopirox olamine (CPX), dimethyloxallyl glycine (DMOG), a novel HIF-PHD inhibitor (FG-4497) and cobalt chloride ($CoCl_2$) with respect to their ability to enhance in vitro proliferation, neurogenesis and dopaminergic differentiation of human fetal mesencephalic NPCs (hmNPCs) in ambient oxygen (21%). Although able to stabilize HIF-1α, iron chelators (DFO and CPX) and DMOG were toxic to hmNPCs. $CoCl_2$ was beneficial only towards neuronal and dopaminergic differentiation, while FG-4497 enhanced proliferation, neurogenesis and dopaminergic differentiation of hmNPCs. Both $CoCl_2$ and FG-4497 were protective to human dopaminergic neurons. Finally, exposure to hyperbaric oxygen (HBO) also stabilized HIF-1α in hmNPCs and induced neurogenesis in vitro. These findings suggest that several HIF stabilizing agents or conditions can rescue impaired neurons and promote neurogenesis in vitro.

Keywords Neural progenitor cells · Hypoxia · Hypoxia-inducible factor-1α (HIF-1α) · Non-hypoxia · Neurogenesis · Neuroprotection · Hyperbaric oxygen (HBO)

Abbreviations

ANOVA	Analysis of variance
CPX	Ciclopirox olamine
DA	Dopamine
DAPI	6′-Diamidino-2-phenylindole
DAT	Dopamine transporter
DCX	Doublecortin
DFO	Deferoxamine mesylate
DMOG	Dimethyloxallyl glycine
EGF	Epidermal growth factor
EPO	Erythropoietin
FCS	Fetal calf serum
FGF-2	Fibroblast growth factor 2
GFAP	Glial fibrillary acidic protein
HBO	Hyperbaric oxygen
HEK	Human embryonic kidney
HIF-1α	Hypoxia-inducible factor-1α
hmNPCs	Human mesencephalic neural progenitor cells

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hfNPCs	Human frontal (cortical) neural progenitor cells
MAP2ab	Microtubule associated protein-2
MPP^+	1-Methyl-4-phenylpyridinium
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium
NSE	Neuron-specific enolase
PHD	Prolyl 4-hydroxylase domain
PHI	PHD inhibitor
TH	Tyrosine hydroxylase
TUJ1	Neuronal class III β-tubulin
VEGF	Vascular endothelial growth factor
pVHL	von Hippel–Lindau tumour suppressor protein

Introduction

Stabilization of the hypoxia-inducible factor-1 (HIF-1) protein is essential for its role as a regulator of gene expression under physiological and pathological conditions in various tissues. HIF-1 has dual effects and can induce either cell survival or cell death (Semenza 2000). HIF-1 is a $\alpha_1\beta_1$ heterodimer and the α subunit (HIF-1α) undergoes a rapid degradation via the prolyl hydroxylase (PHD)/VHL pathway. Under sufficient oxygen supply, the von Hippel–Lindau tumour suppressor protein (pVHL) targets the HIF-1α subunit for rapid ubiquitination and proteasomal degradation (Maxwell et al. 1999). Binding of the pVHL tumour suppressor protein requires the modification of HIF-1α by prolyl-4-hydroxylation at proline residues by the prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins (Bruick and McKnight 2001; Ivan et al. 2001; Jaakkola 2001). The PHD enzymes (PHD1, PHD2 and PHD3) have evolved to sense changes in 2-oxoglutarate, oxygen and iron via changes in enzyme activity. PHD requires oxygen, Fe^{2+}, 2-oxoglutarate and ascorbic acid (see Fig. 1). Reduced oxygen conditions inhibit prolyl hydroxylation by limiting the amount of oxygen available for the hydroxylation reaction. Therefore, the activity of the HIF-1 transcription factor becomes increased by oxygen deprivation (hypoxia), but also by a variety of other agents that can prevent HIF-1α degradation even in non-hypoxic conditions. Hypoxia-mimetics are chemical compounds capable of stabilizing HIF-1α under environmental (normoxic) oxygen levels. They target either PHD enzymes directly, selectively inhibiting their activity, or indirectly by depleting iron or increasing reactive oxygen species (ROS) production, which can oxidize PHD bound iron (Brunelle et al. 2005; Guzy and Schumacker 2006). Paradoxically, pure oxygen–hyperbaric oxygen (HBO) condition might be another non-hypoxic factor responsible for HIF-α subunit stabilization via ROS production. This hypothesis is based on a model of HIF-α stabilization as a result of alterations in oxygen metabolism rather than being dependant solely on environmental oxygen concentration (Lopez-Lazaro 2006).

During real or mimicked hypoxia, HIF-1α is stabilized and binds to DNA as a heterodimer with HIF-1β, resulting in the transcription of a huge number of genes involved in glucose homeostasis, erythropoiesis, angiogenesis, etc. (Wenger et al. 2005). Downstream target genes of HIF-1, such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF), have been shown to promote neurogenesis in vitro and in vivo (Chen et al. 2007b; Jin et al. 2000; Milosevic et al. 2007a) and are neuroprotective following ischemic or excitotoxic injury (Jin et al. 2000; Kilic et al. 2005; Won et al. 2007).

Typically, cell survival is threatened when cells are deprived of oxygen. Surprisingly, reduced oxygen level determines stem cell fate and leads to a better performance of various stem cell types including murine bone marrow cells (Parmar et al. 2007), murine CNS progenitors (Chen et al. 2007a; Milosevic et al. 2005; Studer et al. 2000), human mesenchymal stem cell (Grayson et al. 2006), human hematopoietic cells (Danet et al. 2003; Ivanovic et al. 2000) and human mesencephalic progenitors (Schwarz et al. 2006; Storch et al. 2001).

In this study, we modified cell culture conditions by utilizing various HIF stabilizing agents as supplements during expansion or differentiation of NPCs derived from human fetal mesencephalon in ambient oxygen. Furthermore, we identified a HIF stabilizer capable of inducing neurogenesis and neuroprotection of NPCs in vitro, which may be relevant to neurorepair and neurogenesis in vivo.

Materials and Methods

Propagation and Differentiation of Human Mesencephalic NPCs (hmNPCs)

Tissue from a 10–14-week-old non-infected human fetus was used to generate human mesencephalic (hmNPCs) and cortical (frontal—hfNPCs) neuroprogenitor cultures. Tissue preparation, characterization, propagation in the undifferentiated state and in vitro differentiation were performed as described (Milosevic et al. 2007b). In brief, expansion of hNPCs was carried out in a monolayer via plating onto polyornithine-fibronectin pre-coated culture dishes at a density of 30,000 cells/cm^2 in reduced atmospheric oxygen (3%). hNPCs were expanded in defined media (DMEM/F12) supplemented with epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2; 20 ng/ml each, both from PromoCell, Heidelberg, Germany) and 2% B-27 (Invitrogen, Carlsbad, CA). Prior to immunolabelling

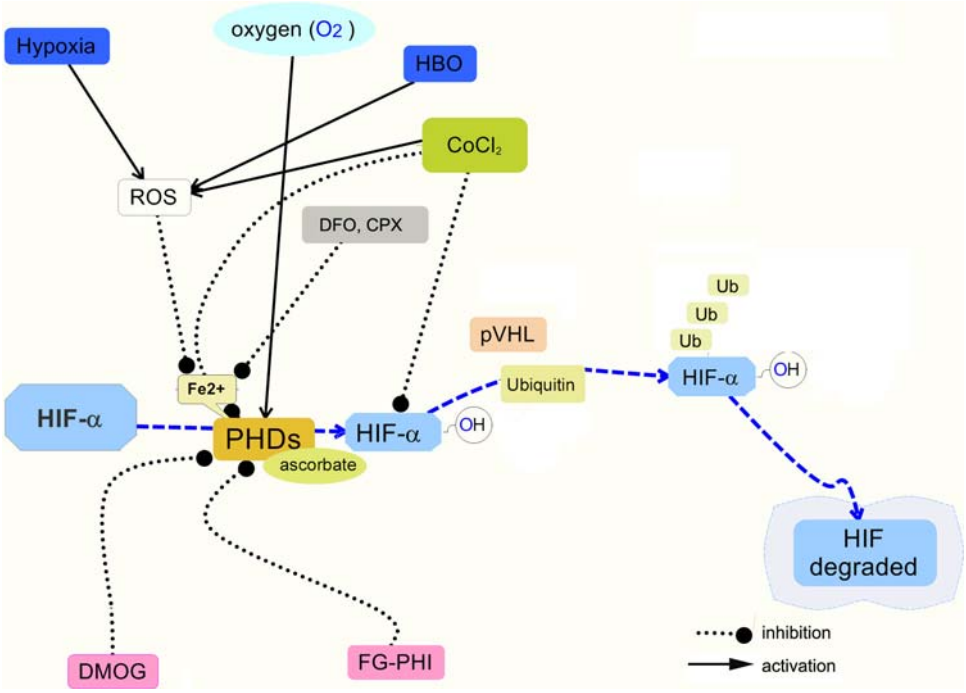

Fig. 1 Schematic model of possible ways to stabilize HIF-α in NPCs. The pyrolyl-4-hydroxylase domain (PHD) oxygen-sensing system plays a pivotal role in HIF-α (HIF-1α and HIF-2α) post-translational modification and subsequent HIF-α proteasomal degradation by controlling HIF-α-pVHL physical interaction. In addition to sufficient oxygen supply, for proper HIF-α hydroxylation, PHDs (PHD1, PHD2 and PHD3) require 2-oxoglutarate, and the cofactors iron and ascorbate. Reduced oxygen supply (hypoxia) contributes to HIF-α stabilization by both directly inhibiting PHD-mediated hydroxylation and indirectly through generation of ROS. Both non-hypoxic HIF-α stabilizers HBO and cobalt chloride act, at least in part, via generation of ROS. Iron chelators (DFO, CPX) inhibit PHD by depleting Fe^{2+}. DMOG and novel small molecule PHD inhibitors (FG-PHI, e.g. FG-4497) are capable of stabilizing HIF-α even in normoxic conditions by direct PHD inhibition

or protein extraction, hmNPCs were allowed to differentiate in vitro for 2 weeks. Differentiation of the cells was induced via replacement of expansion media by defined mitogen-free media supplemented with 2% B-27 and 5 μM forskoline (Sigma, St. Louis, MO).

Cell Culture

Human embryonic kidney cell lines (HEK-293 cells) stably expressing human dopamine transporter (DAT) protein (HEK-DAT cells) were generated as described previously (Storch et al. 1999). The cells were maintained in DMEM high glucose medium supplemented with 10% heat inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 in air. The selection was performed by addition of geneticin (G418) at 400 μg/ml during expansion, but was omitted during incubation of the cells with tested substances.

HIF Stabilizers

In order to stabilize HIF-1α in room air oxygen, both direct and indirect modes of PHD enzyme inhibition were used. The iron chelators ciclopirox olamine (CPX, 1–20 μM) and deferoxamine mesylate (DFO, 10–200 μM), as well as cobalt chloride (10–100 μM) were purchased from Sigma. The novel prolyl hydroxylase inhibitor, FG-4497, was provided by FibroGen Inc. This small molecule inhibitor of PHD enzymes was previously disclosed in patent filing US20040254215A1. It has been reported to induce HIF activity in HeLa and 1G6 cells, to increase plasma erythropoietin (EPO) (100- to 150-fold within 4–6 h) and hemoglobin in rodents (Hsieh et al. 2007; Robinson et al. 2008). Both the novel PHD inhibitor FG-4497 (5–30 μM) and the PHD inhibitor dimethyloxallyl glycine (DMOG, 0.05–1 mM, Cayman Chemical, Ann Arbor, USA) were prepared in dimethyl sulphoxide (DMSO; Sigma) and kept frozen until usage.

Experimental Design

All HIF stabilizing agents were applied in media during hmNPCs cultivation in ambient oxygen (21% O_2, 5% CO_2). DMSO (<0.25% final concentration) was added in media and used as negative control. FG-4497 was applied in media containing no serum (HEK-DAT cells) or without

B-27 supplement (NPCs). FG-4497 has high protein binding affinity, and thus, in the presence of serum protein, the amount of free drug available to enter cells is significantly reduced. For studying neuroprotection, hmNPCs during expansion or after 14 days of differentiation were treated with appropriate HIF stabilizers 1 h prior to addition of the dopaminergic (DA) neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) to ensure that HIF-1 stabilization preceded the toxic insult.

Hyperbaric Oxygen (HBO) Treatment

In order to induce hyperoxia on the cellular-level, exponentially growing hmNPCs were subjected to hyperbaric oxygen administration in a tabletop chamber. The cells were exposed to 100% oxygen at 1.5 atmospheres absolute (ATA) for 60 min in PBS, once daily, continuously for 5 days. After HBO, PBS was replaced with expansion media and the cells were placed back in a 3% O_2 incubator. Controls were kept in 3% oxygen the whole time. Finally, the cells were differentiated for 7 days in 3% oxygen before they were harvested for protein extraction. HBO-induced HIF-1α stabilization was demonstrated on expanded NPCs during incubation in PBS to avoid HIF-1α stabilization by growth factors present in our standard expansion media.

Cell Death/Cell Cycle Assay

The DNA content, as reflected by the fluorescence signal of propidium iodide, was measured using a flow cytometer (Becton Dickinson, Heidelberg, Germany). Control and treated hmNPC samples were prepared for cell cycle analysis by lysing the cells in 300 µl of hypotonic fluorescence solution (HFS) as described (Milosevic et al. 2007b), relying on the method described by Nicoletti et al. (1991). Histograms of DNA content were acquired using the CellQuest software (Becton Dickinson). The number of nuclei present in each peak of the histogram, left to the G1 (sub-G1), G1/G0, S, G2/M, was analyzed by measuring the peak area using the ModFit LT software (Verity, Turramurra, Australia).

In Vitro Viability and Cytotoxicity Assay

Neuroprogenitor cell proliferation and viability were determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium (MTT) assay essentially as described previously (Mosmann 1983; Sabolek et al. 2008). Cytotoxicity has been determined in parallel by measuring lactate dehydrogenase (LDH) levels using commercially available kit (TOX-7, Sigma). LDH is released from cells when the cells are injured, so that LDH in the medium is an indicator of the integrity of cell membrane. The cells were plated in 96-well plates at a density of 20,000 cells/well (in 100 µl medium) and incubated for 24 h. Then, various concentrations of different drugs (HIF stabilizers) were added to the culture. After incubation at 37°C in a 21% oxygen incubator for up to 72 h, 10 µl of MTT reagent (5 mg/ml MTT) was added to each well and incubated in an incubator for additional 3 h. The resulting formazan dye was extracted with acid-isopropanol (0.04 M HCl in absolute isopropanol) and the absorbency was measured spectrophotometrically with a computer-operated immuno reader (Tecan Deutschland GmbH, Crailsheim, Germany) at a wavelength of 570 nm with reference at 630 nm. MTT reduction was expressed as percentage of the untreated control.

Immunocytochemistry

NPCs were either expanded or differentiated on Lab-Tek 4-well-chamber permanox slides (Nalge Nunc International, Rochester, NY). Control and treated hmNPCs (at least two different tissue preparations) were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and rinsed with PBS, counterstained with the DNA-binding dye 4′-6-Diamidino-2-phenylindole (DAPI, 2 µg/ml in PBS) for 15 min at room temperature, twice rinsed in PBS followed by incubation in blocking buffer (10% FCS, 0,2% Triton-X 100 in PBS, pH 7.2) for 30 min at room temperature. After incubation with anti-β-III-tubulin primary antibody (TUJ1; Covance, Berkeley, CA, USA), anti-doublecortin (DCX) primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-microtubule associated protein-2 (MAP2ab; BD Pharmingen, San Diego, CA, USA) or anti-Ki67 antigen antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) for 1 h at room temperature in blocking buffer, the cells were incubated with Alexa Fluor® 488 conjugated or Alexa Fluor® 594 conjugated secondary antibodies (Molecular Probes, Eugene, USA). Coverslips were mounted onto glass slides and examined under a fluorescence microscope (Zeiss Axiovert 200). Acquisition of the immunostained cells was performed using the Image-analysis software AxioVision 4 (Carl Zeiss AG, Jena, Germany).

Immunoblotting

Half-life of HIF-1α was evaluated by immunoblotting. Combined cytoplasmic and nuclear extracts of cultured hmNPCs were prepared in extraction buffer as described previously (Milosevic et al. 2007b). Protein concentrations were determined by the Bradford method, using bovine serum albumin as a standard. Denaturated proteins were resolved on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a Hybond-ECL nitrocellulose

membrane (GE Healthcare, Freiburg, Germany) by semi-dry blotting. Membranes were stained with Ponceau S (Sigma) to confirm equal protein loading and transfer followed by blocking with 5% (wt/vol) nonfat dry milk in PBS-T [PBS, 0.1% (vol/vol) Tween 20] for 2 h at room temperature and subsequent incubation with desired primary antibody (diluted in PBS containing 5% non-fat milk and 0.1% Tween 20) overnight at 4°C with gentle agitation. The antibodies used were as follows: mouse monoclonal anti-actin (C4, ICN Biomedicals), mouse monoclonal anti-HIF-1 alpha (Novus Biologicals, Littleton, CO, USA), rabbit polyclonal anti-tyrosine hydroxylase (anti-TH) antibody (Santa Cruz), mouse monoclonal anti-TUJ1 (Covance) mouse monoclonal anti-neuron specific enolase (NSE, Chemicon International, Hampshire, UK), mouse monoclonal anti-glial fibrillary acidic protein (GFAP, Chemicon) and horseradish peroxidase-coupled secondary antibodies (Pierce, Rockford, IL, USA). Chemiluminescence detection was performed by incubating the membranes with SuperSignal-Dura substrate (Pierce) followed by analyzing on a CCD cooling camera (Fuji LAS-1000plus). The chemiluminescence was quantified using AIDA, a two-dimensional densitometry software (Raytest Isotopenmeb, Straubenhardt, Germany).

Statistical Evaluation

Normally distributed data were statistically assessed using appropriate analysis of variance (ANOVA) followed by Tukey test for multiple comparisons versus control group (SigmaStat software package, Jandel Corp., San Rafael, CA), with significance being defined as $P < 0.05$. All data are expressed as mean ± SEM.

Results

HIF Stabilizers Influence hmNPC Viability

The effect of various HIF stabilizers on both mesencephalic and cortical hNPC viability was demonstrated using the MTT assay (Fig. 2a–e). In general, cells were incubated with various HIF-α stabilizers for a 72-h incubation period in room air oxygen. Drug concentrations sufficient to protect HIF-1α from rapid degradation are indicated with a representative immunoblot underling each histogram. One-way ANOVA was utilized to evaluate the statistical significance of treatment within each tissue preparation. After treatment of hNPCs with DFO for 72 h, cell viability was significantly decreased in comparison to untreated cells ($P < 0.001$, Fig. 2a). In all three cell preparations, treatment of hNPCs with DFO resulted in a dose-dependent reduction in cell number. For example, treatment of DFO decreased the number of viable hmNPC M1 by 22 ± 2% at 10 μM, by 36 ± 2% at 50 μM, by 60 ± 2% at 100 μM and by 65 ± 4% at 200 μM (Fig. 2a). CPX treatment also significantly decreased NPC viability starting from the lowest dose in all three cell preparations ($P < 0.001$, Fig. 2b), reducing cell viability in hmNPC M1 by 56.3 ± 1.7% at 1 μM. Cobalt chloride did not significantly influence cell proliferation or viability within the 72-h incubation period (Fig. 2c), while FG-4497 significantly and dose-dependently increased hNPC number ($P < 0.001$, Fig. 2d). For example, 5 μM and 10 μM FG-4497 increased the number of hmNPC M2 by 35.8 ± 3.6% and 38.6 ± 1.7%, respectively (Fig. 2d). Another PHD inhibitor, DMOG, was cytotoxic to both hNPC cell types at doses required to stabilize HIF-1α ($P < 0.001$). As shown in Fig. 2e, 0.5 mM DMOG reduced viability by 33 ± 2.6% in hmNPC M2 and by 54.2 ± 1.5% in hmNPC M1. A non-toxic dose of 100 μM DMOG was insufficient to stabilize HIF-1α. One-way ANOVA revealed a significant increase in hmNPC number when cells were expanded for 1 week in 3% vs. 21% oxygen ($P < 0.01$, $n = 6$). The same cells expanded in 21% oxygen in the presence of FG-4497 showed no statistical difference in proliferation rates obtained by direct cell counting when compared to cells growing in 3% oxygen (Fig. 2f). No significant difference was observed between 5 and 10 μM FG-4497 treatment in 21% oxygen (Fig. 2f).

HIF-α Stabilizers Regulate NPC Cell Cycle/Cell Death

The HIF-α stabilizer FG-4497 did not affect cell death as measured by the appearance of a sub-G1 peak in NPC cultures (Fig. 3a). In 21% oxygen atmosphere, cell death was 6.0 ± 0.6% and did not exceed 8% in any of treated samples. Following a 1-week incubation of expanding NPCs with the HIF-PHD inhibitor FG-4497, the cell cycle distribution was influenced only with respect to S-phase, which was slightly increased from 8 ± 0.1% in control to 10 ± 0.6 in 20 μM FG-4497 samples (Fig. 3a). Cobalt chloride also affected NPC cell cycle at 50 μM, but cell death was induced at higher concentrations with 9.6 ± 0.1% of sub-G1 cells at 100 μM $CoCl_2$ compared to 5.1 ± 0.3% of sub-G1 cells in untreated cultures (Fig. 3b). The fraction of cells in S-phase was significantly induced with 5.9 ± 0.3% and 8.9 ± 0.3% of cells in S-phase at 50 and 100 μM $CoCl_2$, respectively, versus 3.6 ± 0.3% of S-phase cells observed under control conditions. Corresponding to the increase in S-phase, there were less cells in G1/G0 phase in cultures treated with $CoCl_2$—81.9 ± 0.5% and 73.9 ± 0.1% of cells were in G1/G0 phase at 50 μM and 100 μM $CoCl_2$, respectively, versus 87.4 ± 0.8% of G1/G0 phase cells in control cultures).

Despite a significant decrease of viability decrease measured by MTT reduction, 72 h after hmNPCs incubation with

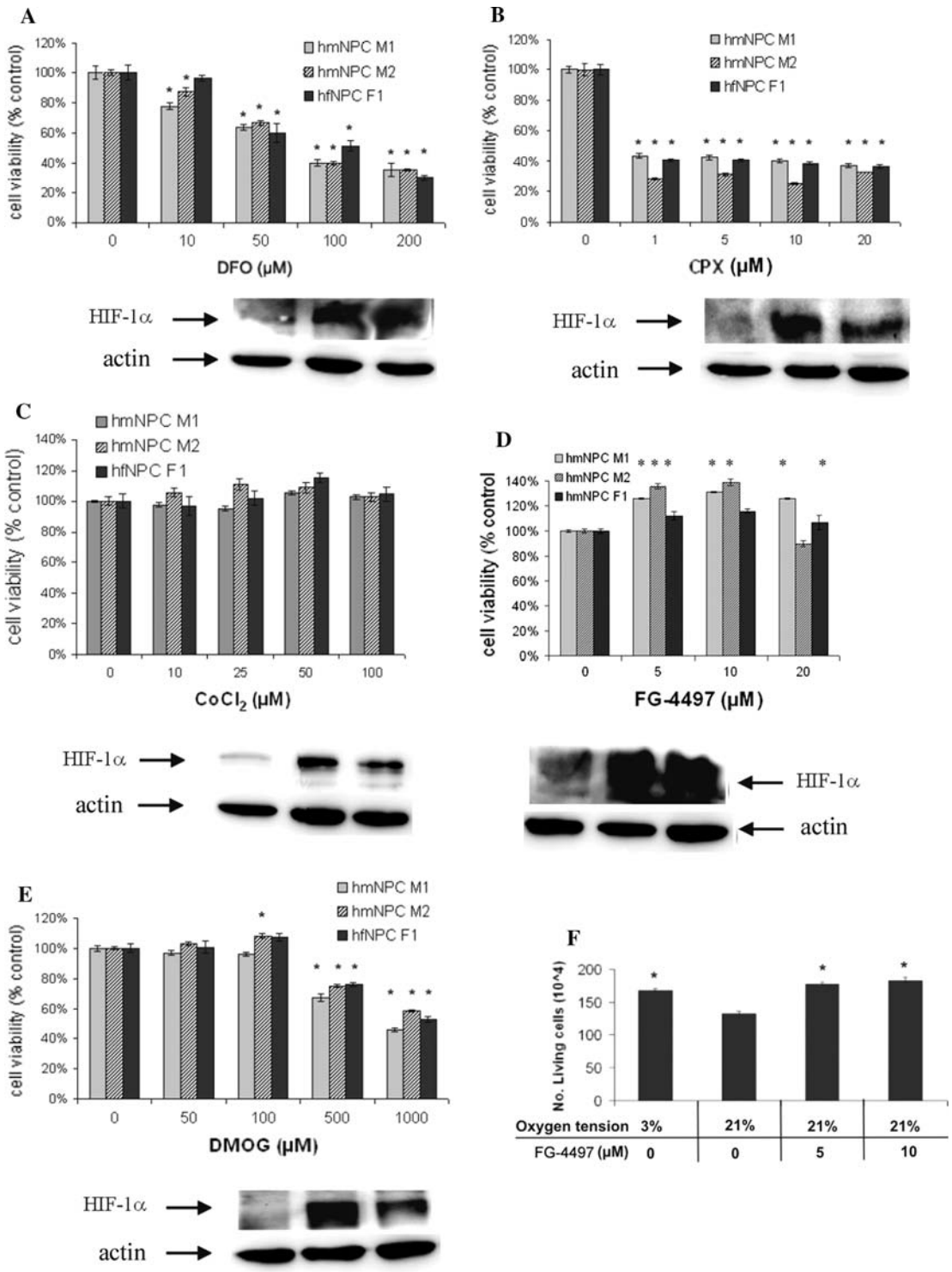

Fig. 2 HIF-α stabilizers affect human neuroprogenitor cell viability. (**a**–**e**) Two midbrain-derived hNPCs (hmNPC M1 and hmNPC M2) and one frontal preparation (hfNPC F1) were used for the MTT cell viability test. hNPCs were seeded in 96-well plates, grown for 24 h in expansion medium in 21% oxygen, and treated with deferoxamine (**a**), ciclopirox olamine (**b**), cobalt chloride (**c**), PHD inhibitor FG-4497 (**d**) or dimethyloxallyl glycine (**e**) in expansion media for 72 h at concentrations as indicated. Values are expressed as cell viability compared to untreated controls and represent mean ± SEM from six independent experiments. **f** Number of living hmNPCs with or without FG-4497 expanded in 21% vs. 3% oxygen for 1 week. Western blots below diagrams show HIF-1α stabilization achieved at the particular drug concentration indicated above it. $*P < 0.05$ when compared to control (no HIF-α stabilizer) in (**a**–**e**) or when compared to untreated 21% condition in (**f**). One-way ANOVA revealed no statistical differences between untreated cells in 3% oxygen compared to FG-4497-treated hmNPCs in 21% oxygen (**f**)

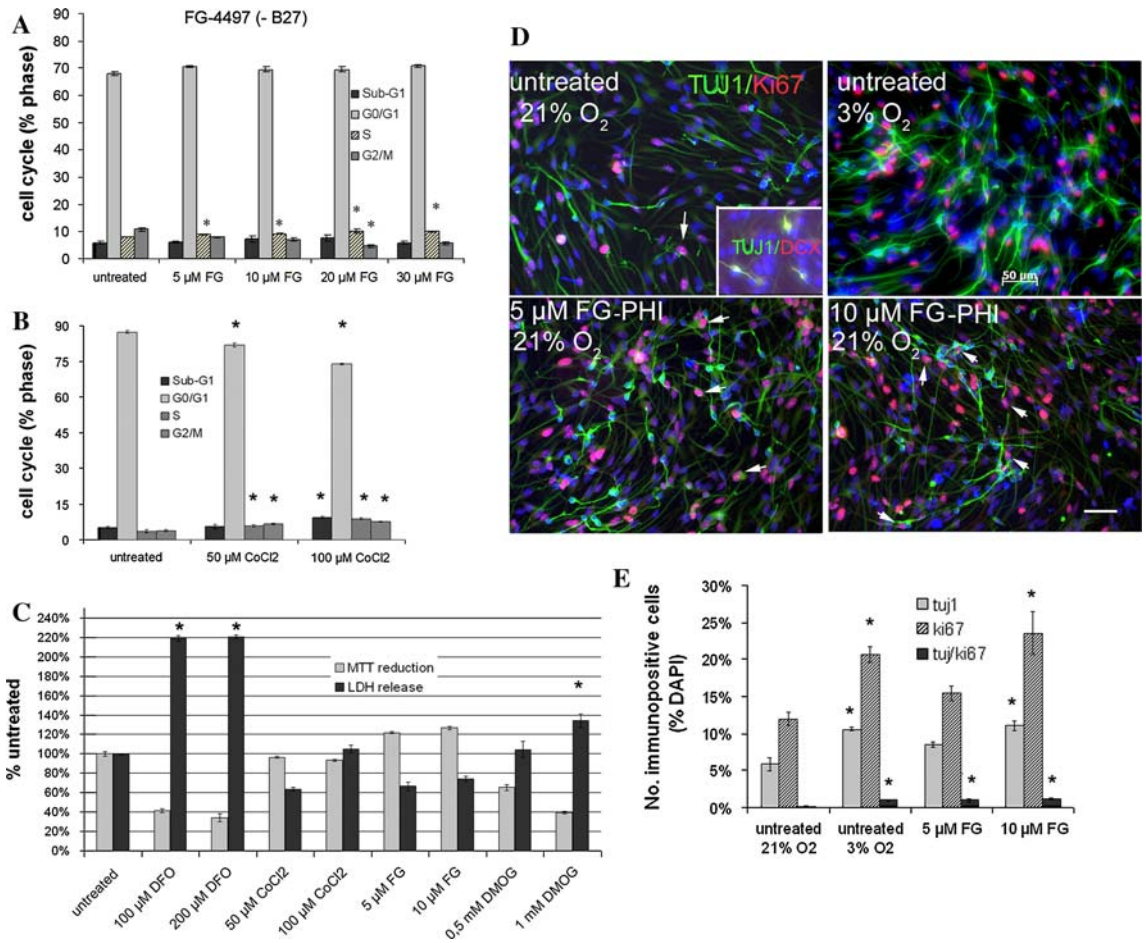

Fig. 3 HIF-α stabilizers enhance proliferation increasing the numbers of newly generated neurons in vitro. Cell-cycle-phase distribution in hmNPCs following 1 week exposure to HIF-α stabilizers FG-4497 in the absence of B-27 supplement (**a**) and cobalt chloride (**b**) in ambient oxygen. **c** Cell viability (MTT reduction)/cytotoxicity (LDH release) assay performed in parallel on hmNPCs incubated for 72 h with HIF-α stabilizers capable of preventing HIF-α subunit degradation in those cells (**d**, **e**): Amplifying neuroprogenitors are positively stained for Ki67 either in untreated NPCs grown in parallel in 3% oxygen (used as a positive control) and as untreated or FG-PHI (FG-4497)-treated samples expanded for 1 week in 21% oxygen (**d**). *Arrows* indicate proliferating neuroblasts ($TUJ1^+/Ki67^+$) in one representative hmNPC preparation. Doublecortin (DCX)/TUJ1 co-expression is highlighted in the insert of (**d**). NPC nuclei are shown in blue (DAPI). Scale bar = 50 μm. The numbers of immunopositive cells ($Ki67^+$ only, $TUJ1^+$ only or $Ki67^+/TUJ1^+$ co-labelled) expressed as the percentages of DAPI stained nuclei are shown as mean ± SEM (**d**). $^*P < 0.05$ when compared to untreated control ($n = 6$)

0.5 mM DMOG (65 ± 3% of control), LDH release was not significantly increased at the same conditions in vitro. However, 1 mM DMOG induced LDH release 34.0 ± 7% over control levels (Fig. 3c). A prominent viability reduction (41 ± 2% of control) parallel with elevated LDH release was obtained with 100 μM DFO (219 ± 3% over control values). LDH values were similar after incubation of the cells with 200 μM DFO (Fig. 3c). $CoCl_2$ and FG-4497 did not cause leakage of LDH from NPCs at concentrations established to be sufficient to induce HIF-α stabilization.

HIF-α Stabilizers Enhance Neuroblast Proliferation in hmNPC Cultures

The effect of HIF stabilizers on neurogenesis was assessed using immunostaining for immature neurons with TUJ1 in parallel with doublecortin (DCX). Proliferating neuroblasts were identified via double-immunostaining for TUJ1 and the proliferation marker Ki67 in cultures growing under 21% atmospheric oxygen tension (Fig. 3d). Virtually all $TUJ1^+$ cells were also positive for DCX (Fig. 3d, insert). The number of $TUJ1^+$, $Ki67^+$ and double-positive ($TUJ1^+/Ki67^+$) cells were determined in control and FG-4497-treated cultures and normalized to the total number of DAPI-stained cells (Fig. 3e). The percentage of immature neurons was increased to 11.1 ± 0.7% in 10 μM FG-4497-treated cultures compared to 5.8 ± 0.9% in 21% untreated cultures (one-way ANOVA, $P < 0.001$, $n = 6$). NPCs expanded in 21% oxygen but those treated with the HIF stabilizer FG-4497 (10 μM) were indistinguishable from untreated cultures grown in 3% oxygen with respect to the number of newly forming neurons (10.6 ± 0.2%; Fig. 3e).

The total rate of expanding cells was also significantly higher in 10 μM FG-4497-treated cultures versus untreated 21% cultures as revealed by increased Ki67 staining (23.6 ± 2.9% vs. 12.0 ± 0.9%, respectively; $P = 0.016$), but insignificantly different from untreated 3% oxygen cultures (20.7 ± 1.1%). $TUJ1^+/Ki67^+$ cells were rare to find in untreated cultures, but in both 5 μM and 10 μM FG-4497-treated cultures, double-positive cells were quantified as 1.0 ± 0.1% and 1.2 ± 0.1%, respectively. Similarly, in 3% oxygen cultures, the percentage of the cells expressing both TUJ1 and Ki67 was 1.0 ± 0.1% ($P < 0.01$; Fig. 3e).

HIF-α Stabilizers Improve Neuronal and Dopaminergic Differentiation

Mesencephalic NPCs were differentiated for 1 week in 21% oxygen with simultaneous exposure to non-toxic HIF-α stabilizers: FG-4497 (5 and 10 μM) and $CoCl_2$ (50 and 100 μM). Parallel differentiation of the cells in 3% oxygen served as a positive control. The cells were fixed and immunostained for the mature neuronal marker MAP2ab and for TH to detect dopaminergic neurons (Fig. 4a). For each sample, untreated and treated, 10 randomly selected fields with approximately 300–500 $DAPI^+$ nuclei were chosen and TH^+ neurons were counted. Dopaminergic differentiation was expressed as a percentage of $TH^+/DAPI^+$ cells. This ratio was 2.9 ± 0.2% in untreated 3% oxygen samples, 1.7 ± 0.1% in untreated 21% oxygen samples and increased to 2.0 ± 0.3% in 50 μM $CoCl_2$ and 4.2 ± 0.3% in 100 μM $CoCl_2$. Similar effects were observed in the presence of 5 μM FG-4497 (2.5 ± 0.3% TH^+) and 10 μM FG-4497 (3.0 ± 3%; Fig. 4b). Thus, treatment of hmNPCs with HIF-α stabilizers during differentiation significantly improved dopaminergic differentiation (one-way ANOVA; $P < 0.001$; $n = 10$), reaching a level observed in reduced oxygen condition (3% oxygen). Multiple comparisons versus the untreated 21% group (Tukey test) revealed a significant effect of 3% oxygen, 100 μM $CoCl_2$ and 10 μM FG-4497. Although it was almost impossible to count all $MAP2ab^+$ cells, an increased neuronal differentiation was also obvious (Fig. 4a—green). Immunoblotting of both mature (NSE) and dopaminergic neuron (TH) markers confirmed their increased expression following treatment with the PHD inhibitors FG-4497 and cobalt chloride (Fig. 4c). Glial differentiation as measured by GFAP immunoblotting was slightly, but not significantly, reduced in FG-4497-treated samples, while in the $CoCl_2$ treated samples, it was markedly reduced by up to 50% (Fig. 4c).

HBO Induces Neurogenesis In Vitro

Transient exposure of hmNPCs to hyperoxia (1 h of HBO once daily for 5 consecutive days) did not cause HIF-1α

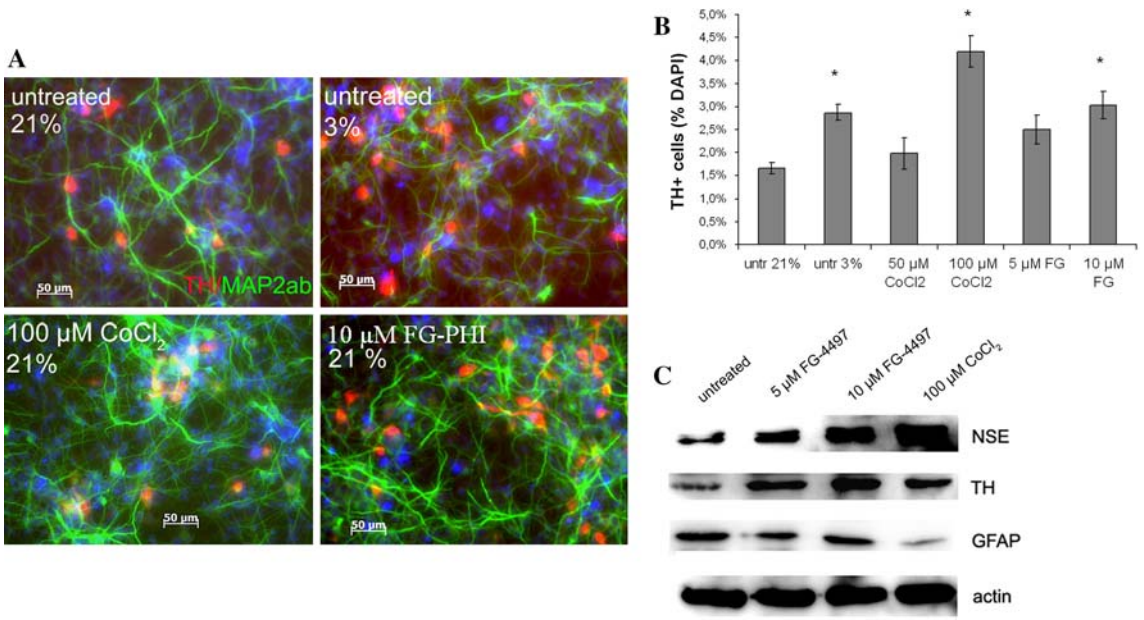

Fig. 4 HIF-α stabilizers improve dopaminergic differentiation. Representative human midbrain-derived NPCs immunostained for neuronal marker (MAP2ab) and dopaminergic marker (TH) upon 1 week of differentiation either in 3% oxygen or in 21% oxygen as untreated or supplemented with HIF stabilizers (FG-4497 and $CoCl_2$) are shown in (**a**). The numbers of immunopositive cells (TH^+ cells) expressed as the percentages of DAPI stained nuclei are counted and presented as mean ± SEM (**b**). Immunoblots representing alterations in the expression of neuronal (NSE), dopaminergic (TH) and glial (GFAP) markers obtained in hmNPCs following differentiation in the presence of HIF stabilizers (**c**). *$P < 0.05$ vs. untreated 21% ($n = 10$) (**b**)

degradation as observed by immunoblotting performed on expanded cells (Fig. 5a). Moreover, such treatment caused a significant augmentation in the expression of neuron-specific class III beta-tubulin (TUJ1) with levels of 142 ± 14% of the control condition (Fig. 5b, c). NSE protein expression revealed mature neurons with an increase under HBO to 179 ± 13% of the levels measured in controls (Fig. 5b, c). As observed by immunoblotting, glial differentiation (GFAP) remained unaffected (Fig. 5b, c).

HIF-α Stabilizers Induce Dopaminergic Neuroprotection

Non-toxic HIF-α stabilizers FG-4497 and $CoCl_2$ were further tested for their ability to protect proliferating and differentiated NPCs from a neurotoxic insult caused by the dopaminergic neurotoxin MPP^+. Relatively high concentrations of MPP^+ (1 mM) for 24 h reduced the viability of expanded NPCs to 47 ± 3% of control (Fig. 6a). Viability was recovered via a short term pretreatment with HIF-α stabilizers (added 1 h prior to MPP^+), followed by a 24-h co-treatment of MPP^+ with the HIF-α stabilizers. FG-4497 of 20 μM counteracted MPP^+-induced toxicity, rendering viabilities of 94 ± 6% cells compared to untreated cells (Fig. 6a, −B27 bars). When B27 was included in the media, FG-4497 was bound to albumin (which is a component of B27) and therefore inactivated (Fig. 6a, +B27 bars). About 100 μM $CoCl_2$ also rescued NPCs such that the viability was increased to 77 ± 6% and was significantly different from MPP^+ alone (Fig. 6a). Furthermore, HIF-α stabilizers rescued differentiated hmNPCs against MPP^+ toxicity (Fig. 6b). Loss of differentiated hmNPCs following 1 mM MPP^+ treatment for 24 h was 41 ± 5% compared to untreated cultures. This loss was ameliorated by FG-4497 or $CoCl_2$ pretreatment by 12 ± 11%.

Treatment with MPP^+ for 24 h resulted in a substantial loss of neurons and decrease in number of TH-positive cells. In addition, a reduction in the neurite network of dopaminergic cells was obvious even when the cell bodies were positively stained for TH (Fig. 6c). Both FG-4497 and the $CoCl_2$-treated dopaminergic neurons showed somewhat preserved processes. FG-4497 but not $CoCl_2$ partially restored the neuronal network (Fig. 6c, upper row).

In order to further investigate the ability of HIF-α stabilizers to protect against MPP^+ toxicity, we investigated modified HEK-293 cells expressing high levels of the DAT protein (HEK-DAT cells). These cells are very sensitive to MPP^+ and this toxicity completely depends on cellular uptake of MPP^+ via the DAT (Storch et al. 1999). A reduction in cell viability of 48 ± 2% compared to untreated samples was observed in HEK-DAT cells following 10 μM MPP^+ treatment for 48 h (Fig. 6d). One-way ANOVA followed by multiple comparisons versus MPP^+ treated group (Bonferroni *t*-test) revealed a significant protection provided by FG-4497 (5 and 10 μM) and 50 μM $CoCl_2$ ($P < 0.05$; $n = 6$).

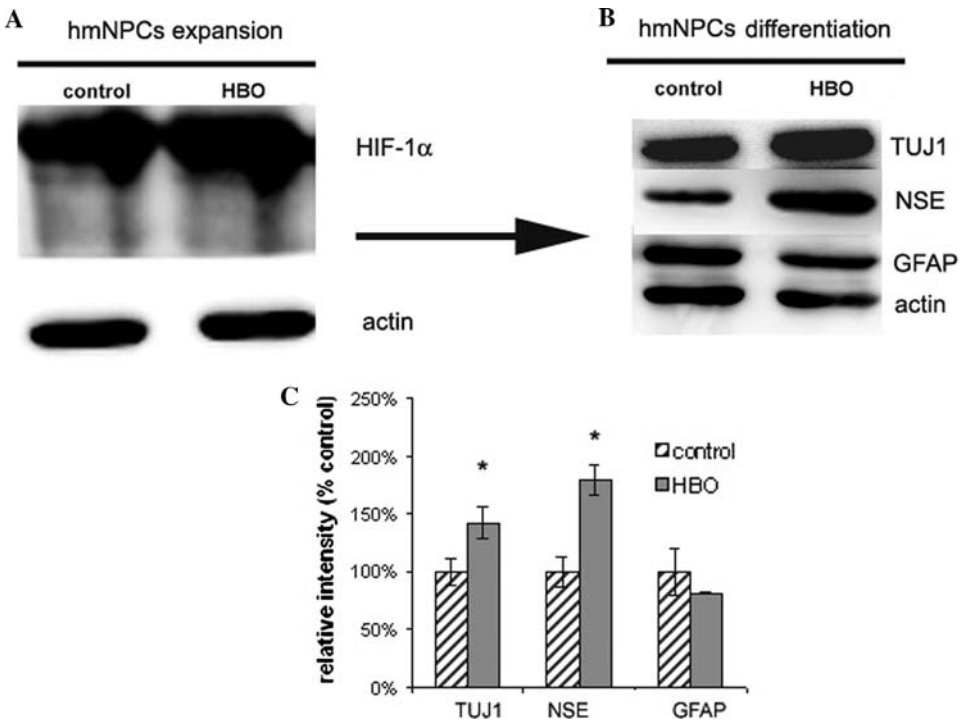

Fig. 5 Hyperbaric oxygen supports neurogenesis in vitro. Once stabilized under condition of limited oxygen supply, HIF-1α remained steady in neuroprogenitors shortly incubated in hyperbaric oxygen conditions (HBO) (**a**). After five continuous HBO cycles, each lasting for 1 h daily, the cells were differentiated in vitro for 1 week and protein extracts were proceeded for immunoblotting. Protein expression of early and late neuronal differentiation (TUJ1 and NSE) and glial differentiation (GFAP) was determined (**b**) and quantified (**c**). *Represents $P < 0.05$ when compared to control (*t*-test; $n = 4$)

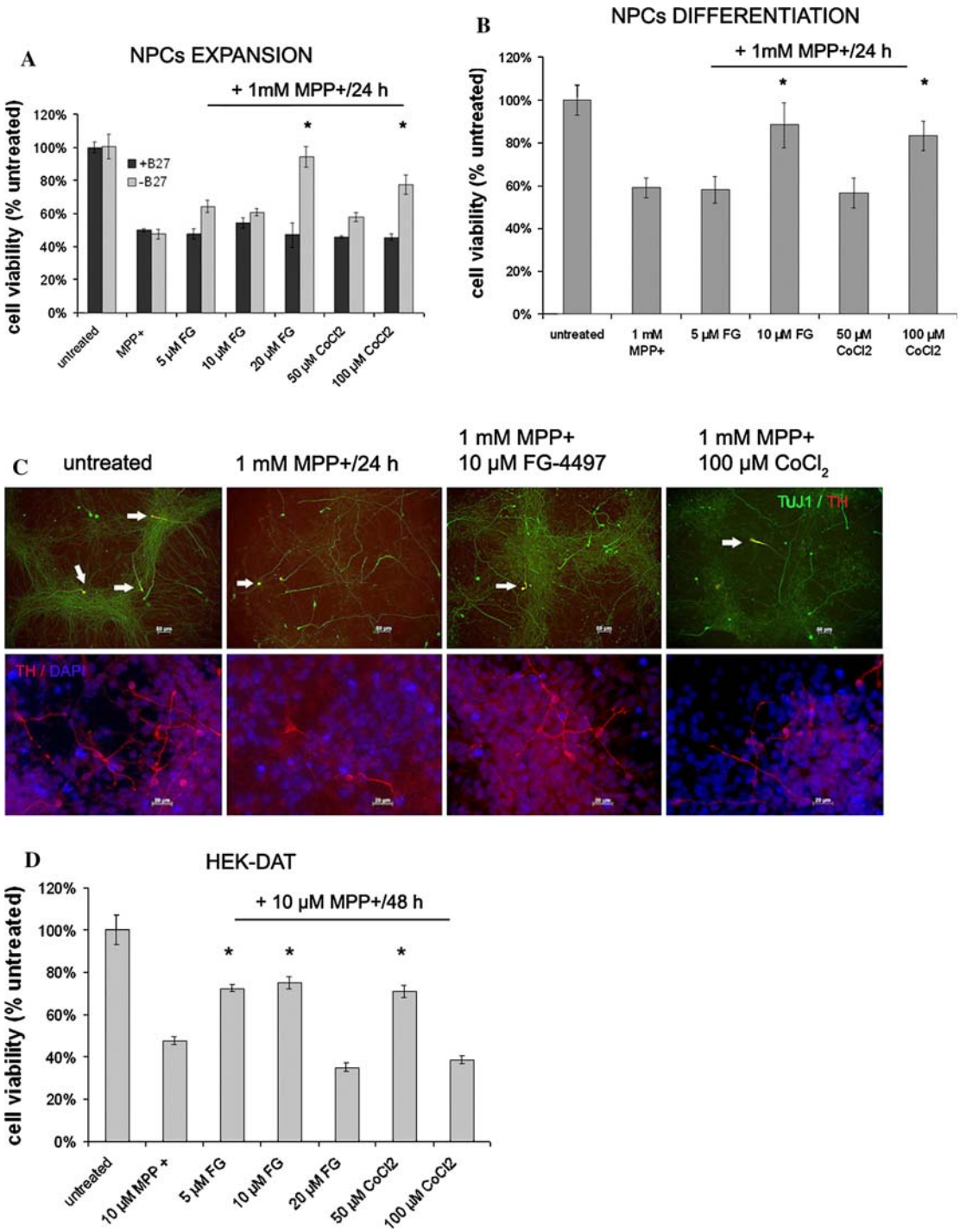

Fig. 6 HIF-α stabilizers protect NPCs from toxic insults. hmNPCs during expansion (**a**) or after differentiation for 2 weeks (**b**, **c**) were exposed to 1 mM MPP^+ for 24 h with or without HIF-α stabilizers (FG-4497 or $CoCl_2$) in ambient oxygen. Two different expansion media were used with or without B-27 supplement (**a**). Media with B-27 was used as a negative control, as HIF-α could not be stabilized due to a reduction in free drug available by albumin binding. Prior to MPP^+ treatment, NPCs were differentiated for 2 weeks in 3% oxygen environment and then transferred to 21% oxygen to continue with the neurotoxic experiments (**b**, **c**). Representative photomicrographs of differentiated hmNPCs illustrating neuronal (*upper row*) and dopaminergic cell loss (*lower row*) following MPP^+-treatment and a partial recovery of neuronal network due to the neuroprotection mediated by hypoxia mimetic FG-4497 (**c**). *Arrows* indicate dopaminergic neurons ($TH^+/TUJ1^+$; yellow). Nuclei were counterstained with DAPI. In (**c**), scale bar = 50 μm (*upper row*) or 20 μm (*lower row*). HEK-DAT cells were expanded in room air oxygen with serum; HIF-α stabilizers were added in media containing no serum but 10 μM MPP^+ for 48 h as indicated (**d**). Cell viability was measured using the MTT assay. $^*P < 0.05$ when compared to MPP^+ alone ($n = 6$)

Discussion

The aim of this study was to elucidate whether non-hypoxic stabilization of HIF-1α in human mesencephalic NPCs could positively influence proliferative/survival responses indirectly or directly, leading to increased numbers of dopaminergic neurons. We thus stabilized HIF-1α protein in ambient oxygen tension (21%) by pharmacological inhibition of HIF-PHDs or extreme hyperoxia (HBO). Being neuroprotective, HIF-1α could prevent injury or contribute to recovery of NPCs or neurons. Reduced oxygen tension (hypoxia) is central to the pathogenesis of several human diseases, including brain and heart infarction and a prominent feature of solid tumour development. In contrast, beneficial effects of hypoxia and HIF-1α on neural stem cells are profound (Chen et al. 2007a; Milosevic et al. 2007a; Zhu et al. 2005). Apart from hypoxia, iron chelating agents and certain transition metals (Co, Mn, Ni) are capable of inducing HIF-1α protein, supporting the hypothesis of ferroprotein involvement in this oxygen sensing system (Goldberg et al. 1988). Cobalt inactivates PHD activity by binding to the iron site of the enzyme, and prevents HIF-1α degradation by direct binding to HIF-1α, thereby impeding its recognition by the pVHL protein (Yuan et al. 2003). Furthermore, $CoCl_2$ stabilizes HIF-1α partially via ROS generation by a non-mitochondrial mechanism (Chachami et al. 2004; Chandel et al. 1998). HIF-1α could be also activated by non-hypoxic factors in a redox-sensitive manner (Lopez-Lazaro 2006). Hence, while ambient oxygen initiates a rapid HIF-1α degradation, HBO might be supporting its stabilization. Mechanism of HBO-induced HIF-1α stabilization remains elusive, but there are some indications that ROS production is likely involved in this process (Sanders et al. 1993). Once stabilized, HIF-1α might be implicated in many biological processes with beneficial outcome.

We utilized several hypoxia-mimetics to provide a long-term HIF-α stabilization in hmNPCs. Various HIF-α stabilizers have different cellular targets and therefore cause different cellular responses (Fig. 1). In addition to the HIF-α stabilizing activity, most of them are not specific inhibitors of the PHD enzyme, possessing certain side effects, consequently acting on vital cellular processes. Some of the drugs demonstrated a prominent cytotoxicity (DFO), while others showed combination of antiprolifeartive and cytotoxic effects (DMOG). As shown in Fig. 2, non-toxic drug concentrations were usually insufficient to stabilize HIF-1α subunit and therefore irrelevant for proposed application. Iron levels are regulated within cells by a host of regulatory proteins that keep iron levels strictly controlled. An excess of iron can lead to the generation of free radicals that can damage cells. We demonstrated here that iron depletion by iron chelators (e.g. DFO, CPX) mimics hypoxia in NPCs. However, iron is an essential element that is required for many processes in mammalian cells and its removal in NPCs, with the aim to stabilize HIF-1α in room air oxygen, initiated numerous side effects that ultimately caused cell death. Indeed, although mentioned in literature as an antioxidant, DFO interferes with proliferation, and therefore, it was used as an antiproliferative and antitumour agent (Lovejoy and Richardson 2002).

Total cell counts performed on exponentially growing cells in 21% O_2 treated with $CoCl_2$ revealed a dose-dependent proliferation inhibition and S-phase arrest. In contrast, FG-4497 induced S-phase of the cell cycle, without being toxic to NPCs and increased total cell numbers in normal oxygen, comparable to those observed in hypoxia. FG-4497 is a novel HIF-PHD inhibitor that has been reported to ameliorate mucosal damage in experimental murine colitis (Robinson et al. 2008) and to protect against hypoxic distal tubular injury in an isolated perfused rat kidney model (Rosenberger et al. 2008). In our experiments, a neuroprogenitor cell type-specific effect (frontal vs. midbrain) of FG-4497 or any drug tested was not observed.

Consistent with recent studies on bone-marrow-derived mesenchymal stem cells (MSCs) that have indicated $CoCl_2$-mediated decrease of cyclin D1 and nestin expression (Pacary et al. 2007), in the present study, $CoCl_2$ was slightly toxic to proliferating NPCs, most likely by causing oxidative cellular damage via ROS generation. Interestingly, cobaltous ions did not harm differentiated hmNPCs, but supported both neuronal and dopaminergic differentiation. On the other hand, glial differentiation was significantly reduced with 100 μM of cobalt chloride. Neurotropic effects of $CoCl_2$ have already been demonstrated by promotion of neurite outgrowth in PC-12 rat pheochromocytoma cells (Kotake-Nara et al. 2005), whereas in MSCs, the successful promotion of neuronal differentiation was followed by an increased dopaminergic specification of the cells (Pacary et al. 2006). The latest is consistent with recent evidences implying HIF-1α as an important factor for the development of dopaminergic system (Kim et al. 2008; Milosevic et al. 2007a). A similar explanation stands for the induction of dopaminergic differentiation observed by the application of another hypoxia mimetic, the novel HIF-PHD inhibitor FG-4497. This non-toxic small molecule appeared as a powerful inducer of in vitro neurogenesis as demonstrated by an increased number of proliferating neuroblasts (TUJ1 and DCX positive) that thereafter differentiated into more mature neuron-like cells (NSE positive). Indeed, both HIF-1α and its target genes (EPO, VEGF) have been implicated in the enhancement of neurogenesis (Chen et al. 2007b; Jin et al. 2002; Schaenzer et al. 2004). FG-4497 deserves further investigation in the area of regenerative medicine with the promise to bring an improvement of in vivo neurogenesis targeting NPCs in the adult brain.

Successful HIF-PHD inhibition represents a target for neuroprotection in the central nervous system (Siddiq et al. 2005). A phenomenon that provides a protection through acquiring tolerance to an otherwise lethal stressor has been termed as preconditioning. It is well established that the hypoxic preconditioning (mild hypoxia for up to several hours) provides protection against ischemic brain injury in animal models of cerebral ischemia as a result of neuroprotective effects of HIF-1 and its downstream genes (Liu et al. 2005; Prass et al. 2003). Hypoxic preconditioning has recently been proposed as an approach to promote survival of transplanted cells (Hu et al. 2008; Pasha et al. 2008; Theus et al. 2008). Similarly, the tolerance of hmNPCs before transplantation or neurons before injury, could be achieved by pharmacological HIF-1α stabilization. HIF-α stabilizers exert their neuroprotective and pro-survival effects by inducing EPO, VEGF, Bcl-2 and Bcl-xL (Tsai et al. 2006). EPO receptors are highly expressed on adult dopaminergic neurons (Csete et al. 2004), which supports the notion regarding EPO-mediated protection towards dopaminergic neurons (Demers et al. 2005). Neuroprotective effects of VEGF towards DA neurons were also described (Yasuhara et al. 2005). Here, proliferating hmNPCs, mostly self-renewing Nestin$^+$ cells (Milosevic et al. 2006), differentiated hmNPCs, and HEK-293 cells expressing the human DAT gene (HEK-DAT cell line) were used for neuroprotection studies. As already described, HEK-DAT cells are highly sensitive to MPP^+ due to the uptake of the neurotoxin by the DAT, followed by the cellular energy depletion (Storch et al. 1999). In general, MPP^+-induced cytotoxicity also involves DAT-independent mechanisms, particularly at higher doses, probably via ROS formation (Jung et al. 2007). The MPP^+-mediated toxicity in HEK-DAT cells is considered to be more specific towards the dopaminergic phenotype and likely does not involve ROS production, as it was independent of the presence of antioxidants (Storch et al. 1999). We demonstrated that both $CoCl_2$ and FG-4497 provide cytoprotection and neuroprotection and most likely also provide protection towards dopaminergic neurons. FG-4497 was more effective in this regard as pre-treatment with this compound helped differentiated hmNPCs to preserve an already formed neuronal network. $CoCl_2$ failed to perform the same way, possibly due to side effects such as synergism between $CoCl_2$ and MPP^+ with respect to ROS generation that might harm the cells. Another reason for better neuroprotection accomplished by FG-4497 might come from its ability to support neurogenesis, a phenomenon that we could not demonstrate with $CoCl_2$. Possessing capability to both protect neurons and to repair them by enhancing neurogenesis, FG-4497 fulfils the requirements for a targeted pharmacotherapeutic for the treatment and/or prevention of acute cerebral ischemia, as well as long-term neurodegeneration (e.g. Parkinson's Disease).

Taken together, our data strongly indicate that pharmacological HIF-α stabilization causes a variety of biological effects on ex vivo expanded human neural progenitor cells holding important therapeutic implications.

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