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Oxygen regulates proliferation of neural stem cells through Wnt/β-catenin signalling

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ABSTRACT

Reduced oxygen levels (1–5% O_2, named herein 'physioxia') are beneficial for stem cell cultures leading to enhanced proliferation, better survival and higher differentiation potential, but the underlying molecular mechanisms remain elusive. A potential link between physioxia and the canonical Wnt pathway was found recently, but the differential involvement of this signalling pathway for the various stem cell properties such as proliferation, stem cell maintenance, and differentiation capacity remains enigmatic. We here demonstrate increased Wnt target gene transcription and stabilised active β-catenin upon physioxic cell culture in primary tissue-specific foetal mouse neural stem cells. Knock-out of the main oxygen sensing molecule, hypoxia-inducible factor-1α (Hif-1α), had no impact on Wnt activation assuming that physioxia induces the Wnt pathway independently of Hif-1α. To determine the physiological relevance of physioxia-induced Wnt/β-catenin signalling, we examined proliferation, cell cycle kinetics, survival and stem cell maintenance upon Wnt activation and inhibition. Whereas survival and stem cell maintenance seem to be independent of the Wnt pathway, our studies provide first evidence that Wnt/β-catenin signalling positively stimulates proliferation of physioxic cells by affecting cell cycle regulation. Together, our results provide mechanistic insight into oxygen-mediated regulation of the self-renewal activity of neural stem cells.

1. Introduction

Neural stem cells (NSCs) are multipotent cells capable of self-renewing and generating various cell types like neurons, astrocytes and oligodendrocytes. NSCs can be isolated from the developing or adult brain and expanded in vitro in serum-free medium containing mitogenic factors such as fibroblast growth factor and epidermal growth factor (Fisher, 1997; McKay, 1997). Standard cell culture is commonly performed at atmospheric O_2 tension of 21% O_2, however in mammalian brain, most tissues are physiologically exposed to O_2 levels ranging from 1% to 5% O_2 (Silver and Erecinska, 1998). Therefore, for this physiological level of oxygenation in vitro the new term of "physioxia" was recently introduced (Carreau et al., 2011). It has been shown in previous studies that reduced O_2 is vital to in vitro cultures of foetal NSCs along with proliferation, survival and potential to dopaminergic differentiation of midbrain-derived NSCs (Storch et al., 2001; Studer et al., 2000). The response to low O_2 involves activation of hypoxia-inducible factors (Hifs), of which Hif-1 is the most explored one (Semenza and Wang, 1992). Hif-1 is composed of the O_2-dependent α-subunit (Hif-1α) and the O_2-independent aryl hydrocarbon receptor nuclear translocator (ARNT or Hif-1β). Hif-1α protein synthesis is positively regulated by the PI3K/AKT and the MAPK pathway (Semenza, 2003). Under air conditions, however, proline and asparagine residues of Hif-1α are hydroxylated inhibiting binding of Hif-1α to its coactivators and inducing Von Hippel–Lindau protein (pVHL)-mediated ubiquitination and degradation of Hif-1α. Under physioxic conditions, however, hydroxylations are reduced and thus, Hif-1α proteins are stabilised. Hif-1α then binds together with Hif-1β to hypoxia response elements (HREs) in promoter and enhancer regions of various known target genes to activate their transcription (Pouyssegur and Mechta-Grigoriou, 2006; Schofield and Ratcliffe, 2004; Semenza, 1998). Expression of Hif-1α target genes such as *vascular endothelial growth factor* (*Vegf*) or *phosphoglycerate kinase 1* (*Pgk1*) induces the adaptations to low O_2 involving i.a. increased angiogenesis or altered glucose metabolism (Forsythe et al., 1996; Liu et al., 1995; Pugh et al., 1991; Semenza et al., 1994). It was previously shown for cultured embryonic stem cells, that physioxia also induces canonical Wnt/β-catenin signalling (Mazumdar et al., 2010).

Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; BrdU, 5-bromo-2′-deoxyuridine; GFAP, glial fibrillary acidic protein; Hif, hypoxia-inducible factor; IWP, inhibitor of Wnt production; IWR, inhibitor of Wnt response; Map2, microtubule-associated protein 2; NSC, neural stem cells; pVHL, von Hippel–Lindau protein.

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The canonical Wnt signalling pathway, unlike other Wnt pathways, involves stabilisation of β-catenin. In the absence of Wnt signals, a destruction protein complex consisting of glycogen synthase kinase-3β (GSK-3β), axin, adenomatous polyposis coli and casein kinase 1 constantly phosphorylates the protein β-catenin and thereby targets it for ubiquitination and degradation by the proteasome. Upon binding of a Wnt protein to the Frizzled receptor, GSK-3β is inhibited and in turn phosphorylation and degradation of β-catenin is blocked. GSK-3β kinase activity can be also reduced by the PI3K/AKT pathway leading to accumulation of β-catenin (Voronkov and Krauss, 2013). Stabilised β-catenin localised to the nucleus, where it dimerises with Tcf/Lef transcription factors allowing transcription of Wnt downstream gene targets and induction of a cellular response (Clevers, 2006; Verheyen and Gottardi, 2010). The physiological relevance of physioxia-induced Wnt/β-catenin signalling, however, remains elusive.

In the present study, we investigated O_2-dependent activation of Wnt/β-catenin signalling in NSCs and defined the role of Hif-1α by means of Hif-1α conditional knockout mouse model. Also, we were the first to identify which stem cell features observed in physioxic cell cultures – proliferation, cell cycle regulation, survival or stem cell maintenance – are mediated by direct involvement of Wnt/β-catenin signalling.

2. Material and methods

2.1. Generation and analysis of mice

Wildtype C57Bl/6J mice were purchased from Charles River, Sulzfeld, Germany and Hif-1α$^{flox/flox}$ mice were a kind gift from Shuhei Tomita, MD, PhD. Generation and analysis of *Nes*-Cre Hif-1α conditional knockout embryos have been described previously (Tomita et al., 2003). Animal procedures were approved by the Animal Rights Committee.

2.2. Neural stem cell isolation, culture and treatments

Mesencephalic and cortical regions were dissected from mouse E14 embryos and the meninges were removed. To achieve a single cell suspension, the tissue samples were trypsinised (2.5 ng/ml, Sigma-Aldrich, Seelze, Germany) for 10 min at room temperature, incubated in DNase (40 μg/ml, Sigma-Aldrich, Seelze, Germany) for 10 min at 37 °C and triturated. For monolayer cultures, cells were plated onto poly-L-ornithine/fibronectin-pre-coated culture dishes. Cultures were maintained in expansion medium composed of DMEM (high glucose) supplemented with 32% F12, 2% B27 and 1% penicillin/streptomycin (all Life Technologies, Darmstadt, Germany) and 20 ng/ml EGF and FGF2 (Sigma-Aldrich, Hamburg, Germany) with medium change three times per week. For physioxic culture, cells were maintained in a gas mixture composed of 92% N_2 5% CO_2 and 3% O_2. To preserve constant O_2 levels, medium was pre-equilibrated by exposure to the gas mixture for at least 24 h and cells were not passaged.

To activate β-catenin signalling, the GSK3 inhibitor Chir99021 (Chir, 3 μM, Axon Medchem, Groningen, The Netherlands) was added to the cells with every medium change and in addition 3 h prior to cell harvest. To block Wnt signalling, cells were grown in the presence of the inhibitor of Wnt production (IWP-2, 40 nM) or the inhibitor of Wnt response (IWR-1, 200 nM, both Sigma-Aldrich, St. Louis, MO, USA). Compounds were renewed with every medium change and before cells were harvested.

2.3. Cell death and proliferation

For apoptosis studies, cells were cultured in 4-well plates for the indicated time period. Dead cells were visualised by staining with propidium iodide (PI) and PI^+ cells were enumerated as a percentage of total number of cells detected by Hoechst 33342 (Invitrogen, Darmstadt, Germany) nuclear staining. Ten randomly chosen fields per well were analysed. For cell proliferation studies, 2×10^4 cells were seeded into 6-well plates under different growth conditions. After the indicated time period, cells were trypsinised in 1 ml of accutase (Sigma-Aldrich, Seelze, Germany) and counted in a Neubauer hemocytometer.

2.4. Cumulative BrdU labelling

Following isolation and proliferation for 5 days in respective O_2 condition, 10 μM BrdU was added to the culture and cells were fixed after 2 h, 4 h, 6 h, 8 h, 12 h, 16 h, 24 h and 36 h with Accustain for 30 s. Cells were permeabilised with HCl and immunostained for rat anti-BrdU (1:200, Abcam, Cambridge, UK) and Hoechst 33342. By analysing the BrdU incorporation rate (Nowakowski et al., 1989), the total cell cycle length (T_C), the length of the S-phase (T_S) and the portion of proliferating cells in a population, called growth fraction (GF), could be estimated.

2.5. RNA isolation and qRT-PCR

Total RNA was extracted using the Qiagen RNeasy Kit according to the manufacturer's instructions. Complementary DNA was generated from 1 μg of total RNA with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using SYBR Green PCR Kit (Qiagen, Hilden, Germany) and the Stratagene MX3000P thermocycler with following programme: 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. *Hydroxymethylbilane synthase* (*Hmbs*) was used as housekeeping genes for normalisation. Following primers were used: *Lef1* F, 5′- TCCT GAAATCCCCACCTTCT -3′; *Lef1* R, 5′- TGGGATAAACAGGCTGACCT -3′; *Tcf1* F, 5′- CCAGTGTGCACCCTTCCTAT -3′; *Tcf1* R, 5′- AGCCCCACAGAG AAACTGAA -3′; *Axin2* F, 5′- GAGAGTGAGCGGCAGAGC -3′; *Axin2* R, 5′- CGGCTGACTCGTTCTCCT -3′; *Dkk4* F, 5′- CTCGCTGTGTGCATCAGACA -3′; *Dkk4* R, 5′- TACTGCTTTGTGATTTCTTCGTA -3′; *Fzd7* F, 5′- GGGTAT CTCTGTGTAGCCCTGA -3′; *Fzd7* R, 5′- AGAGGCAGGTGGATGTCTGT -3′; *Sfrp5* F, 5′- GATCTGTGCCCAGTGTGAGA -3′; *Sfrp5* R, 5′- TTCAGCTGCC CCATAGAAA -3′; *Vegf* F, 5′- GCTACTGCCGTCCGATTG -3′; *Vegf* R, 5′- CTCCAGGGCTTCATCGTTAC -3′; *Pgk1* F, 5′- TACCTGCTGGCTGGATGG -3′; *Pgk1* R, 5′- CACAGCCTCGGCATATTTCT -3′; *Hmbs* F, 5′- TGTATGCT GTGGGTCAGGGAG -3′; *Hmbs* R, 5′- CTCCTTCCAGGTGCCTCAGA -3′ (all Eurofins MWG Operon, Ebersberg, Germany).

2.6. Microarray gene chip analysis

For the microarray gene chip assay, cells were maintained in expansion medium for 48 h and 13 days after dissection and medium change was done three times per week. For physioxic culture, cells were maintained in a gas mixture composed of 92% N_2 5% CO_2 and 3% O_2. To preserve constant O_2 levels, medium was pre-equilibrated and cells were not passaged. Isolation of RNA was carried out using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured using Nanodrop spectrometer (Agilent Technologies, Santa Clara, CA, USA) and RNA quality was approved by Agilent 2100 Bioanalyzer. Hybridization to whole mouse genome microarray gene expression chips (Gene ST 1.0 Arrays, Affymetrix, Santa Clara, CA, USA) was performed following the manufacturer's protocol. Microarray chips were then immediately scanned using the Agilent Microarray Scanner (Agilent Technologies, Santa Clara, CA, USA).

2.7. Western blotting

Cytoplasmic and nuclear proteins were isolated with the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to manufacturer's instructions. Protein concentrations were determined using the Quant-iT™ Protein Assay Kit (Molecular Probes, Darmstadt, Germany). Samples were subjected to SDS-PAGE (12% gels), before transfer onto nitrocellulose membrane. Immunoblotting for total β-catenin (1:500,