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O₂ regulates stem cells through Wnt/β-catenin signalling

Jolly Mazumdar^{1,6}, W. Timothy O'Brien², Randall S. Johnson³, Joseph C. LaManna⁴, Juan C. Chavez⁵, Peter S. Klein², and M. Celeste Simon^{1,6,*}

¹Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, U C San Diego, La Jolla, CA 92093

²Division of Hematology-Oncology, University of Pennsylvania School of Medicine, U C San Diego, La Jolla, CA 92093

³Division of Biological Sciences, U C San Diego, La Jolla, CA 92093

⁴Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106

⁵Translational Medicine, Wyeth Research, Collegeville, Pennsylvania, PA 19426

⁶Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

Abstract

Stem cells reside in specialized microenvironments or “niches” which regulate their function. *In vitro* studies employing hypoxic culture conditions ($\leq 5\%$ O₂) have revealed strong regulatory links between O₂ availability and stem/precursor cell functions^{1–6}. Therefore, while some stem cells are perivascular, others may occupy hypoxic niches and be regulated by O₂ gradients. However, the underlying mechanisms remain unclear. Here, we show that Hypoxia Inducible Factor-1α (HIF-1α), a principal mediator of hypoxic adaptations, modulates Wnt/β-catenin signalling in hypoxic embryonic stem (ES) cells by enhancing β-catenin activation and expression of downstream effectors LEF-1 and TCF-1. This regulation extends to primary cells, including isolated neural stem cells (NSCs), and is not observed in differentiated cells. *In vivo*, Wnt/β-catenin activity is closely associated with low O₂ regions in the subgranular zone (SGZ) of the hippocampus, a key NSC niche⁷. *Hif-1α* deletion impairs hippocampal Wnt-dependent processes, including NSC proliferation, differentiation and neuronal maturation. This decline correlates with reduced Wnt/β-catenin signalling in the SGZ. Therefore, O₂ availability may have a direct role in stem cell regulation via HIF-1α modulation of Wnt/β-catenin signalling.

Keywords

Embryonic stem cells; hypoxic niche; HIF-1α/ARNT; Wnt/β-catenin signalling; neurogenesis

*Address correspondence to: M. Celeste. Simon, Ph.D. Abramson Family Cancer Research Institute University of Pennsylvania BRB II/III, Room 456 421 Curie Boulevard Philadelphia, PA 19104 Telephone: (215) 746-5532 Fax:(215)746-5511 (celeste2@mail.med.upenn.edu).

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Wnt/ β -catenin pathway activation is characterized by (i) stabilization of cytoplasmic β -catenin upon receptor engagement by Wnt ligands, (ii) β -catenin nuclear translocation, (iii) β -catenin interaction with lymphoid enhancer-binding factor-1/T-cell factor-1 (LEF/TCF) transcription factors, and (iv) stimulation of target genes^{8,9}. To determine if this pathway is modulated by O₂ availability, we transiently transfected murine ES cells and P19 embryonal carcinoma (EC) cells with a luciferase-based TOP-Flash (TCF optimal promoter) Wnt reporter plasmid, and compared reporter activity under “normoxia” (21% O₂), and “hypoxia” (1.5% O₂). Exposure to hypoxia significantly enhanced reporter activity (2 fold) in both embryonic cell lines (Fig. 1a and see Supplementary Information, Fig. S1a, b). Hypoxic exposure also increased levels of nuclear β -catenin, LEF-1 and TCF-1 proteins (Fig. 1b). Quantitative real time PCR (qRT-PCR) analyses of hypoxic cells showed increased expression (2–6 fold) of Wnt target genes, such as *Axin2* and *Dkk-4* (Dickkopf-4), and β -catenin activators, *Lef-1* and *Tcf-1* (Fig. 1c). This is consistent with our previous data showing increased *Lef-1* expression in hypoxic murine ES cells¹⁰. HIF-1 α protein stabilization and upregulation of the HIF-1 α target *Pgk-1* confirmed the induction of a hypoxic response (Fig. 1b, c).

Hypoxia exerted a similar effect in stimulated cells. While Wnt pathway stimulators, including 6-Bromindirubin-3' oxime (BIO), Lithium Chloride (LiCl) or Wnt-3a conditioned medium (Wnt-3a CM), enhanced reporter activity ~20 fold, exposure to hypoxia increased TOP-Flash activity 50–80 fold in stimulated cells relative to untreated controls (Fig. 1d and Supplementary Information, Fig. S2a, b). Hypoxic exposure also further increased expression of Wnt target genes *Lef-1* and *Dkk-4* in stimulated cells (Fig. 1e). TOP-Flash assays in RNAi-mediated β -catenin depleted cells confirmed the involvement of β -catenin in hypoxia induced luciferase activity (Fig. 1f). We excluded the possible involvement of other signalling pathways proposed to promote β -catenin stabilization (e.g. Akt/PDK) by inhibiting glycogen synthase kinase-3 β (GSK-3 β)¹¹, by assessing GSK3 β phosphorylation levels, which remained unchanged under hypoxia (Supplementary Information, Fig. S1c). Collectively, these data indicate that ES and P19 EC cells maintain constitutively active Wnt signalling that is β -catenin dependent, and markedly enhanced by hypoxia.

Hypoxic induction of Wnt signalling was also evident in cell proliferation assays. Hypoxic ES cells displayed increased numbers (based on cell counts) compared to normoxic cells (Fig. 1g). This reflected increased cell survival, as hypoxic exposure had modest effects on ES cell cycle, but significantly reduced apoptotic cell death (Supplementary information, Fig. S2c, d). Addition of Wnt-3a CM, which stimulates cell expansion/self-renewal¹², increased the numbers of both normoxic and hypoxic cells relative to untreated controls. In contrast, treatment with Dickkopf-1 (DKK-1), an extracellular Wnt pathway inhibitor, exclusively decreased cell numbers under hypoxia (Fig. 1g). Of note, DKK-1 treatment downregulated TOP-Flash activity in both normoxic and hypoxic ES cells (Fig. 1h), suggesting that hypoxia sensitizes ES cells to the growth effects of Wnt/ β -catenin signalling.

One of the primary mediators of hypoxic responses is HIF-1, a heterodimeric transcription factor containing an O₂ sensitive α subunit (HIF-1 α) and a constitutively expressed β subunit (HIF-1 β , also known as ARNT). To determine whether hypoxia activates Wnt signalling via HIF-1, we analyzed TOP-Flash activity in *Hif-1 α ^{-/-}* ES cells. In contrast to a report showing HIF-1 α mediated repression of Wnt signalling in colon cancer cells¹³, *Hif-1 α* deletion significantly downregulated TOP-Flash activity in hypoxic ES cells, but had minimal effect on basal activity (Fig. 2a). Combined treatment of Wnt-3a CM and hypoxia did not superinduce TOP-Flash activity in *Hif-1 α ^{-/-}* cells (Fig. 2b), and loss of *Hif-1 α* had no effect on normoxic responses to Wnt stimulation (Fig. 2b). However, normoxic HIF-1 α stabilization in WT ES cells by the hypoxia mimetic deferoxamine (DFX) significantly

restored reporter activity (> 1.5 fold) (Fig. 2a). We observed a similar regulatory link between hypoxic ARNT activity and Wnt/ β -catenin signalling (Fig. 2c). Deletion of *Hif-1 α* and *Arnt* also diminished expression of Wnt target genes including *Dkk-4*, *Lef-1* and *Tcf-1* under hypoxia (Fig. 2d, e and Supplementary Information, Fig. S3a). Thus, hypoxic induction of Wnt/ β -catenin signalling is mediated by HIF-1 α /ARNT complexes. Moreover, as expected from increased levels of both β -catenin and LEF-1 in hypoxic cells (Fig. 1b), we detected increased association of nuclear β -catenin extracted from hypoxic ES cells with immunoprecipitated LEF-1 (Fig. 2f). Intriguingly, we noted reduced levels of β -catenin in whole cell extracts of hypoxic *Hif-1 α ^{-/-}* cells as compared to hypoxic *Hif-1 α ^{+/+}* (Fig. 2e).

Since hypoxic induction of *Lef-1* and *Tcf-1* mRNA and corresponding proteins strongly correlated with HIF-1 α protein accumulation, we examined whether HIF-1 α directly contributes to increased transcription of *Lef/Tcf* genes. Analysis of murine *Lef-1* and *Tcf-1* gene sequences revealed multiple putative HREs (hypoxia response elements) spanning exon 1 and the upstream promoter and enhancer regions (+3000 bp) (Fig. 2g upper). Subsequently, in chromatin IP (ChIP) assays, compared to normoxia, hypoxic ES cells exhibited increased (4–10 fold) HIF-1 α association at each genomic region tested (Fig. 2g lower). Therefore, HIF-1 α regulates LEF-1/TCF-1 protein abundance and function in embryonic cells. In proliferation assays, neither *Hif-1 α ^{-/-}* nor *Arnt^{-/-}* cells displayed growth advantage under hypoxia compared to the corresponding wild type *Hif-1 α ^{+/+}* and *Arnt^{+/+}* cells (Supplementary Information, Fig. S3b, c).

Stem and committed progenitor cells can respond differently to low O₂ signals⁵. Therefore, we asked if hypoxic effects on Wnt/ β -catenin signalling are a function of differentiation stage. Embryonic cells were stimulated with neuronal supplements (N2B27) to form neuronal precursors¹⁴. Thereafter, we subjected ES and P19 EC cell-derived neurons expressing the neuronal markers β -tubulin III (β -tubIII) and doublecortin (DCX) (Fig. 3a and Supplementary Information, Fig. S4a) to TOP-Flash assays. The transfection efficiency was monitored using GFP as a reporter (data not shown). Whereas hypoxia enhanced TOP-Flash activity in undifferentiated ES and P19 cells (Fig. 3b), it did not have any effect on the embryonic cell-derived neurons. This observation implied that hypoxia selectively activates Wnt/ β -catenin signalling in undifferentiated cells. We also observed hypoxic induction of *Lef-1* and *Tcf-1* genes exclusively in undifferentiated cells (Fig. 3c). Furthermore, neuronal differentiation coincided with a significant loss of baseline *Lef-1/Tcf-1* levels (3–5 fold) (Fig. 3c). We suggest that the *Lef-1* and *Tcf-1* loci become epigenetically modified and therefore inaccessible to HIF-1 in differentiated cells.

It has been shown that LEF/TCF over-expression increases β -catenin nuclear translocation, and activates transcription in multiple embryonic cell types^{15,16}. To investigate if β -catenin accumulation in hypoxic ES cells involved *Lef-1* induction, we engineered LEF-1 overexpressing ES cells (ES-*Lef-1*). LEF-1 overexpression resulted in increased levels of nuclear β -catenin under normoxia relative to vector controls (VC) (Fig. 3d). Also, ES-*Lef-1* cells displayed enhanced cell proliferation, and were sensitive to DKK-1 inhibition (Supplementary Information, Fig. S4c). This observation recapitulated Wnt/ β -catenin dependent hypoxic effects on ES cell proliferation (Fig. 1g). Moreover, we reproduced nuclear β -catenin-HIF-1 α interaction in hypoxic ES cells (see Supplementary Information, Fig. S4b), similar to previous observations in differentiated neoplastic cells¹³, further highlighting LEF-1's involvement in differentiation-stage specific responses of the Wnt/ β -catenin pathway to hypoxia. Taken together, we propose a model where HIF-1 α regulates *Lef-1* and *Tcf-1* in undifferentiated cells, leading to increased nuclear β -catenin-LEF/TCF interaction, and activation of Wnt/ β -catenin targets (Fig. 3e).

To determine the physiological relevance of the above model, we next investigated whether decreased O₂ levels correlate with Wnt/ β -catenin activity *in vivo*. We screened for areas of Wnt/ β -catenin activity in a Wnt reporter (*BAT-GAL*) mouse strain with β -galactosidase immunostaining¹⁷, and compared its distribution with that of low O₂ regions detected by immunostaining with the hypoxia marker pimonidazole hydrochloride. Pimonidazole detects O₂ partial pressures of less than 10 mm Hg (~1.3% O₂)¹⁸. Active Wnt/ β -catenin signalling was detected in close proximity to low O₂ regions in both embryonic and the adult brain (Fig. 4a, b and Supplementary Information, Fig. S5 a–d). One such region positive for Wnt/ β -catenin activity was the subgranular zone (SGZ) of the dentate gyrus (DG) (Fig. 4a, and Supplementary Information, Fig. S5 e–g), a primary neurogenic niche in which adult hippocampal progenitors (AHPs) proliferate and differentiate into dentate granule neurons.⁷ Pimonidazole clearly marked the SGZ, as identified by its colocalization with the NSC marker Sox2 (Fig. 4c, d). Importantly, enumeration of blood vessels marked by laminin immunostaining revealed a much higher nuclei:blood vessel ratio within the granule cell layer of the DG in comparison to other areas in the brain including the adjacent hippocampal Oriens (Or) layer, and the cerebellar granule cell (CG) layer (Fig. 4e–g). Other vascular markers such as CD31 and India ink showed a similar trend (Supplementary Information, Fig. S5h–k). This suggests that hypoxia within the dentate gyrus, as independently confirmed by HIF-1 α stabilization and the expression of hypoxia responsive genes carbonic anhydrase IX (CAIX) and vascular endothelial growth factor (VEGF), (Supplementary Information, Fig. S5l–q), is likely a consequence of O₂ limitation due to fewer blood vessels.

To further examine the potential link between HIF-1 α and Wnt/ β -catenin signalling *in vivo*, we engineered a *BAT-GAL* reporter line in the background of conditional global HIF-1 α mutants (*gHif-1 $\alpha^{\Delta/\Delta}$* , *BAT-GAL^{Tg}*). Because global *Hif-1 α* deletion results in embryonic lethality¹⁹, we crossed mice homozygous for the *loxP*-flanked (floxed) *Hif-1 α* allele (*Hif-1 $\alpha^{fl/f}$*) with *Hif-1 $\alpha^{fl/f}$* mice expressing tamoxifen inducible Cre driven by a Ubiquitin C (*UbC*) promoter, such that tamoxifen administration will result in global postnatal *Hif-1 α* (*gHif-1 $\alpha^{\Delta/\Delta}$*) deletion (Supplementary Information, Fig. S6a, b). *Hif-1 α* deletion reduced hippocampal Wnt signalling, based on loss of *BAT-GAL* reporter activity in mice expressing the *BAT-GAL* transgene (*n*=3) (Fig. 4h, i and Supplementary Information, Fig. S6c, d). *Hif-1 α* deletion also decreased expression of Wnt target genes such as *Dkk-4* (undetected), *Lef-1* and *Axin2* (2.5 fold) (*n*=5) in *gHif-1 $\alpha^{\Delta/\Delta}$* animals (Fig. 4j).

Because Wnt/ β -catenin signalling has been identified as a positive regulator of neurogenesis occurring in the SGZ of adult hippocampus⁷, we employed the SGZ as a model stem cell niche to evaluate the role of HIF-1 α in stem cell functions. Based on the SGZ HIF-1 α -Wnt / β -catenin connection, we hypothesized that loss of HIF-1 α may result in aberrant neurogenesis *in vivo*. We analyzed the dentate gyrus of young adult (7–8 weeks) *gHif-1 $\alpha^{\Delta/\Delta}$* or control mice lacking Cre (*gHif-1 $\alpha^{fl/f}$*) for cells expressing the early neuronal marker doublecortin (DCX). *gHif-1 $\alpha^{\Delta/\Delta}$* dentate gyrus displayed a 3.4 fold reduction in DCX⁺ cells as compared to *gHif-1 $\alpha^{fl/f}$* control animals (*n*=7) (Fig. 4k, o). Moreover, DCX⁺ neurons in the *gHif-1 $\alpha^{\Delta/\Delta}$* mice were severely diminished (4 fold) in their capacity to form neurites (*n*=1) (Fig. 4l, p), a phenotype critical for integration of the newborn neurons into the existing neuronal circuitry.

To distinguish between cell-intrinsic versus -extrinsic effects underlying HIF-1 α 's impact on hippocampal neurogenesis, we further analyzed adult neurogenesis in a neuron specific *Hif-1 α* deletion model. To selectively delete HIF-1 α from murine neurons, we crossed *Hif-1 $\alpha^{fl/f}$* mice with R1ag#5 (R1) mice expressing Cre driven by the calcium/calmodulin-dependent Kinase (α CamKII) promoter^{20,21}. Cre expression in the R1 animals results in postnatal neuronal *Hif-1 α* (*nHif-1 $\alpha^{\Delta/\Delta}$*) deletion in the forebrain²¹, including Sox2⁺ cells of

the SGZ of the DG (Supplementary Information, Fig. S5r–t). Similar to global *Hif-1α* deletion, neuronal *Hif-1α* loss significantly reduced BrdU⁺ (which labels proliferating NSCs and progenitors) cell numbers (2.2 fold) and DCX⁺ newborn neurons (2 fold) in the SGZ of *nHif-1α*^{Δ/Δ} mice as compared to *nHif-1α*^{fl/fl} control animals (*n*=6) (Fig. 4m,n and q). That the DCX⁺ cells were derived from NSCs and AHPs was confirmed by double-immunostaining with BrdU and DCX (Supplementary Information, Fig. S6e). Moreover, similar to *gHif-1α*^{Δ/Δ}DCX⁺ cells, the DCX⁺ cells of *nHif-1α*^{Δ/Δ} mice were severely diminished in their capacity to form neurites (3 fold) (*n*=3) (Supplementary Information, Fig. S6f, g). Importantly, the neurogenesis defects were rescued pharmacologically in *nHif-1α*^{Δ/Δ} mice treated with GSK-3 inhibitor SB 216763 (2mg Kg⁻¹) as compared to vehicle-treated *nHif-1α*^{Δ/Δ} mice (*n*=6) (Fig. 4q). We also made similar observations in *nHif-1α*^{Δ/Δ} mice transduced with lentiviruses expressing a constitutively active form of β-catenin (Δ-GSK-β-catenin)²² (*n* = 3–5 per group) (Supplementary Information, Fig. S6h, i). These models cannot exclude a potential role for HIF-1α in regulating trophic signals provided by the postmitotic mature granule neurons of the DG in the survival and integration of NSCs into hippocampal circuitry. Nevertheless, HIF-1α clearly regulates adult neurogenesis by promoting NSC/progenitor cell proliferation and differentiation via Wnt/β-catenin signalling.

We also observed that the hypoxic regulation of Wnt/β-catenin activity exhibited by ES cells is shared by primary cells of the nervous system. Isolated embryonic and adult neural stem cells (E-NSC and A-NSC) transfected with TOP-Flash plasmid displayed significantly increased reporter activity under hypoxia (Fig. 5a, b and c), and this effect was lost in differentiated neurospheres (Fig. 5d and f). Of note, transient transfection with a normoxia-active HIF-1α triple mutant (HTF-1α TM[P402A/P577A/N815A]) plasmid²³ increased *Lef-1* levels ~ 12 fold exclusively in the undifferentiated neurospheres (Fig. 5e). Moreover, differentiated neurospheres exhibited significantly reduced baseline *Lef-1* levels as compared to undifferentiated control neurospheres (Fig. 5e), reminiscent of reduced basal *Lef-1* expression observed in ES cell-derived neurons (Fig. 3c). We conclude that the regulation of Wnt/β-catenin by HIF-1α through *Lef-1* induction represents an important mechanism of stem cell homeostasis, and may be applicable to stem cell microenvironments beyond the hippocampal neurogenic niche.

Numerous adult stem cells have been found closely associated with the vasculature^{24–27}. Interestingly, we now demonstrate that low O₂ levels may also contribute to stem cell microenvironments. This suggests that whereas some adult stem cells occupy well-oxygenated perivascular domains, others reside within hypoxic niches and are regulated by O₂ deprivation. The induction of Wnt/β-catenin signalling by HIF-1α represents a molecular mechanism underlying this regulation. These studies extend the positive effects of HIFs previously shown for other stem cell pathways, including Oct-4 and Notch-1^{28,29}. The opposite result was reported for HCT116 colon cancer cells, in which HIF-1α inhibited Wnt/β-catenin activity¹³, suggesting that the interaction between HIF-1α and Wnt/β-catenin in undifferentiated stem/progenitor cells and more differentiated cell types (including neoplastic cells) is functionally distinct. In summary, the present findings confirm the emerging concept that HIFs not only act in metabolic adaptations, but can also regulate critical stem cell phenotypes.

METHODS

Cell culture and treatments

Arnt^{-/-}, *Arnt*^{Res} and corresponding WT (*Arnt*^{+/+}) ES cells have been described previously^{30, 31}. ES cells were cultured as described⁴. P19 EC cells (ATCC) were cultured as per manufacturer's protocol. Hypoxia (0.5%, 1.5% and 3% O₂) was achieved using a

HERAcell 240 hypoxic workstation with O₂ control. Deferoxamine (DFX) (Calbiochem) was used as a hypoxia mimetic at a final concentration of 200 μ M for 16 h. Wnt-3a conditioned medium (Wnt-3a CM) and corresponding control medium were harvested from L Wnt-3A and L Cells respectively (ATCC). LiCl and BIO were purchased (Sigma) and used in final concentration as follows; LiCl (20 mM) and BIO (200 nM).

Reporter assay

Transient transfections of cells with TOP-Flash or FOP-Flash plasmids were performed with Lipofectamine 2000 (Invitrogen) in 24-well plates. Luciferase assays were performed using the dual luciferase protocol (Promega) with the following modification for siRNA: Cells were first transfected with siRNA against β -catenin (ON-TARGET plus SMART pool L-040628-00-0005, Dharmacon), and after 24 h was followed by a second co-transfection with firefly (200 ng) and *Renilla* luciferase (24 ng) reporter gene expression plasmids. β -catenin knockdown was verified 48–72 h post transfection by standard western blot procedure.

Apoptosis and cell proliferation

For apoptosis studies, undifferentiated ES monolayers were grown on gelatin coated coverslips, and cultured under hypoxia for indicated time period. TUNEL⁺ cells were visualized using an ApopTag kit (Millipore). TUNEL⁺ cells were enumerated as percentage of total cells in the field detected by DAPI nuclear staining. 3 randomly chosen fields per plate ($n=3$) were analyzed and averaged per condition. For proliferation analysis, 10⁴ cells were seeded on 6 cm² plates, and two plates were counted per time point in a hemocytometer over 6 days. Recombinant mouse DKK-1 (R&D systems) was used at a final concentration of 300 ng ml⁻¹. For cell cycle analysis, ES monolayers were cultured either under normoxia or hypoxia, briefly pulsed with 5'-bromo-2'-3'-deoxyuridine (BrdU; 10 μ M for 20 min), stained with propidium iodide (PI) and analyzed by flow cytometry.

qRT-PCR analyses

Total RNA (2 μ g of each sample isolated using RNeasy [Qiagen]) was reverse transcribed using High Capacity cDNA Reverse Transcription kit (ABI) and assayed for gene expression by SYBR-GREEN technology (ABI). The following primer sequences were used: Lef-1F: 5' TCCTGAAATCCCCACCTTCT 3', Lef-1R: 5' TGCGATAAACAGGCTGACCT 3'; Tcf-1/7F: 5' CAGCTCCCCCATACTGTGAG 3', Tcf-1/7R: 5' TGCTGTCTATATCCGCAGGAA 3'; Dkk-4F: 5' ACGAAGAAATCACAAAGCAGTAAG 3', Dkk-4R: 5' AAAAATGGCGAGCACAGC 3'; Axin2F: 5' GAGAGTGAGCGGCAGAGC 3', Axin2R: 5' CGGCTGACTCGTTCTCCT 3'; Fzd7F: 5' GGGTATCTCTGTGTAGCCCTGA 3', Fzd7R: 5' AGAGGCAGGTGGATGTCTGT 3', Pgk1F: 5' TACCTGCTGGCTGGATGG 3', Pgk1R: 5' CACAGCCTCGGCATATTTCT 3'; HIF-1 α KOF: 5' GCACTAGACAAAGTTCACCTGAGA 3', HIF-1 α KOR: 5' CGCTATCCACATCAAAGCAA 3'. ChIP primers used were as follows: Lef-1 P1F: 5' TTCCCAGCGCTCATCATCA 3', Lef-1 P1R: 5' CCTTTCGCTTCGGTTTTCCT 3'; Lef-1 P2F: 5' AAAACAAAACCCCAAATCACC 3', Lef-1 P2R: 5' TCACCGTGCAAAACCTCTC 3'; Lef-1 P3F: 5' CGGCGTAGACGCTCTCAG 3', Lef-1 P3R: 5' CGCTTTCCCACTTAGAAGGAC 3'; Tcf-1 P1F: 5' ACACCGAAACGTTCTTGAGGC 3', Tcf-1 P1R: 5' TCACCACGACCGATCACTGTT 3', Tcf-1 P2F: 5' GGATGCAACTTCCCAGACTGAG 3', Tcf-1 P2R: 5' GCTTAGAACCTGCTGTCCAGGA 3'

Immunoprecipitation assay

For Chromatin immunoprecipitation (ChIP) experiments, the sonicated nuclear extracts of hypoxic cells were immunoprecipitated with anti-HIF-1 α monoclonal antibody (Novus Biologicals), reverse cross-linked and analyzed by qRT-PCR. For immunoprecipitation experiments, 500 μ g of hypoxic ES nuclear protein extracts were cleared with either 10 μ l of anti-HTF-1 α polyclonal antibody (Novus Biologicals) pre-coupled to protein-G-sepharose (Roche) or 5–10 μ l of anti-LEF-1 polyclonal antibody (Cell Signaling) pre-coupled to protein-A sepharose (Roche).

Generation and Analysis of Mice

Generation and analysis of *Hif-1 α^{ff}* ¹⁹, *CamKIIalpha-Cre*²⁰, and *Ubc-Cre-ER*^{T2 32} has been described previously. *BAT-GAL* reporter mice were obtained from the Jackson laboratory and genotyped as previously described¹⁷. To generate global *Hif-1 $\alpha^{\Delta\Delta}$* knockout mice (*gHif-1 $\alpha^{\Delta\Delta}$*) *Hif-1 α^{ff}* mice were crossed with *Hif-1 α^{ff}* mice expressing *Ubc-Cre-ER*^{T2} and offsprings were screened for the presence of *Ilox P* using the genotyping primers P1: 5' GCAGTTAAGAGCACTAGTTG 3', P2: 5' GGAGCTATCTCTCTAGACC and P3: TTGGGGATGAAAACATCTGC 3'. Cre mediated recombination between the *loxP* sites in the *2loxP* allele produces the *IloxP* allele, which lacks exon 2 and results in a mutant mRNA transcript containing multiple in frame stop codon downstream of exon 1 sequences. Tamoxifen free base (MP Biomedicals) was administered to nursing mothers at a concentration of 200 mg Kg⁻¹ as previously described³³ to induce recombination. Neuronal deletion of *Hif-1 α^{ff}* (*nHif-1 $\alpha^{\Delta\Delta}$*) was achieved by crossing mice carrying *Hif-1 α^{ff}* alleles with *Hif-1 α^{ff}* mice expressing Cre recombinase under the control of the calcium/calmodulin-dependent kinase Cam KIIalpha promoter (CamKII-Cre) as described previously²¹. All mice were maintained in microisolator cages and treated in accordance with NIH and American Association of Laboratory Animal Care Standards, and consistent with the animal care and use regulations of the University of Pennsylvania, Philadelphia.

LacZ and Hypoxyprobe Detection

Dissected embryos, or sections from adult organs were washed in PBS, fixed for 30 minutes in 4% paraformaldehyde, and incubated in the 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-Gal) staining solution for 30 minutes –16 h. Hypoxic regions in developing mouse embryos and adults were detected using pimonidazole hydrochloride detection kit (Chemicon), per manufacturer's protocol. Briefly, pimonidazole in water was administered intraperitoneally at a dosage of 60 mg Kg⁻¹. To minimize Hypoxyprobe background, a washout period of 1 h was allowed. Animals were sacrificed and brains were harvested following cardiac perfusion.

Antibodies for Immunoblotting, Immunofluorescence and Quantitation

Primary antibodies were obtained as mentioned: Lef-1, Tcf-1, HIF-1 α , ARNT, CREB, actin, GSK-3 β and pGSK-3 β (Cell signal), β -tubulin III and Doublecortin (Abeam), BrdU (USBiologicals), HIF-1 α (Cayman for IHC), β -catenin (BD Transduction Laboratories), Cre recombinase monoclonal (Covance), Sox2 (Chemicon and R&D Systems) and β -galactosidase polyclonal (Fitzgerald).

To examine the effects of *Hif-1 α* deletion, young adult (7–8 weeks) neuronal or global *Hif-1 $\alpha^{\Delta\Delta}$* or control mice lacking Cre (*Hif-1 α^{ff}*) were injected with BrdU (5-Bromo-2'-deoxyuridine; 100 mg Kg⁻¹ for four consecutive days) (Roche) to label proliferating AHPs and their progeny. Mice were perfused after the final BrdU injection. For rescue analysis of neuronal defects, animals were injected with either GSK3 inhibitor SB216763 (2mg Kg⁻¹), or vehicle (DMSO) every alternate day for 7 doses as previously described³⁴. For tissue

staining, organs were harvested from perfused experimental animals and embedded in Tissue Tek Optimal Cutting Temperature (OCT) media (Fisher), then frozen to -80°C for storage. $14\text{ }\mu\text{m}$ frozen sections were cut using a Microm HM 550 Cryostat. Sections were fixed with 5% buffered formalin for 2 minutes, washed three times in PBS and incubated in protein blocking agent for thirty minutes at room temperature. Primary antibody cocktails of BrdU and DCX diluted in 2% BSA in PBS, or per manufacturer's protocol were then added on the tissue sections and incubated at 4°C overnight. Slides were washed three times in PBS and then incubated in secondary antibody cocktail in 3% BSA in PBS for one hour. Slides were mounted in Vectashield mounting medium (Vector Laboratories) and visualized on a Leica Leitz 500 microscope. Images were acquired and processed using Photoshop CS3 (Adobe).

For quantitation of BrdU⁺, DCX⁺ and β -galactosidase⁺ cells, and for the enumeration of laminin positive vessels, 5–7 tissue sections offering an anterior to posterior coverage of the DG from each animal were stained. Positive cells were counted per field (at $10\times$ magnifications) and correlated per mice. The results were pooled to generate average values. For neurite quantification DCX⁺ cells with processes longer than $20\text{ }\mu\text{m}$ extensions were counted as positive.

Neural stem cell Isolation, Culture, Differentiation and Transfection

Mouse Ventral Mesencephalon E14 neurospheres were purchased from STEMCELL Technologies. Isolated neural stem cells and embryonic neurospheres (STEMCELL Technologies) were cultured using STEMCELL Technologies' kit (05715). Briefly, 5–6 hippocampus dissected from 4–6 week young adult mice were collected in a dish containing neurocult tissue collection solution. The tissue was chopped into small pieces using a razor, subjected to brief enzymatic disassociation in Neurocult disassociation solution at 37°C , mechanically disassociated by gentle pipetting, and resuspended in Neurocult resuspension solution. Primary cells were seeded at a density of 1×10^5 cells in T-25cm² flasks, and growth of neurospheres monitored under the microscope. For transfection, 1:3 diluted culture of 3–4 day old neurospheres, generated from a seeding density of 2×10^6 cells 10 ml^{-1} were plated into 24 or 6-well dish ($500\text{ }\mu\text{l}$ – 3ml), and transfected with 500 ng – $2\text{ }\mu\text{g}$ plasmid using Lipofectamine 2000 (Invitrogen), and samples treated for mRNA or reporter activity analysis 48 h post-transfection. For differentiation assay, neurospheres were seeded on Poly-ornithine coated glass coverslips and cultured in Neurocult Differentiation media (STEMCELL Technologies). For immunostaining of neurospheres, the floating spheres were first attached to glass coverslips using Cell-Tak cell tissue adhesive (BD) and then processed similar to adherent cells.

Generation of ES-*lef-1* cells

Lef-1 cDNA (Open Biosystems: MMM1013-9334706) was subcloned into pLKO.1 neo (Addgene: Plasmid 13425, PI: Shiela Stewart) with Age-1 and Eco-R1. LEF-1 overexpressing lentivirus was prepared in HEK293T cells using the pMDLg/pRRE, pRSV-Rev and Envelope system. ES cells were transduced with virus supernatant and selected with 0.4 mg ml^{-1} Geneticin. ES cells transduced with empty pLKO.1 neo virus (VC) served as control.

Generation of SA- β -catenin lentivirus and stereotactic injection

The control vector was pHIV-IRES-ZsGreen (Addgene: Plasmid 18983, PI: Zena Warb, Bryan Welms). $\Delta\text{GSK } \beta$ -catenin insert has been described before²². The insert was subcloned into pHIV-IRES-ZsGreen. $1\text{ }\mu\text{l}$ of high titer lentivirus ($\sim 2\times 10^9$ transducing units ml^{-1}) was injected into the DG of 10–12 week old mice bilaterally (AP -2 , ML ± 1.5 , DV -1.8 from Bregma, $n=3$ – 5 per group) as previously described³⁴. Viral spread was assessed by

visualizing the presence of Zsreen positive cells along the anterior–posterior axis of the DG (about 1.2–1.6 mm). Following 2 weeks of recovery, mice were injected with BrdU (100 mg Kg⁻¹) i.p daily for 4 days. The mice were then perfused with 4% paraformaldehyde and the hippocampal sections processed for BrdU and DCX staining.

Statistical analyses

Statistical significance was computed using Student's *t* test or one-way ANOVA, and significance was set at *P* < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Hypoxia activates Wnt/β-catenin signalling in mouse embryonic cells

a, ES and P19 EC cells transiently transfected with TOP-Flash and pRL-SV40 plasmids were grown either in normoxia (N, 21% O₂) or hypoxia (H, 1.5% O₂ for 16h) ($n=9$). **b**, β-catenin and LEF-1/TCF-1 analysis in ES cells under indicated O₂ conditions by western blot analysis of nuclear extracts. CREB served as a loading control, **c**, qRT-PCR analysis of Wnt target gene levels in hypoxic ES cells relative to 18S rRNA levels, normalized to normoxic cells ($n=9$). **d**, TOP-Flash activity in ES and P19 EC cells treated with 200 nM Broraoindirubin-3' oxime (B) and cultured either in hypoxia or normoxia ($n=9$). **e**, qRT-PCR analysis of Wnt-3a CM (W) treated ES cells show increased induction of Wnt target genes under hypoxia compared to normoxia ($n=6$). **f**, Severe reduction of reporter activity in ES and P19 EC cells transfected with pools of siRNA against β-catenin at indicated O₂ levels (UT: untransfected, tSi: target siRNA, cSi: control siRNA) ($n=6$) (left), and western blot analysis of silenced β-catenin 48 h post transfection (right), **g**, Growth rate of ES cells treated with Wnt-3a CM, or Wnt inhibitor DKK-1 (300 ng ml⁻¹) under indicated O₂ conditions ($n=3$). **h**, TOP-Flash activity in ES cells treated with DKK-1 (300 ng ml⁻¹) ($n=3$). * = $P < 0.05$, ** = $P < 0.005$, Student's t -test. Error bars represent S.D..

Figure 2. HIF-1 (HIF-1 α /ARNT complex) mediates hypoxia induced Wnt signalling in embryonic cells

a, *Hif-1 α ^{-/-}* ES cells (middle) show attenuated TOP-Flash activity under hypoxia as compared with hypoxic *Hif-1 α ^{+/+}* cells (left). Increased reporter activity in normoxic ES cells treated with DFX (16 h) (right) ($n=9$). **b**, TOP-Flash activity in *Hif-1 α ^{+/+}* and *Hrf-1 α ^{-/-}* cells stimulated with Wnt-3a CM (W) and cultured under indicated O₂ conditions ($n=9$). **c**, TOP-Flash activity is attenuated in hypoxic *Arnt*^{-/-} ES cells, and rescued in hypoxic *Arnt*^{Res} cells (*Arnt*^{-/-} cells with restored *Arnt* expression) compared to hypoxic *Arnt*^{+/+} cells ($n=9$). **d**, qRT-PCR analysis of *Hif-1 α ^{-/-}* cell extracts for Wnt target gene expression ($n=6$). **e**, Western blot analysis of β -catenin, LEF-1 and TCF-1 proteins in whole cell extracts of null cells (indicated by the absence of specific protein) or corresponding wild type cells cultured either under normoxia or hypoxia. Actin served as the loading control. **f**, Immunoprecipitation with LEF-1 antibody was performed on nuclear extracts of normoxic or hypoxic ES cells. CREB served as the loading control. **g**, ES cells were cultured under 21% or 1.5% O₂ for 16 h and then assayed by ChIP. Following IP with antibody against HIF-1 α or isotype control, DNA extracts were assessed by qRT-PCR. Results of each genomic region tested (upper) are expressed as fold difference between HIF-1 α IP and mouse IgG control (lower). *Leif-1* coding region (I.C) served as a control ($n=4$). * = $P < 0.05$, ** = $P < 0.005$, Student's t -test and one-way ANOVA (b). Error bars represent S.D..

Figure 3. O₂ regulation of Wnt/β-catenin signalling is differentiation stage specific

a, ES and P19 EC cells were treated with (+) or without (–) N2B27 neuronal growth and differentiation supplements, and monitored for the formation of neuronal progenitors (NP) through the expression of neuronal marker β-tub III. **b**, ES and P19 EC cell-derived NPs demonstrate attenuated TOP-Flash reporter activity under hypoxia compared to hypoxic undifferentiated controls ($n=3$). **c**, ES cell derived-NPs were cultured under normoxia or hypoxia for 16 h and assessed for *Lef/Tcf* gene expression by qRT-PCR. ($n=3$). **= $P < 0.005$, Student's t -test. Error bars (**b–c**) represent S.D.. **d**, Western blot analysis of nuclear extracts of ES-*Lef-1* and the corresponding control virus (VC) transduced ES cells cultured under normoxia. CREB served as the loading control. **e**, Schematic representation of a model wherein hypoxia (via HIF-1α) induces β-catenin transcriptional effectors, *Lef-1* and *Tcf-1*, resulting in increased nuclear translocation of β-catenin (? indicates mechanism is unknown), subsequent interaction between β-catenin and LEF-1/TCF-1 and activation of Wnt target genes.

Figure 5. Neuronal *Hif-1α* regulates neurogenesis through Wnt/β-catenin signalling

a, E14 NSCs (E-NSC) or adult NSCs (A-NSC) isolated from 4–6 week old hippocampus were cultured as neurospheres, and employed for TOP-Flash assay under normoxia or hypoxia ($n=6$). **b**, Transfection efficiency for NSC TOP-Flash assays was monitored with GFP in 2-day post dissociation neurospheres (white arrows in phase). **c**, Neurospheres were assayed for progenitor markers including Nestin (**a upper panel**) and Sox2 (**a lower panel**). **d**, TOP-Flash assay in differentiated E14 embryonic (dE-NSC), or adult NSCs (dA-NSC) cultured under normoxia or hypoxia ($n=6$). **e**, qRT-PCR analysis of *Lef-1* levels in A-NSCs or differentiated A-NSCs (dA-NSC) transfected with HIF-1α TM plasmid or corresponding vector control (VC) ($n=3$). **f**, Differentiation of neurospheres was assessed by the expression of differentiation markers including β-tubIII (**f upper panel**) and DCX (**f lower panel**). * = $P < 0.05$, ** = $P < 0.005$, Student's *t*-test (**a**, **d–e**). Error bars (**a**, **d–e**) represent S.D..