Published in final edited form as:

Cell Mol Neurobiol. 2011 January ; 31(1): 119–133. doi:10.1007/s10571-010-9561-5.

Continuous Expression of HIF-1 α in Neural Stem/Progenitor Cells

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Abstract

Hypoxia-inducible factor-1 alpha subunit (HIF-1 α) is a transcriptional activator mediating adaptive cellular response to hypoxia. Normally degraded in most cell types and tissues, HIF-1 α becomes stable and transcriptionally active under conditions of hypoxia. In contrast, we found that HIF-1 α is continuously expressed in adult brain neurogenic zones, as well as in neural stem/progenitor cells (NSPCs) from the embryonic and postnatal mouse brain. Our *in vitro* studies suggest that HIF-1 α does not undergo typical hydroxylation, ubiquitination and degradation within NSPCs under normoxic conditions. Based on immunofluorescence and cell fractionation, HIF-1 α is primarily sequestered in membranous cytoplasmic structures, identified by immunoelectron microscopy as HIF-1 α -bearing vesicles (HBV), which may prevent HIF-1 α from degradation within the cytoplasm. HIF-1 α shRNAi-mediated knockdown reduced the resistance of NSPCs to hypoxia, and markedly altered the expression levels of Notch-1 and β -catenin, which influence NSPC differentiation. These findings indicate a unique regulation of HIF-1 α protein stability in NSPCs, which may have importance in NSPCs properties and function.

INTRODUCTION

The external signals and intracellular mechanisms that control neural stem/progenitor cell (NSPC) generation, function and behavior following injury have been studied intensely. It is well established that oxygen is an important signal in all major aspects of stem cell biology. Oxygen levels have a profound effect on stem cell niche and significantly affect proliferation, self-renewal and differentiation of multipotent progenitor cells, including NSPCs (Csete 2005; Lin et al. 2006; Simon et al. 2008; Panchision 2009). *In vivo* studies utilizing experimental models of ischemia showed that NSPCs strongly respond to hypoxia by massive proliferation and migration towards the stroke-induced brain lesion (Kokaia et al. 2006) indicating the importance of the NSPCs in the adaptation and possible recovery following acute brain damage or prolonged pathological conditions. The hypoxic control of stem cell behavior is mediated by the hypoxia-inducible factor (HIF)-dependent pathways (Simon et al. 2008) (Zhu et al. 2005; Panchision 2009).

HIF-1 is a transcriptional activator mediating adaptive cellular response to hypoxia. It is a heterodimeric complex composed of two subunits HIF-1 α and HIF-1 β (ARNT). While the

β -subunit is a continuously expressed nuclear protein, the stability, sub-cellular localization and transcriptional activity of the α -subunit are oxygen-regulated. In presence of oxygen, HIF-1 α undergoes prolyl hydroxylation by prolyl-4-hydroxylases (PHDs) and binds to VHL (Von Hippel-Lindau tumor suppressor protein), a component of E3 ubiquitin ligase complex. Following polyubiquitination, HIF-1 α is subjected to proteosomal degradation (Lee et al. 2004; Ke et al. 2006). Recent studies suggest that HIF-1 α proteosomal degradation is regulated by the subcellular localization of HIF-1 α , such that degradation mainly occurs in the cytoplasm. Interestingly, this localization-dependent regulation seems to be cell-type-specific and is not completely understood (Tanimoto et al. 2000; Berra et al. 2001; Zheng et al. 2006). In contrast, under hypoxic conditions, HIF-1 α becomes stabilized and translocates from the cytoplasm. In the nucleus, it dimerizes with HIF-1 β , becomes transcriptionally active and upregulates genes, including pro-angiogenic, cell proliferation and survival factors, including enzymes of the glycolytic pathway and glucose transporters (Kietzmann et al. 2001; Rossant et al. 2002; Lee et al. 2004; Ke et al. 2006).

A number of studies have described an oxygen-independent stabilization of HIF-1 α induced by growth factors and cytokines (Lee et al. 2004), association with HSP-90 protein (Liu et al. 2007), mTOR (mammalian target of rapamycin) signaling (Land et al. 2007) or Ang II-mediated oxidation (Page et al. 2008). These aspects of HIF regulation likely account for different levels of HIF-1 α that are detected in different mouse organs under normoxic conditions (Stroka et al. 2001). In addition, inactivating mutations of the VHL gene that prevent association with HIF-1 α , result in non-hypoxic stabilization of HIF-1 α mediating Warburg effect in clear cell renal carcinoma, and progression of retinal angioma (Liu et al. 2007; Kaelin 2008). Neuron-specific knockdown of HIF-1 α in mice results in increased tissue damage and reduced survival rate following transient focal cerebral ischemia (Baranova et al. 2007). Information regarding HIF expression in neural stem cells is limited and is mostly associated with the embryonic development and hypoxia. HIF-1 α is expressed in embryonic tissues and plays an important role in development: systemic deletion of the HIF-1 α gene is embryonic lethal, and associated with malformation of the heart and cardiovascular system (Iyer et al. 1998). Conditional gene deletion of HIF-1 α within stem cells of the developing nervous system results in hydrocephalus, massive neuronal apoptosis and regression of vasculature (Tomita et al. 2003).

In our previous studies we demonstrated that NSPCs isolated from the embryonic E14 mouse brain continuously express HIF-1 α , and regulate the expression and release of the angiogenic factor VEGF. We also showed that the HIF-1 α /VEGF pathway played an important role in neural progenitor-mediated protection of both endothelial cells and neurons following *in vitro* and *in vivo* hypoxia (Roitbak et al. 2008; Harms et al. 2010). Here, we analyze stabilization and subcellular distribution of HIF-1 α protein in NSPCs. We also suggest that NSPC-expressed HIF-1 α is biologically active, affecting the resistance of NSPCs to hypoxia and influencing expression levels of the major components of Notch and Wnt signaling pathways.

MATERIALS AND METHODS

Cell Culture

Neural stem/progenitor cells from the mouse embryonic brain (eNSPCs) were established from telencephalon of gestational day 14 mouse embryos (Roitbak et al. 2008). Briefly, embryonic telencephalons were dissected, and after removal of meninges, mechanically dissociated by titration with a P-1000 pipetman in Hank's balanced salt solution (HBSS). After brief centrifugation (3 min, 1300 rpm), the cells were resuspended in culture medium and plated into 6-well poly-L-lysine coated tissue culture dishes (Becton-Dickinson, Franklin Lakes, NJ) at the density of approximately one embryonic telencephalon per well.

Neural stem/progenitor cells from P28 mouse brain (pNSPCs) were isolated from the SVZ of 28-days old C57BL/6 strain mice (The Jackson Laboratory). Animals were deeply anesthetized with isoflurane, whole brains were removed and placed in ice-cold HBSS medium (GIBCO). Each brain was cut with a microtome blade and SVZ were removed under a dissection microscope. The pieces of brain tissue were incubated with trypsin/enzyme solution at 37°C for 15 min (1.3 mg/ml trypsin + 0.01% papain, and 0.01% DNase I), followed by brief trituration and incubation in the same solution for another 10 min. The samples were centrifuged, triturated again and subsequently centrifuged (adopted from (Parmar et al. 2003)). NSPCs were expanded in defined serum-free DMEM/F12 medium containing 15 mM HEPES, 2.5 mM L-glutamine (Gibco), 3 mM sodium bicarbonate, 25 µg/ml insulin, 16 µg/ml putrescine, 30 nM sodium selenite, 100 µg/ml apo-transferrin and 20 µM progesterone (as previously described in (Reynolds et al. 1992) plus 10 ng/ml EGF and 10 ng/ml bFGF (Invitrogen). NSCs cells were grown under normal growth conditions (20% oxygen, 75% nitrogen and 5% CO₂), at 37°C. To induce NSPC differentiation, the growth factors were withdrawn from the media for 7 days. The neural stem cells from both embryonic (eNSPCs) and postnatal day 28 (pNSPCs) mice fulfilled criteria of multipotentiality and self-renewal ((Roitbak et al. 2008) and the Supplementary Figure 1).

Oxygen-glucose deprivation (OGD)

Cell cultures were subjected to oxygen-glucose deprivation injury as previously described (Wetzel et al. 2008). Cultures were placed in an anaerobic chamber (Coy Laboratories) containing a gas mixture of 5% H₂, 5% CO₂ and 90% N₂. The chamber maintains a strict < 0.2% oxygen atmosphere through the hydrogen gas reacting with a palladium catalyst to remove oxygen. Normal culture medium was replaced with deoxygenated, glucose-free Earle's Balanced Salt Solution (EBSS), and cells were exposed to glucose-free anaerobic conditions for 1–3 h at 37°C. Control cell cultures were placed in EBSS containing 25 mM glucose and incubated under normal tissue culture conditions for the same period.

Antibody for HIF-1α detection—In our previous immunofluorescence studies we observed that HIF-1α was expressed both in the nucleus and cytoplasm ((Roitbak et al. 2008) and Supplementary Figure 2A). Based on the analysis of five different sources of HIF-1α antibody (Supplementary Figure 2A and E), in the present study we chose to use a biotinylated anti-HIF-1α antibody provided in the in the Surveyortm IC Intracellular HIF-1α immunoassay kit (R&D Systems). Specificity of this antibody for immunofluorescence, electron microscopy and Western blot was examined (Supplementary Figure 2, panels B–F). Panel F on Supplementary Figure 2 demonstrates that anti-HIF-1α antibody (recognizing human, mouse and probably, rat HIF-1α) detected on blots a clear ~120kD band in NSPC lysates, but not the mouse brain endothelial cells and rat primary astrocytes (Genlantis). HIF-1α immunofluorescence in the brain tissue was accompanied by a strong background staining (probably due to secondary streptavidin antibody), therefore confocal microscopy imaging required significant decrease in signal intensity and background elimination. However, our preabsorption controls demonstrated validity of the IF tissue staining (Supplementary Figure 2, panel C).

Immunofluorescence Microscopy

Cells were fixed with 4% paraformaldehyde solution and quenched with 50 mM ammonium chloride. Following permeabilization with 0.1% (v/v) Triton X-100 and blocking with 1% horse serum, cells were incubated with primary antibodies (in 1% horse serum) for 1h followed by fluorophor-conjugated secondary antibodies for 1h, at room temperature.

Mouse brain floating sections were prepared and subjected to immunofluorescence staining as previously described (Roitbak et al. 2008). Sections were permeabilized with 0.1% (v/v) Triton X-100, blocked with 5 % donkey serum, and incubated with primary antibodies

overnight at 4°C. Primary antibodies and dilutions were as follows: anti-HIF-1 α from the Surveyor[™] IC HIF-1 α immunoassay kit (R&D Systems, 60–100 ng/ml), mouse monoclonal anti-nestin (1:1000, BD Biosciences), mouse monoclonal and rabbit polyclonal (1:1000) anti-GFAP (Accurate Chem. & Sci. Corp.), goat polyclonal anti-doublecortin (1:300, Santa Cruz Biotech), goat polyclonal anti-Sox-2 (1:200, Santa Cruz Biotech), mouse monoclonal anti- β III tubulin/Tuj1 (1:300, Promega), rabbit polyclonal anti-TGN38 (1:100 Novus Biologicals), mouse monoclonal anti-clathrin (1:100, Millipore), mouse monoclonal anti- β -COP (1:100, Santa Cruz), rabbit polyclonal anti-LC3B (1:400, Cell Signaling) and rabbit polyclonal anti-Lamp3 (1:50, Santa Cruz Biotech). Coated vesicle sampler kit (BD Transduction labs) containing the antibodies against adaptins α , β , γ , and δ , AP180, EEA1, β -NAP and Rab4, was used according to manufacturer's recommendations. Cy3- and FITC-conjugated secondary antibodies, and Cy3-conjugated streptavidin were used at a 1:250 dilution (Jackson ImmunoResearch Laboratories). DAPI staining was used for detection of nuclei. All samples were imaged on Zeiss LSM510 and Zeiss LSM510-META confocal imaging systems.

Electron Microscopy

Pre-embedding labeling was performed on E14 NSPCs grown on 0.4 μ m cell culture filters (Falcon). The cells were fixed with 4% Paraformaldehyde + 0.05% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4), quenched with 50 mM ammonium chloride and permeabilized with 0.1% (v/v) Triton X-100. Following blocking with the mixture of 1% BSA and 1% donkey serum, the cells were incubated with biotinylated anti-HIF-1 α (R&D Systems, 60–100 ng/ml, overnight at 4°C) followed by streptavidin-gold (Sigma, 1h RT). Control samples were immunostained with streptavidin-gold only. The cells were post-fixed with 2% glutaraldehyde in 0.1M PB and osmicated for 15 min in 0.5% OsO₄ in 0.1M PB. The samples were dehydrated in graded series of ETOH (50-70-80-95-100%, 10 min/ea, RT), infiltrated in graded mixtures of ETOH:EmBed 812 (75:25, 50:50, 25:75; 100%, 30 min, RT) and incubated in 100% EmBed 812 (Electron microscopy Sciences). The filters with cells were embedded and polymerized (60°C, 24 hours). Digital images of 70 nm thin sections (stained with uranyl acetate) were acquired on Hitachi H-7500 transmission electron microscope.

Subcellular fractionation experiments were performed on eNSPC lysates:

Nuclear and post-nuclear fractions—Nuclear and cytoplasmic (post-nuclear) extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Pierce/Thermo Sci.), according to manufacturer's recommendations.

Cytosolic and membrane fractions—NSPCs ($\sim 4 \times 10^6$ cells) were scraped with ice-cold PBS, centrifuged and resuspended in 1 ml of 50 mM Tris-HCl (pH 7.5) containing 2 mM PMSF and disrupted by repeated freeze-thaw. After that the cells were homogenized in a Dounce tissue homogenizer. Homogenates were centrifuged at $600 \times g$ for 10 min, and supernatant representing postnuclear extract (PN), was saved. After centrifugation of the postnuclear extract at $100,000 \times g$ for 60 min at 4°C using an Optima TL Ultracentrifuge (Beckman Instruments), supernatant (cytosolic fraction) and pellet (membrane fraction), were collected. The pellet was then resuspended in lysis buffer containing 1% (vol/vol) TX-100, 150 mM NaCl, 10mM Tris-HCl pH 7.4, and a protease inhibitor cocktail. The final total protein concentration in the samples was normalized based on the Bradford protein assay. 2XSDS (1:1 vol/vol) was added and the samples were subjected to Western blot analysis.

Sucrose density gradient centrifugation was performed according to (Surviladze et al. 1998). NSPCs ($\sim 10^7$) were scraped with ice-cold PBS, centrifuged, resuspended in 300 μ l of the hypotonic buffer (10mM Tris-HCl pH 7.5, 10 mM KCl and 5 mM EDTA with addition of the protease inhibitor cocktail) and disrupted by repeated freeze-thaw. The cells then were passed three times through 26G syringe needle and sonicated three times for 20sec. Cell lysates were then adjusted to 40% (w/v) sucrose using 80% stock sucrose in 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and protease inhibitor cocktail. 60% sucrose (bottom of the gradient) was overlaid with 40% sucrose containing the cell lysate mixture, followed by the step gradient of 30, 20 and 10% sucrose solutions in Tris buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA). Samples were centrifuged at $100,000 \times g$ for 6 hours at 4°C using an Optima TL Ultracentrifuge (Beckman Instruments). Eleven fractions (100 μ l) were collected from the top, except the bottom 100 μ l and sedimented pellet containing cell debris and unbroken cells. Proteins were resolved on SDS-PAGE gels and detected by immunoblotting with antibody specific to HIF-1 α .

Gel chromatography—Sucrose gradient fractions 5–9 were combined and used to determine the size of HIF-1 α containing structures or complexes by employing gel chromatography procedure (Bohuslav et al. 1995). Small column (0.8 cm $\times$ 5 cm) of Sepharose 4B (Sigma) was washed with 2 volumes of the lysis buffer. 0.3 ml of the cell lysate was applied at the top and left to enter the gel for 5 min: the 0.3 ml of the eluate was collected as fraction 1. Then 0.3ml of the hypotonic Tris buffer (10 mM Tris-HCl pH 7.5, 10 mM KCl and 5 mM EDTA and a protease inhibitor cocktail) was applied, fraction 2 collected in 5 min and so on. The resulting fractions were then used for Western blotting. Calibration of the column was performed using 29–2 000 kDa molecular weights kit for gel filtration chromatography (Sigma). The calibration showed that the elution volumes were in the fraction 10 for alcohol dehydrogenase (150 kDa), fraction 8 for thyroglobulin (669 kDa) and fraction 4–5 for blue dextran (2 000 kDa).

Western Blot analysis

Western Blot was performed as described in (Roitbak et al. 2008). The cells were scraped from cell the culture dishes with 2xSDS sample buffer (200 μ l per well, 6 well plates), or with lysis buffer (250 μ l per well) containing 1% (vol/vol) TX-100, 150 mM NaCl, 10 mM Tris-HCl pH 7.4, and a protease inhibitor cocktail. Total protein concentration was measured using Bradford protein assay, and the loading was additionally confirmed by comparing actin or GAPDH immunoreactivity across the lanes. The proteins were separated on 4–20% gradient Criterion precast gels (Bio-Rad). A broad range molecular weight calibration marker from 10,000 to 250,000 MW (Bio-Rad) was used as a standard. The proteins were identified using mouse monoclonal anti-VEGF (1:100, Abcam), rabbit polyclonal anti-cleaved Notch 1 (1:1000, Cell Signaling), mouse monoclonal anti- β -catenin (1:500, BD Biosciences), mouse monoclonal anti-ubiquitin (1:500, Millipore), mouse monoclonal anti-VHL (1:500, Santa Cruz Biotech.), rabbit polyclonal anti-actin (1:1000, Sigma) and anti-clathrin (1:100, Millipore), mouse monoclonal anti-GAPDH (1:500, Millipore), goat polyclonal anti-Sox-2 (1:500, Santa Cruz Biotech) and rabbit polyclonal anti-P564 hydroxylated HIF-1 α antibody (1:500, Novus Biologicals). Horseradish peroxidase-labeled secondary antibodies from Amersham Biosciences were used in dilution 1:3000. Incubation with both primary and secondary antibodies was performed for 1 h at room temperature. For HIF-1 α detection, we used a biotinylated anti-HIF-1 α antibody (60–100 ng/ml) provided in the in the Surveyortm IC Intracellular HIF-1 α immunoassay kit (R&D Systems), followed by horseradish peroxidase-conjugated streptavidin (1:200). The expression and activity of the mTOR pathway components were detected using mTOR Pathway and mTOR Substates Antibody Sampler Kits (Cell Signaling), according to manufacturer's recommendations. The density of the protein bands was determined using a GS-800 Calibrated Densitometer and

Quantity One software (Bio-Rad), normalized by actin or GAPDH expression and quantified using GraphPad Prism software.

HIF-1 α immunoprecipitation

Cells were scraped from the dishes in lysis buffer provided in the Surveyortm IC Intracellular HIF-1 α immunoassay kit (R&D Systems; 250 μ l per well, 6-well plate), supplemented with 1mM DTT, PMSF, and the protease inhibitor cocktail. A biotinylated anti-HIF-1 α antibody (200 ng/ml) provided in the Surveyortm IC Intracellular HIF-1 α immunoassay kit (R&D Systems) was used to immunoprecipitate (IP) HIF-1 α . The IP was performed using streptavidin-agarose beads (30 μ l per IP, Sigma). 1000–1500 μ l of cell lysate ($\sim 5 \times 10^6$ cells) was used for each immunoprecipitation. The final immunoprecipitates were resuspended in an equal (40 μ l) volume of 2 $\times$ SDS sample buffer. In some experiments, the cell lysates were preincubated with streptavidin-agarose as a control for any nonspecific binding to the beads.

In vitro HIF-1 α ubiquitination assay

eNSPC lysates were prepared using lysis buffer provided in the Surveyortm IC Intracellular HIF-1 α immunoassay kit, supplemented with 1 mM PMSF (no DTT), and the protease inhibitor cocktail. Reaction were performed in the presence of 50 mM TrisHCl, pH 7.5, 2.5 mM MgCl₂, 2 mM ATP and 30 μ g ubiquitin (Sigma), added to the cell lysate. The reaction was carried out in darkness at 37°C. After 2 hours, the cell lysates (control and ubiquitination reaction mix) were subjected to the immunoprecipitation with anti-HIF-1 α antibody. No non-specific binding of ubiquitin to streptavidin beads was observed.

In vitro HIF-1 α hydroxylation assay

eNSPC lysates were prepared using 1% Triton-X100 containing lysis buffer supplemented with the protease inhibitor cocktail. Cell extracts (0.5 mg of protein/ml) were incubated in the reaction buffer (0.5 mM dithiothreitol, 50 μ M FeCl₂, 1 mM ascorbate, 2 mg/ml bovine serum albumin and 0.4 mg/ml catalase in 40 mM Tris-Cl at pH 7.5). After 30 min of incubation at 37 °C, the reaction mix was resuspended in 2 $\times$ SDS sample buffer, and the samples (control and hydroxylation reaction mix) were subjected to Western blot analysis using anti-hydroxylated HIF-1 α antibody (recognizing HIF-1 α hydroxylated at P564, Novus Biologicals).

MTT colorimetric cell viability assay

Cells were incubated with 0.5 mg/ml methyltetrazolium (MTT, Sigma) for 4 h at 37°C, and treated with 1:1 ethanol:DMSO for 20 minutes at room temperature. The ability of cells to convert MTT into purple formazan provides an indication of the mitochondrial integrity and activity, interpreted as a degree of cell viability. The optical density was measured at 570 nm (with background subtraction at 630 nm) using a microplate reader (Dynex Technologies). Abs_{570 nm} is directly proportional to the number of viable cells. Data were quantified using GraphPad Prism software; n=24 measurements per group.

HIF-1 α gene silencing *in vitro*

Lentivector shRNAi was used to knock down HIF-1 α expression in cultured NSPCs. NSPCs established from telencephalon of E14 C57BL/6 mouse embryos, were expanded and passaged prior to transduction with shRNAi Lentiviral particles. Mouse HIF-1 α Mission TRC shRNA Lentiviral particles target set was purchased from Sigma. The protocol provides a system for long-term silencing of HIF-1 α and a stable cell selection using puromycin (PM) resistance. 60–70% confluent NSCs were incubated with five different HIF-1 α shRNAi and non-target shRNA (control) lentiviral particles (concentration range 1–

15 μ l/100 μ l medium) for 20h, in the presence of 8 μ g/ml Hexadimethrine Bromide. The puromycin titration experiments showed a high sensitivity on NSCs to PM, therefore the PM-resistant cell selection was effective at the PM concentration as low as 0.25 μ g/ml. Five days following selection, the PM-resistant colonies were passaged, expanded and, at 3 weeks after transduction, were subjected to Western blot analysis.

Immunochemical detection of tissue hypoxia was performed using HypoxyprobeTM-1 Plus Kit (Hypoxyprobe, Inc), according to manufacturers recommendations. HypoxyprobeTM-1 is a substituted pimonidazole hydrochloride, which is reductively activated in hypoxic cells and forms stable adducts with thiol groups in proteins, peptides and amino acids. FITC-MAb1 binds to these adducts, allowing their immunochemical detection. Two month-old C57BL/6 mice were injected via lateral tail vein with 60mg/kg pimonidazole hydrochloride. 20 minutes after HypoxyprobeTM-1 administration, mice were overdosed with isoflurane and transcardially perfused with PBS, followed by 4% paraformaldehyde. Brains were postfixed overnight, cryoprotected in 30% sucrose, and sectioned with cryostat (Leica) at 30 μ m thickness in the coronal plane. Mouse brain floating sections were quenched with 50 mM ammonium chloride, permeabilized with 0.1% (v/v) Triton X-100, blocked with 1 % BSA, and incubated with FITC-conjugated mouse monoclonal antibody provided by the manufacturer (clone 4.3.11.3, 1:50 dilution), overnight at 4°C. The images were acquired on a Zeiss LSM510 confocal imaging system.

RESULTS

Stabilized HIF-1 α is expressed in postnatal NSPCs and in neurogenic zones of adult mouse brain

We previously demonstrated constitutive expression of HIF-1 α in E14 embryonic NSPCs (eNSPCs) (Roitbak et al. 2008). In the present study, immunofluorescence staining and Western blot analyses demonstrate that HIF-1 α is also stabilized in NSPCs isolated from the postnatal mouse brain (pNSPCs, Figure 1A, B and C). Figure 1B shows HIF-1 α immunoreactivity in pNSPCs expressing the neural stem cell marker nestin. Expression of HIF-1 α was not influenced by exposure to growth factors EGF and bFGF as HIF-1 α expression remained prominent after 7 days of growth factor withdrawal (Figure 1C, bottom immunoblot, lane GF-).

HIF-1 α is also continuously expressed in endogenous neural progenitors *in vivo*. Intense HIF-1 α immunofluorescence was localized to the adult mouse brain neurogenic regions of SVZ and SGZ (Figure 2a and 2b). Within the neurogenic zones, HIF-1 α was localized to the cells positive to stem cell-specific markers nestin (Craig et al. 1996) and Sox-2 (Suh et al. 2007), and within GFAP-positive astrocytes (Figure 2c–h). Within the adult SVZ, HIF-1 α was localized to nestin-positive cells (Figure 2c) suggesting that the transcription factor was primarily expressed by endogenous neural progenitors. Interestingly, HIF-1 α was also expressed in the subventricular GFAP+ astrocytes (Figure 2e) possibly representing endogenous neural stem cells (Doetsch et al. 1999; Pastrana et al. 2009). HIF-1 α was not observed in Dcx-positive SVZ neuroblasts (Figure 2d). Within hippocampal SGZ, HIF-1 α immunofluorescence was co-localized with nestin and Sox-2-positive cells (Figure 2f and 2h), but not expressed in doublecortin-positive neuroblasts (Figure 2g). Additional analysis of HIF-1 α expression in the adult neurogenic zones is presented on Supplementary Figure 3A.

NSPC-expressed HIF-1 α is stable and not ubiquitinated

HIF-1 α expression in NSPCs was not affected by withdrawal of growth factors or insulin from the cell culture medium (Figure 1C, Figure 3A and (Roitbak et al. 2008)). We

examined a possible stabilizing influence of other signaling molecules such as mTOR and Hsp-90. mTOR signaling cascade activity was analyzed using mTOR Pathway and mTOR Substates Antibody Sampler Kits (Cell Signaling). The major components of the mTOR pathway including mTOR protein, regulatory associated protein Raptor and rapamycin-insensitive companion of mTOR Rictor, are expressed and active (phosphorylated) in NSPCs in the presence of growth factors (Figure 3A, GF lanes). Growth factor withdrawal for 4 days induced partial degradation of the mTOR and Rictor proteins (Figure 3A, -GF lanes), as well as significant decrease in phosphorylation levels of the mTOR protein and its substrate 4E-BP1 (by 37 % and 89% respectively, Figure 3A, P-mTOR and P-4E-BP1). Thus, activity of mTOR and the key elements of both rapamycin-sensitive (with P-4E-BP1 as a substrate) and rapamycin-insensitive (with Rictor as a major regulatory protein) components of mTOR signaling cascade are significantly downregulated upon growth factor removal. Since the expression levels and stability of HIF-1 α protein in the examined samples remain unchanged (Figure 3A), we conclude that mTOR signaling does not influence HIF-1 α protein expression in NSPCs. A possible stabilizing effect of the Hsp-90 protein was also examined. We observed barely detectable levels of Hsp-90 in NSPCs and found no association between Hsp-90 and HIF-1 α in the co-immunoprecipitation experiments (not shown), suggesting that HIF-1 α stabilization is not influenced by Hsp-90 binding.

To further elucidate the underlying mechanism of HIF-1 α stabilization in NSPCs, we asked whether or not the HIF-1 α degradation machinery was functional in NSPCs. As described in the Introduction, HIF-1 α is degraded via the VHL-mediated ubiquitin-proteasomal pathway. Stability of the HIF-1 α transcriptional subunit is regulated by various post-transcriptional modifications such as hydroxylation and ubiquitination, as well as its association with VHL E3 ubiquitin ligase complex. Immunoblot analyses showed that both VHL active isoforms (19 and 30 kD) are expressed in the progenitor cells (Figure 3B, lane 1). Immunoprecipitation experiments did not detect an association between HIF-1 α and VHL (Figure 3B, lane 2). The lack of the HIF-1 α /VHL association indicates that HIF-1 α protein is not hydroxylated. Concurrently anti-hydroxylated HIF-1 α antibody (recognizing HIF-1 α hydroxylated at P564) did not detect a hydroxylated 115–120 kDa form of HIF-1 α protein in NSPC lysates (Figure 3C, lane 1), as well as in HIF-1 α immunoprecipitates (not shown). *In vitro* hydroxylation assay on NSPC lysates (prepared in detergent-containing lysis buffer), followed by immunoblot with anti-hydroxylated HIF-1 α antibody, detected a hydroxylated full length HIF-1 α protein (Figure 3C, lane 2). Together these observations indicate that a stabilized HIF-1 α protein is not hydroxylated despite the endogenous PHD hydroxylase activity in NSPCs. Immunoprecipitation with anti-HIF-1 α followed by immunoblot with anti-ubiquitin antibody revealed that HIF-1 α is not ubiquitinated in NSPCs (Figure 3D, lane 3). *In vitro* ubiquitination assay on NSPC lysates (prepared in detergent-containing lysis buffer), followed by immunoprecipitation with anti-HIF-1 α and subsequent immunoblot with anti-ubiquitin antibody, detected heavily ubiquitinated HIF-1 α protein (Figure 3D, lane 4). Interestingly, *in vitro* ubiquitination assay was significantly less effective when performed on the cell extracts prepared in detergent-free conditions, in which the cells were disrupted mechanically and by freeze-thawing (Figure 3E, lane 2). Both *In vitro* hydroxylation and ubiquitination assays were performed without addition of exogenous VHL, as well as ubiquitin-activating enzymes, such as E1 or UBCH7, or exogenous PHD-1 or PHD-2. Therefore, our results reveal that endogenous components of HIF-1 α degradation process are present and functional in NSPCs. However, both HIF-1 α hydroxylation and ubiquitination can only be triggered after detergent-induced release of the protein into the cytosol, indicating that access of the protein degradation machinery to HIF-1 α is normally limited.

Analysis of the subcellular distribution of HIF-1 α

A number of studies suggest that in many cell types, degradation of HIF-1 α is influenced by its subcellular compartmentalization (Groulx et al. 2002; Zheng et al. 2006). To determine if the same is true for the NSPCs, we performed detailed analyses of the intracellular localization of HIF-1 α , using immunofluorescence staining and cell fractionation techniques. Immunofluorescence and electron microscopy detected insignificant amounts of HIF-1 α in the nucleus (Figure 4E and F, Supplementary Figure 2B), with much more prominent staining in the cytoplasm both in cultured cells and in the SVZ area of the brain sections (Figure 4A–C, Supplementary Figure 2B). This observation was later supported by subcellular fractionation studies, showing a low (approximately 10% of the amount in the cytoplasm) amount of HIF-1 α in the nuclear (N) fraction (Control lanes, WB on Figure 5). Thus, NSPCs express HIF-1 α under normoxic conditions, and a low amount of the protein is present in the nucleus-associated, transcriptionally active pool.

Both in eNSPCs (Figure 4A and 4B) and pNSPCs (Figure 4C), as well as in adult neurogenic zones, HIF-1 α was not diffusely distributed throughout cytoplasm but mostly accumulated in the aggregates localized in close vicinity to Golgi (Figure 4B, Golgi marker TGN38 is shown in green). Panel D on Figure 4 demonstrates that no HIF-1 α expression was observed in the mouse brain endothelial cells (bEnd.3 cell line, ATCC). Following 2 hours of oxygen-glucose deprivation (OGD), HIF-1 α immunofluorescence was dispersed throughout the cytoplasm, and returned more evenly to the pre-OGD pattern at 24h after re-oxygenation (Figure 5). Subcellular fractionation and immunoblot with anti-HIF-1 α antibody demonstrated that 2h OGD treatment induced significant (2.5 fold) increase of HIF-1 α expression in the nuclear compartment. Immunofluorescence staining also detected HIF-1 α nuclear expression at the same time point of the OGD treatment (Figure 5, 2 h OGD panels). Thus, hypoxia leads to the increased nuclear expression of HIF-1 α in NSPCs.

HIF-1 α is associated with the cytoplasmic membrane fraction—To further analyze subcellular localization of HIF-1 α , we performed an additional fractionation and isolation of cellular sub-fractions within the post-nuclear, cytoplasmic compartment (referred as PN). The PN consists of the cytosolic (so-called soluble) and membrane (associated with membrane-enclosed organelles and vesicles) fractions. In order to preserve membranes and keep the membrane-associated protein pool separately from cytosolic proteins, the cells were gently disrupted by repeated freeze-thaw and homogenization, and the cytosol was separated from the membranes using high spin (100,000 $\times$ g) centrifugation. The experiments detected significant amount of HIF-1 α associated with membrane fraction, or in other words, localized to membranous structures (Figure 6A, top immunoblot).

HIF-1 α is associated with large subcellular structures/aggregates—

Subsequently we determined the properties and the approximate size of the HIF-1 α associated membranous structures within the NSPC cytoplasmic compartment. Cell lysates were first subjected to sucrose density gradient-based cell fractionation followed by Western blot analysis (see Experimental procedures). HIF-1 α was most abundant in the high-density (heavy) fractions 5–11 collected from the middle and bottom parts of the sucrose gradient (Figure 6A, middle immunoblot). Our next step was to identify the size of the cell structures or complexes associated with HIF-1 α protein. Fractions 5–9 obtained following sucrose gradient centrifugation were pooled and subsequently size-fractionated by gel filtration using a Sepharose 4B column (Bohuslav et al. 1995). Gel filtration and Western blot analysis (Figure 6A, bottom immunoblot) revealed that HIF-1 α was most abundant in fraction 4 (corresponding to 2 000 kDa blue dextran elution) and fraction 10 (corresponding to ~ 150 kDa molecules). Thus, in NSPCs, HIF-1 α is associated with large, high molecular weight structures or complexes, as well as distributed in the cytosol as a monomer possibly

released from the membranes during preparation procedures (molecular weight of HIF-1 α is ~120 kDa).

HIF-1 α -associated structures were identified using immuno-electron microscopy as HIF-1 α -bearing vesicles (HBV) with size ranging from 50 to 500 nm (Figure 6B and EM micrograph on 6C). Immunofluorescence analysis was performed for HBV characterization. From cell fractionation experiments, HIF-1 α -enriched fraction 4 was also enriched with clathrin (Figure 6A, bottom immunoblot), a major component of clathrin-coated vesicles involved in protein trafficking from Golgi towards the endosomal/lysosomal system and back (Pearse 1987; Ohno 2006). However, immunofluorescence analysis of the cultured NSPCs (Figure 6C), using the markers for clathrin-coated vesicles such as clathrin, adaptins α , β and γ , as well as AP180, adaptin δ and β -NAP (not shown) shared a common distribution, but only partial co-localization with HIF-1 α -containing aggregates (occasional yellow dots on Clathrin, Ad α , Ad β and Ad γ panels). No co-localization was found between HIF-1 α and the markers for coatamer-coated COP-vesicles (β -COP), early endosomes/recycling vesicles (Rab 4 and EEA1 (not shown)), and lysosomes (Lamp3). In some cells, HIF-1 α co-localized with autophagy marker LC3B (Figure 6C, insert on LC3B panel), however no definitive autophagosome structures were observed in undifferentiated NSPCs.

Thus, we did not find a definitive association of the HBV with traditional components of the vesicular transport machinery including clathrin-coated vesicles, COP-vesicles, endosomes and lysosomes, suggesting that HIF-1 α is localized to the specialized vesicles, possibly serving as HIF-1 α storage compartments. The sequestration within HBV may prevent hydroxylation, ubiquitination and proteosomal degradation of HIF-1 α and lead to its stabilization.

Stabilized HIF-1 α is functionally active in NSPCs

In our previous studies we showed that in NSPCs HIF-1 α governs VEGF expression and mediates vasculotropic properties of neural progenitors (Roitbak et al. 2008). In addition, a clear link between hypoxia, HIFs and key regulatory proteins such as Notch and β -catenin has been demonstrated in several stem/precursor cell populations (Simon et al. 2008; Panchision 2009). In the present study we analysed whether stabilized HIF-1 α influences expression of these hypoxia-regulated proteins.

HIF-1 α knockdown using shRNAi alters VEGF, active Notch-1 and β -catenin expression—

Analysis of HIF-1 α gene silencing using western blot showed that shRNA induced 85% decrease in HIF-1 α and ~ 80% reduction in VEGF expression (Figure 7A), supporting our previous finding that VEGF expression in NSPCs is regulated by continuously expressed HIF-1 α . Noticeably, inhibition of HIF-1 α expression in NSPCs eliminated to non-detectable level the expression of C-terminal domain of the intracellular Notch-1 protein (active cleaved intracellular domain, NICD) and resulted in two-fold increase in the expression of β -catenin (Figure 7A). The decreased expression of Notch C-terminus may indicate the inhibition of Notch signaling pathway, whereas stabilization of intracellular β -catenin may imply the upregulation of Wnt/ β -catenin signaling pathway. Both pathways play an important role in neural stem cell differentiation.

HIF-1 α knockdown using shRNAi impairs NSPC resistance to hypoxia—

HIF-1 α gene silencing did not affect the viability of NSPCs in normoxic conditions (not shown), however it significantly affected their resistance to oxygen-glucose deprivation (OGD). NSPCs were resistant to 3h OGD, moreover, their number increased by ~35% at 24 hours following OGD (Figure 7B, left graph). These results are in agreement with previous observations of the increased progenitor cell proliferation under hypoxic conditions (Studer

et al. 2000; Zhu et al. 2005). Infection with non-target shRNA did not affect these properties (Figure 7B, middle graph), however inhibition of HIF-1 α expression by 85% resulted in significant (~ 30%) decrease of the NSPC number and thus, their resistance to hypoxia (Figure 7B, right graph). These results are in agreement with previous findings (Simon et al. 2008), and indicate that HIF-1 α regulates resistance of NSCs to hypoxia.

Overall the shRNA results demonstrate that stabilized HIF-1 α is functionally active in NSPCs: it significantly affects NSPCs response to hypoxia and the expression of signaling molecules such as VEGF (implicated in vasculotrophic properties of NSPCs) and cleaved Notch-1 and β -catenin, actively involved in neural stem cell differentiation.

DISCUSSION

Our findings from this study are consistent with our earlier observation that transcriptional factor HIF-1 α is stabilized in E14 embryonic NSPCs (Roitbak et al. 2008). Since HIF-1 α is often expressed in the embryonic tissues, our previous findings did not fully establish that HIF-1 α stabilization is a characteristic feature of NSPCs. Here we demonstrate that HIF-1 α is expressed *in vitro* (in the neural progenitors isolated both from embryonic and postnatal mouse brain) and *in vivo* (in the neural stem cell marker-positive cells of the adult neurogenic zones). Our finding is in agreement with the perception that adult neurogenic zones have similar characteristic features of the developing embryonic brain, and the findings that HIF-1 α is expressed in embryonic tissues (Tramontin et al. 2003; Alvarez-Buylla et al. 2004). Our analysis of tissue hypoxia using pimonidazole (HypoxyprobeTM-1) administration did not identify the signs of hypoxia in SVZ and SGZ of the normal adult mouse brain (Supplementary Figure 3B). These results are similar to other investigation where SVZ (ipsilateral but not contralateral to ischemic injury) was found transiently hypoxic only following MCAO (Thored et al. 2007). It is possible that the method is not sensitive to oxygen tensions within the range of so-called physiological hypoxia (1–6%) characteristic for the brain tissue (Li et al. 2005), since 10 mm Hg (~1.3%) is a threshold value for pimonidazole binding in solid tissue (www.hypoxyprobe.com). Future advanced non-invasive imaging methods could possibly establish whether *in-vivo* HIF-1 α expression is associated with moderate hypoxia in the brain neurogenic zones.

In this study we provide evidence for unique stabilization of the HIF-1 α in the NSPCs under normoxic conditions *in vitro*. HIF-1 α expression was not affected by withdrawal of growth factors or insulin from the cell culture medium. We excluded the possible stabilizing effect of the Hsp-90 protein, as well as of mTOR signaling pathway because: 1) we did not find the association between Hsp-90 with HIF-1 α , and 2) HIF-1 α protein expression and stability was not influenced by the downregulation of mTOR signaling pathway. The second observation is in agreement with the suggestion that mTOR does not affect HIF-1 α stability, but may influence HIF-1 α transcriptional activity (Land et al. 2007). HIF-1 α was not hydroxylated, ubiquitinated or degraded in NSPCs, despite the presence and activity of components of the oxygen-dependent degradation cascade. Interestingly, HIF-1 α hydroxylation and ubiquitination were only observed after NSPCs were treated and lysed with detergent, which disrupts all cell membranes.

There is now a growing body of evidence that inducible transcription factors can constantly shuttle between the cytoplasm and the nucleus, and that their cytoplasmic retention can be achieved by binding to cytoplasmic structures, which masks their nuclear localization or proteasomal degradation signals (Ziegler et al. 2005). Our cell fractionation studies lead us to conclude that HIF-1 α is associated with large membranous structures, identified as HIF-1 α -bearing vesicles (HBV). We propose that HIF-1 α is localized to specialized vesicles, and that this localization makes HIF-1 α molecules inaccessible for the components

of the hydroxylation and ubiquitination pathways (including PHDs and VHL/E3 ubiquitin ligase complex). The HBV may resemble stimulus-induced Weibell-Palade bodies that serve as a storage compartment for von Willebrand factor (van Mourik et al. 2002), or glucose transporter GLUT4 storage vesicles (Dugani et al. 2005). The existence of an extensive tubular endosomal network (TEN) that sorts cargoes to various destinations including the specialized storage vesicles, was recently proposed (Bonifacino et al. 2006). A partial co-localization of HIF-1 α -positive structures with clathrin and its adapter proteins (essential components of TEN) might indicate a possible association of HBV with this transporting network.

We also analyzed the biological activity of HIF-1 α within NSPCs using a knockdown approach. Even though nuclear HIF-1 α was almost undetectable by immunofluorescence under normoxic conditions, a small nuclear (transcriptionally active) pool was demonstrated by cell fractionation and Western blot. Most importantly, nuclear HIF-1 α was significantly increased following OGD. shRNA experiments supported our previous studies, showing that HIF regulates VEGF expression and release, and thus is transcriptionally active in NSPCs, which is in agreement with our previous work (Roitbak et al. 2008). In the present study we further demonstrate that HIF-1 α gene silencing results in decreased expression of activated Notch-1 (cleaved intracellular domain, NICD) and increased expression of β -catenin. Thus, HIF-1 α expression levels within NSPCs may also regulate Notch and Wnt signaling pathways known to modulate NSPC self-renewal and differentiation.

In summary, we propose that stabilization of HIF-1 α protein is a unique feature of NSPCs. Continuously sustained basic level of HIF-1 α allows NSPCs to a) maintain a constant small transcriptionally active pool of HIF-1 α in the nucleus and 2) withhold and rapidly respond to the external stimuli such as hypoxia, as well as to perform their protective functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Angela Welford for performing electron microscopy; Drs. Jane Rodman and Angela Wandering-Ness and Vojo Deretic for sharing their expertise in the analysis of HIF-1 α subcellular distribution. This work was supported by NIH P20 RR15636, NIH/NINDS R21 NS064185 and AHA 09GRNT2290178. Confocal images were generated in the UNM Cancer Center Fluorescence Microscopy Facility supported as detailed on the webpage <http://hsc.unm.edu/crtc/microscopy/instru.html>.

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Figure 1. HIF-1 α is expressed in NSPCs isolated from the embryonic and postnatal mouse brain
 A - neural progenitor cells isolated from postnatal day 28 mouse brain (pNSPCs) were subjected to immunofluorescence staining with antibody against HIF-1 α (red). B - dual immunofluorescence staining of pNSPCs with the antibodies against HIF-1 α (red) and neural stem cell marker nestin (green). C- Western blot analysis of eNSPCs and pNSPC cell lysates (top immunoblot) and samples prepared from NSPCs grown in the presence (+GF) and absence (-GF) of growth factors for 7 days (bottom immunoblot). D - preabsorption control: HIF-1 α antibody was pre-incubated with recombinant HIF-1 α polypeptide (Prospect Protein Specialists) for 30 min at RT. NSPCs were immunostained with preabsorbed HIF-1 α antibody, followed by Cy3-conjugated streptavidin secondary antibody. E – control immunostaining with only Cy3-conjugated streptavidin secondary antibody.. Nuclei were visualized using DAPI staining (blue). Cells were imaged on a Zeiss LSM510-META confocal imaging system. Bars = 10 μ m.

Figure 2. HIF-1 α is expressed in the neurogenic zones of adult mouse brain

Micrographs demonstrating HIF-1 α immunofluorescence staining in SVZ (a, c, d, e) and SGZ (b, f, g, h) of adult mouse brain. Coronal sections of 8 week-old mouse brain were immunostained with antibodies against HIF-1 α (a–h, red), nestin (c and f, green), doublecortin (Dcx, d and g, green), GFAP (e, green) and Sox-2 (h, green). Nuclei were visualized using DAPI staining (blue). Brain sections were imaged on a Zeiss LSM510-META confocal imaging system. Bars=20 μ m (a,b), 10 μ m (c–g) and 5 μ m (h).

Figure 3. HIF-1 α is not ubiquitinated in NSPCs

A: Cell lysates were prepared from the NSPCs grown in the presence of growth factors (GF lanes) and 4 days following growth factor withdrawal (–GF lanes). The samples were subjected to immunoblot analysis using the antibodies against major components of mTOR pathway: anti-mTOR, phospho-mTOR (P-mTOR), Raptor, Rictor, and Phospho-4E-BP1. The samples were also probed with the anti-HIF-1 α antibody. GAPDH expression was used as a loading control. B: NSPC lysates (lane 1) and immunoprecipitates with anti-HIF-1 α antibody (lane 2) were subjected to Western blot and probed with the antibodies against HIF-1 α and VHL. C: Analysis of HIF-1 α hydroxylation. eNSPC lysates were prepared using 1% Triton-X100 containing lysis buffer and subjected to *in-vitro* hydroxylation assay. The samples: control (lane 1) and hydroxylation reaction mix (lane 2), were subjected to Western blot analysis using anti-hydroxylated HIF-1 α antibody (H-HIF-1 α) and anti-HIF-1 α antibody (shown in the box). D: Analysis of HIF-1 α ubiquitination. Lane 1: control sample, NSPC lysate. Lane 2: NSCs lysate prepared using detergent-containing lysis buffer was subjected to *in vitro*-ubiquitination assay. Control sample (lane 1) and sample subjected to *in vitro* ubiquitination assay (lane 2) were immunoprecipitated with anti-HIF-1 α antibody

and subsequently probed with anti-ubiquitin antibody (lanes 3 and 4, respectively) – to specifically check for HIF-1 α ubiquitination. The same samples were also probed with anti-HIF-1 α antibody to demonstrate HIF-1 α expression levels in each sample as a loading and IP control (shown in box). E: *In vitro* ubiquitination assay was performed on the cell extracts prepared using detergent-containing lysis buffer (lane 1), or using detergent-free conditions, when the cells are disrupted mechanically and by freeze-thawing (lane 2). The samples were probed with anti-ubiquitin (lanes 1 and 2) and anti-HIF-1 α (shown in box) antibodies.

Figure 4. Confocal microscopy analyses of the subcellular distribution of HIF-1 α . eNSPCs (A, B and E), pNSPCs (C) and mouse brain endothelial cells (D, negative control) were immunostained with antibody against HIF-1 α (red). Samples on B and D panels were co-stained with the antibody against Golgi marker TGN38 (green). Orthogonal image projections on panel B demonstrate the localization of HIF-1 α -positive structures around the Golgi compartment; nuclei were visualized using DAPI staining. E – Orthogonal image projection demonstrating the presence of small amounts of HIF-1 α in the nuclei. Cells were imaged on a Zeiss LSM510-META confocal imaging system. Bars = 10 μ m (A,C,D), and 5 μ m (B and E). F-Electron micrograph of the eNSPC immunostained with anti-HIF-1 α /10 nm streptavidin-gold. The images were acquired on Hitachi H-7500 transmission electron microscope. Bar = 250 nm.

Figure 5. HIF-1 α localization changes following *in-vitro* hypoxia

Micrographs: NSPCs were subjected to 1h and 2h OGD. Subcellular distribution of HIF-1 α was detected in NSPCs immediately after 1 and 2 hours of OGD, as well as at 24 hours after re-oxygenation following 2h OGD. Orthogonal image projections demonstrate nuclear expression of HIF-1 α at 2h of OGD. Zeiss LSM510-META confocal imaging system. Bars = 5 μ m. Western blot: at 2h following OGD NSPC samples were subjected to cell fractionation. Nuclear (N) and cytoplasmic post-nuclear (PN) fractions of the control and OGD samples were immunoblotted with anti-HIF-1 α antibody. Sox-2 (nuclear protein expressed in NSPCs) was used as a loading control for nuclear fractions and GAPDH - as a loading control for post-nuclear fractions.

Figure 6. HIF-1 α is associated with large subcellular structures

A- Top immunoblot: NSPC lysates were subjected to subcellular fractionation, and the post-nuclear (cytoplasmic) extract was centrifuged at $100,000 \times g$ for 60 min to obtain cytosolic (C, supernatant) and membrane (M, pellet) fractions. The collected samples were immunoblotted with anti-HIF-1 α . Middle immunoblot: NSPC lysates were subjected to sucrose gradient-based centrifugation and eleven fractions were collected from the top. Proteins were resolved on SDS-PAGE gels and detected by immunoblotting with antibody specific to HIF-1 α . Bottom immunoblot: Fractions 5–9 collected following sucrose gradient centrifugation were combined and subjected to gel filtration on Sepharose 4B column. The resulting fractions were immunoblotted with anti-HIF-1 α and anti-clathrin antibodies. B - Electron micrographs of the eNSPC immunostained with anti-HIF-1 α /10 nm streptavidin-gold. N - nucleus, G - Golgi, HBV - HIF-1 α -bearing vesicles. The images were acquired on Hitachi H-7500 transmission electron microscope. Bar = 250 nm. C- Electron micrograph: NSPC were immunostained with anti-HIF-1 α /10 nm streptavidin-gold and images were acquired on Hitachi H-7500 transmission electron microscope. Bar = 250 nm. Immunofluorescence images: NSPCs were immunostained with the antibodies against HIF-1 α (red) and markers for clathrin-coated vesicles (clathrin, adaptins α , β , γ , and δ , green), COP-vesicles (β -COP, green), endosomes (Rab 4, green) autophagosomes (LC3B,

green) and lysosomes (Lamp3, green). Cells were imaged on a Zeiss LSM510-META confocal imaging system. Bar =5 μ m.

Figure 7. HIF-1 α knockdown using shRNAi alters VEGF, active Notch-1 and β -catenin expression and impairs NSPC resistance to hypoxia

A - HIF-1 α shRNAi lentiviral particle-treated and non-target shRNA-treated (control) NSPCs were subjected to Western blot and probed with the antibodies against HIF-1 α , VEGF, active Notch-1 (recognizing cleaved cytosolic domain of Notch-1, NICD), β -catenin and actin (loading control). B - HIF-1 α shRNAi lentiviral particle-treated and non-target shRNA-treated NSPCs were plated at the same density. MTT viability assay was performed at 24 hours following 3h OGD; NSPC viability at normoxic (control, open bars, set to 100%) vs hypoxic (OGD, black bars) conditions was compared in three experimental groups: 1) non-infected intact NSPCs (left graph), 2) cells infected with non-target shRNA (middle graph) and 3) cells infected with HIF-1 α shRNA (right graph). * - $p < 0.01$; *** - $p < 0.0001$, Student's t-test; n=24 cultures per group.