# Hypoxia induces early neurogenesis in human fetal neural stem cells by activating the WNT pathway

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# Abstract

Fetal neural stem cells (FNSCs) present in the human fetal brain, differentiate into cells of neuronal and glial lineages. The developing fetus is exposed to lower oxygen concentrations compared to adults and this physiological hypoxia may influence the growth and differentiation of the FNSCs. This study aimed to evaluate the effect of hypoxia on the differentiation potential of human FNSCs isolated from the sub-ventricular zone of aborted fetal brains (n = 5). FNSCs were isolated, expanded, and characterized by Nestin and Sox2 expression, using immunocytochemistry and flowcytometry respectively. These FNSCs were exposed to 20% oxygen (normoxia) and 0.2% oxygen (hypoxia) concentrations for 48 hours, and hypoxia exposure (n = 5) was validated. Whole transcriptome analyses (Genespring GX13) of FNSCs exposed to hypoxia (Agilent 4x44K human array slides), highlighted that genes associated with neurogenesis were enriched on exposure to hypoxia. The pathway analysis of these enriched genes (using Metacore) showed that the involvement of WNT signaling pathway. Microarray analyses was validated using neuronal and glial lineage commitment markers, namely *NEUROG1*, *NEUROG2*, *ASCL1*, *DCX*, *GFAP*, *OLIG2* and *NKX2.2* using qPCR (n = 9). DCX and GFAP protein expression were analysed by western blotting (n = 3). This demonstrated upregulation of the neuronal commitment markers on hypoxia exposure, while no change was observed in astrocytic and oligodendrocyte lineage commitment markers. Increased expression of downstream targets of the WNT signaling pathway, *TCF4 and ID2*, by qPCR (n = 9), and increased protein expression of CTNNB1 (β-catenin) and ID2 by western blot (n = 3), indicated its involvement in mediating neuronal differentiation on exposure to hypoxia.


# Introduction

Neural stem cells (NSC) are multipotent cells that can differentiate into neurons, astrocytes and oligodendrocytes. Fetal neural stem cells (FNSCs) are located in the subventricular zone and dentate gyrus of the fetal brain. NSC are also found in the cortex, striatum and subependymal zone of adult brain [1, 2, 3]. In addition to cues provided by the NSC niche, oxygen concentration can also influence the growth and differentiation potential of NSCs, and plays a critical role during embryonic development [4, 5]. As the fetus develops inside the uterus, the difference in oxygen concentration between maternal and fetal circulation shows that the developing fetus is normally exposed to lower oxygen concentrations, and thus, despite fetal hemoglobin having a greater affinity for oxygen, and existence of other compensatory mechanisms, there is a possibility that the fetal brain may be exposed to hypoxic environment *in utero* [6, 7, 8]. Studies have shown that hypoxia may influence NSC development and plasticity [5, 9]. It has been reported that mild hypoxia (5% $O_2$) activates molecular pathways like Wnt/beta-catenin and Notch, which regulate self-renewal and proliferation of stem cells, including NSCs [5, 10, 11].

This study aimed to understand the role of hypoxia on the differentiation potential of human FNSCs. It also elucidates the possible mechanism by which hypoxia may influence lineage commitment in human FNSCs.

# Methodology

# Sample collection

Aborted fetal samples were collected from the Department of Obstetrics and Gynecology, AIIMS, New Delhi, India. Informed consent was obtained from mothers undergoing Medical Termination of Pregnancy (MTP) in their second trimester of pregnancy (12–20 weeks) for maternal indications. Mothers undergoing MTP for fetal indications (such as chromosomal anomalies) were excluded from the study. Approvals were taken from Institutional Ethics Committee and Institutional Committee for Stem Cell Research, before starting the study. The study was carried out in conformation with the Helsinki Declaration.

# Isolation of human fetal neural stem cells (FNSCs)

Isolation of human FNSCs from the brain of aborted fetuses was done as per published protocol [12]. Briefly, tissue from subventricular zone of brain was isolated and plated onto poly-D-lysine coated culture flasks in neural stem cell media containing neurobasal media (GIBCO, NY, USA) with 1% N2 supplement (GIBCO, NY, USA), 2% Neural survival factor-1 (Lonza, IA, USA), 1% Glutamax (GIBCO, NY, USA), 5mg/mL of bovine serum albumin (Sigma, MO, USA), penicillin (50 IU/ml), streptomycin (50 µg/ml) and gentamicin (2 µg/ml). Tissue demonstrating cells radiating from the core, were gently dissociated and subcultured onto poly-D-lysine coated flasks, to generate monolayers of FNSCs. For neurosphere assay, human FNSCs at passage 2–3 were plated onto non-adherent culture dishes at a density of 1000 cells/$cm^2$ in neural stem cell media (prepared as described above).

# Flow cytometry

Human FNSCs were fixed with 2% paraformaldehyde, permeabilized with 1% BSA containing 0.1% Triton X-100. Cells were blocked with 2% BSA for half an hour and subsequently stained with (intracytoplasmic) mouse anti-human SOX2 antibody conjugated with V450 (BD Biosciences, cat. no. 561610) using appropriate controls. Cells were washed, resuspended in 2% paraformaldehyde, and data was acquired using BD LSR Fortessa (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo v10 software.

# Immunocytochemistry

Human FNSCs (at passage 3–4) were plated onto coverslips coated with poly-D-Lysine. They were washed with PBS and fixed with 2% PFA. Cells were incubated for 1 hour in blocking solution (1% BSA with 0.1% Triton X-100) and then washed with PBS. The cells were incubated overnight at 4˚C with primary antibody (Mouse anti-Nestin 1:1000, Cat no. 33475, CST, MA, USA; Rabbit anti-SOX2 1:1000, Cat no. 23064, CST, MA, USA). The cells were washed thrice with PBS and then incubated with secondary antibody (Mouse anti-Rabbit FITC, 1:1000: Cat no. A11008, Invitrogen; Goat anti-Mouse Alexa Fluor 594, Cat no. A-11005, Invitrogen) for 1 hour at room temperature. Cells were then washed thrice with PBS, and mounted onto glass slides using Vectashield mountant containing DAPI. The slide was allowed to dry overnight. Images were taken on Nikon Eclipse Ti-S fluorescent microscope (Tokyo, Japan) and analyzed with NIS-Elements BR software.

# Exposure of human FNSCs to different oxygen concentrations

Human FNSCs were exposed to oxygen concentrations mimicking normoxia (20% oxygen) and hypoxia (0.2% oxygen) for 48 hours, at 37˚C and 5% $CO_2$, that was created using an Anoxomat hypoxia induction system (Advanced Instruments, Norwood, MA, USA). Hypoxia exposure was validated by evaluating the expression of hypoxia-responsive genes *CA9, VEGF and PGK-1*.

# RNA isolation, cDNA synthesis and qPCR

Total RNA was extracted from the cells after exposure to different oxygen concentrations, using Tri-Reagent (Sigma, MO, USA) and quantified by Nano-Drop ND-1000 spectrophotometer (Thermo-Fisher Scientific, MA, USA). cDNA was synthesized with 1µg total RNA using M-MuLV-RT (Thermo-Fisher Scientific, MA, USA) and random hexamer primers (IDT, IL, USA). The expression of various genes was evaluated in the cells (in triplicates) using gene-specific primers (IDT, IL, USA) (Table 1) and DyNAmo Flash SYBR Green qPCR kit (Thermo-Fisher Scientific, MA, USA) using CFX96 Touch™ Real-Time PCR Detection System (BioRad, CA, USA). 18S rRNA was used as an internal reference gene for normalization. Relative fold change in gene expression was calculated using $2^{-\Delta\Delta CT}$ method. Human FNSCs exposed to normoxia (20% oxygen) were used as controls for hypoxia experiments.

Table 1
Sequence of primers used for gene expression analyses

| Gene | Forward primer | Reverse primer | Product size (bp) |
|---|---|---|---|
| 18S rRNA | GTAACCCGTTGAACCCCATT | CCATCCAATCGGTAGTAGCG | 151 |
| DCX | GGGGGTGTGGGCATAAAGAA | CCTGCTCTTTACCAGCCTCC | 149 |
| ASCL1 | TCCCCCAACTACTCCAACGA | GTTGTGCGATCACCCTGCTT | 193 |
| Ngn1 | TCTTGGTCTGTTTCTCCGGC | GGGTCAGTTCTGAGCCAGTC | 120 |
| Ngn2 | TGACTGACAGACAGACACGC | TGACGAACATCTTAGTTGGCTC | 257 |
| Olig2 | TCGCATCCAGATTTTCGGGT | AAAAGGTCATCGGGCTCTGG | 135 |
| Nkx2.2 | TTCAGTACTCCCTGCACGGTC | TGTCATTGTCCGGTGACTCG | 99 |
| GFAP | AGCCCACTCCTTCATAAAGCC | ATGCGTCTCCTCTCCATCCT | 72 |
| CA-9 | CTTTGAATGGGCGAGTGATT | CTTCTGTGCTGCCTTCTCATCT | 184 |
| ID2 | TTGTCAGCCTGCATCACCAG | AGGGAATTCAGAAGCCTGCAA | 110 |
| TCF4 | CCCGTCCAGGAACTATGGAG | CACCTCCAAGGAGACTCTGG | 188 |
| B-catenin | GTAACCCGTTGAACCCCATT | CATCTACACAGTTTGATGCTGCT | 151 |

# Western blots

Fetal neural stem cells exposed to different concentrations of oxygen were lysed in RIPA lysis buffer with protease-phosphatase inhibitors and protein concentration in lysates was determined using the bicinchoninic acid assay (BCA). 50 µg of lysate was loaded into each well for SDS-PAGE (4% stacking gel, 12–15% resolving gel). Normoxic (20%) and hypoxic (0.2%) samples were run on the same gel. Proteins were blotted onto 0.22 µm nitrocellulose membrane, followed by blocking in 5% non-fat milk (NFM) in TTBS. Blots were incubated overnight at 4˚C with primary antibody (Table 2) diluted in 1% NFM, followed by three washes with TTBS. Blots were then incubated for 1 hour, with corresponding secondary antibody diluted in 1% NFM. This was followed by five washes in TTBS. The blot was incubated with Luminol and peroxidase (Abbkine SuperLumia ECL Plus Kit, Hubei, China) and chemiluminescence detection was done using Azure Biosystems C280 gel documentation sytem (Dublin, CA, USA), followed by analysis with Image J software. Normalization was done using β-actin protein levels.

Table 2
Technical details of primary and secondary antibodies used for western blot experiments

| Antibody | Molecular weight | Antibody specifications | Antibody dilution | Procured from |
|---|---|---|---|---|
| Anti-GFAP (Catalog #ABP54511) | 50 kDa | Polyclonal Rabbit IgG | 1:1000 | Abbkine, China |
| Anti-DCX (Catalog #4604S) | 45 kDa | Polyclonal Rabbit IgG | 1:1000 | CST, MA, USA |
| Anti- HIF1α (Catalog #36169S) | 120 kDa | Monoclonal Rabbit IgG | 1:1000 | CST, MA, USA |
| Anti- β-catenin (Catalog #9582) | 92 kDa | Monoclonal Rabbit IgG | 1:1000 | CST, MA, USA |
| Anti-ID2 (Catalog #3431S) | 15 kDa | Monoclonal Rabbit IgG | 1:500 | CST, MA, USA |
| Anti β-actin (Catalog #STJ94020) | 42 kDa | Polyclonal Rabbit IgG | 1:2000 | St. John's Laboratory, UK |
| HRP-tagged Anti-rabbit secondary antibody (Catalog #7074S) | - | Goat Anti-rabbit IgG | 1:2000 | CST, MA, USA |
| HRP-tagged anti-mouse secondary antibody (Catalog #7076S) | - | Horse Anti-mouse IgG | 1:2000 | CST, MA, USA |

# Gene expression microarrays

RNA concentration and integrity were analyzed using Nanodrop (Thermo-Fisher Scientific, MA, USA) and Bioanalyzer (Agilent, Santa Clara, CA, USA) respectively. Expression microarrays were done on two biological replicates each, of human FNSCs exposed to normoxia and hypoxia, on 4 x 44K human expression array slides (G2519F) (Agilent, Santa Clara, CA, USA), following manufacturer's protocol. Briefly, a total of 200 ng total RNA per sample (n = 2) was subjected to cDNA conversion and linear amplification with fluorescent labeling for preparing Cy3 labelled cRNA. Complementary hybridization (17 hours at 65°C) was followed by washing. Slides were scanned at 3 μm resolution, followed by feature extraction using feature extraction software version 10.7.1.1 (Agilent technologies, Santa Clara, USA). Analysis was done using Genespring Software v14.9.1 (Agilent technologies, Santa Clara, USA). Principal component analysis and Hierarchical clustering analysis were done to identify gene target distribution, that showed a consistent difference in expression between normoxia and hypoxia exposed cells. This was followed by differential gene expression analysis, which was done using unpaired t-test using Genespring software (GX v14.9.1). The list of differentially expressed genes were imported into gene ontology consortium and Metacore for gene ontology and pathway analysis.

# Statistical analysis

Statistical analysis was done using Graph Pad Prism v6. Statistical differences between normoxia and hypoxia exposed groups was estimated using Mann Whitney test. p-value < 0.05 was considered statistically significant.

## Results

# Isolation and characterization of human fetal neural stem cells

Human fetal neural stem cells (FNSCs) were observed to be radiating out from the core of tissue isolated from the subventricular zone of the brain (Fig. 1a). On dissociating and subculturing these, small, unipolar monolayer of human FNSCs were obtained at passage 2–3 (Fig. 1b). Neurosphere assay displayed their ability to form neurospheres after 3–4 days of plating onto non-adherent surface (Fig. 1c). Immunocytochemical staining at passage 3–4 helped characterize the human FNSCs, and demonstrated expression of NESTIN and SOX2 (Fig. 1.d). Flow cytometry indicated that more than 90% of FNSCs expressed SOX2 (Fig. 1e and 1f).

# Exposure of FNSCs to different oxygen concentrations for 48 hours

Human FNSCs were exposed to different oxygen concentrations mimicking normoxia (20%), and hypoxia (0.2%) for 48 hours. Hypoxia exposure was validated by evaluating *CA9, PGK1* and *VEGF* expression by qPCR. The mean fold change ± SD in *CA-9, PGK1* and *VEGF* expression in the FNSCs exposed to hypoxia were 345.91 ± 38.29 ($p < 0.0001$), 27.61 ± 11.48 ($p < 0.001$) and 6.45 ± 2.56 ($p < 0.01$) respectively

(Fig. 2a,b,c). Exposure to hypoxia was also validated by measuring HIF1α protein expression by western blot (n = 3) (Fig. 2d). Normalised protein expression of HIF1α increased from 0.09 ± 0.04 in normoxic controls to 1.75 ± 0.62 (p = 0.10) in FNSCs exposed to 0.2% hypoxia (Fig. 2e).

# Gene ontology and pathway analysis

The analyses of differentially expressed genes (DEGS) in FNSCs exposed to hypoxia as compared to normoxia, showed that 974 genes were upregulated while 368 genes were downregulated (Supplementary Tables 1 and 2). Gene ontology analysis of differentially expressed genes in FNSCs exposed to hypoxia (Supplementary Tables 3–6) showed that pathways pertaining to cell development and cell differentiation were getting enriched. The genes involved in these GO terms were further analyzed for biological processes, and showed that regulation of neuron projection development, positive regulation of neurogenesis, neuron projection guidance, cell morphogenesis involved in neuron differentiation, regulation of neuron differentiation, neuron projection morphogenesis, neuron projection development, neuron development, and generation of neurons were getting enriched and had role in cell differentiation of human FNSCs exposed to hypoxia (Fig. 3a).

The genes involved in the neuron development, neuron differentiation and generation of neurons were then evaluated for enriched pathways (Table 3) and networks using Metacore software. This analysis showed that Wnt-beta catenin canonical network was involved, with candidate genes such as Wnt and Frizzled being up-regulated (Fig. 3b). It was also seen that a sub-node of a VEGF pathway was linked with the Wnt-beta catenin canonical network (Fig. 3c).

Table 3
Key pathways upregulated in fetal neural stem cells (FNSCs) exposed to hypoxia with adjusted p-values

| Pathway Name | Adj. p-values |
|---|---|
| Transcription_HIF-1 targets | 2.687E-08 |
| Inhibition of oligodendrocyte precursor cells differentiation by Wnt signaling in multiple sclerosis | 4.468E-05 |
| Bone metastases in Prostate Cancer | 6.552E-05 |
| Canonical WNT signaling pathway in colorectal cancer | 1.398E-04 |
| Development_MAG-dependent inhibition of neurite outgrowth | 1.398E-04 |
| Breakdown of CD4 + T cell peripheral tolerance in type 1 diabetes mellitus | 4.859E-04 |
| Colorectal cancer (general schema) | 1.329E-03 |
| Development_Embryonal epaxial myogenesis | 1.330E-03 |
| WNT signaling in gastric cancer | 2.171E-03 |
| Role of activation of WNT signaling in the progression of lung cancer | 2.703E-03 |

# Expression of lineage commitment markers in human FNSCs exposed to hypoxia

*ASCL1*, *DCX*, *NEUROG1* and *NEUROG2* are markers for early neurogenesis, and their expression was checked by qPCR, after exposing human FNSCs to hypoxia for 48 hours. The mean fold change ± SEM for *NEUROG1, NEUROG2, ASCL1*, and *DCX* expression in the FNSCs exposed to hypoxia were 36.08 ± 21.88 ($p = 0.0035$); 0.65 ± 0.13 ($p = 0.0035$); 3.23 ± 1.45 ($p = 0.4042$); and 3.08 ± 1.34 ($p = 0.7090$) respectively (Fig. 4a-d). DCX protein expression (n = 3), as measured by western blot, showed a slight increase from 1.0 ± 0.2 in normoxia to 1.1 ± 0.14 in hypoxia ($p = 0.70$) (Fig. 4h and i). Astrocytic lineage marker, *GFAP*, and oligodendrocyte lineage markers, *OLIG2* and *NKX2.2*, did not show any significant change when evaluated by qPCR. The mean fold change ± SEM for *GFAP* expression in the FNSCs exposed to hypoxia, was 1.02 ± 0.20 ($p = 0.6818$) (Fig. 4e), while for *OLIG2* and *NKX2.2*, it was 0.98 ± 0.38 ($p = 0.2235$) and 1.23 ± 0.30 ($p = 0.7059$) respectively (Fig. 4f and g). GFAP protein expression (n = 3) decreased from 1.17 ± 0.29 at normoxia to 0.76 ± 0.2 in FNSCs exposed to hypoxia ($p = 0.40$) (Fig. 4h and j).

# Expression of downstream targets of the Wnt signaling pathway

Downstream targets of the Wnt signaling pathway, *CTNNB1* (beta catenin), *TCF4* and *ID2* were analyzed using qPCR. The mean fold change ± SEM for *CTNNB1* (beta catenin) (Fig. 5a), *TCF4* (Fig. 5b), and *ID2* (Fig. 5c) expression in the FNSCs exposed to hypoxia, were 0.55 ± 0.11 ($p = 0.0426$); 4.19 ± 0.77 ($p = 0.0352$); and 4.10 ± 1.48 ($p = 0.22$) respectively. Protein expression (n = 3) of CTNNB1 (beta catenin) (Fig. 5d-e) increased from 0.6 ± 0.3 (in normoxia) to 1.16 ± 0.27 in hypoxic FNSCs ($p = 0.40$). ID2 (Fig. 5d and f) protein expression (n = 3) also increased from normoxic levels of 0.72 ± 0.1 to 1.27 ± 0.4 after hypoxia ($p = 0.40$).

## Discussion

This study used fetal neural stem cells as a model system to partially mimic the physiological hypoxic conditions *in utero* and its possible influence on the development of the human fetal brain. In this study, human fetal neural stem cells (FNSCs) were isolated from subventricular zone of the aborted fetal brains. These multipotent stem cells, derived from neuroectoderm, have the potential to differentiate into neurons, astrocytes and oligodendrocytes. The isolated FNSCs displayed ability to form neurospheres, and expressed characteristic neural stem cell markers, Nestin and Sox2, as reported earlier [12, 13].

Despite existence of compensatory mechanisms, there is a possibility of physiological hypoxia in the developing fetal brain *in utero* [6, 8]. To partially mimic the physiological hypoxia *in utero*, human FNSCs were exposed to normoxia (20% oxygen) and hypoxia (0.2% oxygen), for 48 hours. Hypoxia mediates its action through HIF1α therefore its elevated protein levels and increased gene expression of its downstream targets, carbonic anhydrase (CA9), phosphoglycerate kinase (PGK-1) and vascular

endothelial growth factor (VEGF) indicate that the FNSCs were exposed to hypoxic environment. It also agrees with previous published reports related to downstream targets of HIF1α [14].

This study investigated the expression of early markers of neurogenesis like *NEUROG1*, *NEUROG2*, *ASCL1*, *DCX*, in human FNSCs exposed to hypoxia. These are also considered to be the lineage commitment markers of neurogenesis. It was observed that the expression of *NEUROG1*, *ASCL1* and *DCX* were found to be increased in human FNSCs exposed to hypoxia. However, the expression of *NEUROG2* was found to be slightly decreased. Interestingly, the increase in *NEUROG1* expression, was much more than the decrease in *NEUROG2* expression. This composite picture showing increased expression of *NEUROG1*, *ASCL1* and *DCX*, supported by increase in protein expression of DCX, signify that hypoxia exposure in human FNSCs may be responsible for initiating neurogenesis, and thus promoting FNSCs to commit to neuronal lineage. Our findings are supported by reports indicating the essential role of *NEUROG1*, *ASCL1* and *DCX* in neurogenesis [15, 16, 17]. Interestingly, there are reports of different hypoxic conditions enhancing the expression of *NEUROG1* and *DCX* [15, 18, 19]. However, there are also a few studies reporting the downregulation of neurogenesis and *ASCL1* by hypoxia, that are contrary to our findings [4, 20]. Again, studies also corroborate our findings by reporting that hypoxia or HIF-1α stimulates neurogenesis [21, 22].

Our findings also indicate that the protein expression of GFAP, an astrocyte lineage commitment marker were decreased, and gene expression of *OLIG2* and *NKX2.2*, oligodendrocyte lineage commitment markers, were not influenced by exposing FNSCs to hypoxia. This indicates hypoxia not inciting FNSCs to commit to glial cell lineages. Our findings are further supported by the fact that even though there are studies reporting reactive astrocytosis and gliosis in hypoxic injury, there are no reports suggesting promotion of neurogenesis in neural stem cells [23, 24].

As FNSCs were exposed to hypoxia, whole genome transcriptomic changes and Gene ontology analysis revealed that genes related to cell development and differentiation were being modulated. On further analysis of these genes for biological processes, it was found that genes involved in neurogenesis were found to be up-regulated when FNSCs were exposed to hypoxia. Considering that the human brain is more evolved and complex than the rodent brain, our study is the first to report induction of neurogenesis in human FNSCs on exposure to hypoxia. Few studies have reported that hypoxia stimulates neural stem cell proliferation but have not identified its influence on neurogenesis [24, 25, 26].

Pathway analysis of our data indicated that the Wnt-beta-catenin signaling pathway may be implicated during this differentiation. This corroborates previous reports implicating this pathway in hypoxia mediated proliferation of neural stem cells [5, 26, 27].

Pathway analysis of microarray data displaying genes enriched in cell development and neurogenesis in our study, showed that canonical Wnt-beta catenin signaling pathway was involved in promoting commitment of FNSCs to neuronal lineage after exposing FNSCs to hypoxia. In this study, the expression of some critical regulators of Wnt-beta catenin signaling pathway, viz. beta catenin, TCF4 and ID2 were elucidated. The protein expression of beta-catenin was found to be increased, even though its gene

expression was slightly downregulated. The expression of downstream effector targets of the Wnt signaling pathway, TCF4 and ID2, were also increased in FNSCs exposed to hypoxia, indicating its involvement in mediating the effects of hypoxia. Interestingly, a few studies have reported that Wnt signaling is responsible for stem cell maintenance as well as neuronal differentiation, lineage commitment, axon guidance, and neurite outgrowth [28, 29]. It has also been reported that Wnt signaling facilitates neurological recovery in experimental stroke, thereby establishing its role in neurogenesis [28, 30].

Beta catenin one of the regulators of the Wnt-signaling pathway, translocates to the nucleus and interacts with TCF to activate the transcriptionally active complex. Interestingly, target genes of this complex are *NEUROG1* and *NEUROG2* [31]. *NEUROG1/2* then binds to p300/CBP co-activator proteins to promote neuronal differentiation [32]. The expression of TCF4 was found to be increased in this study, which might also explain the increase seen in the composite picture of increased expression of lineage commitment markers of neurogenesis like *NEUROG1*, *DCX* and *ASCL1*. This is the likely mechanism involved in influencing FNSCs to differentiate into neurons, after they are exposed to hypoxia. Recent studies have also confirmed the involvement of the Wnt-signaling pathway in mediating neurological recovery in stroke and epilepsy, lending credence to our findings of hypoxia promoting neurogenesis in FNSCs [10, 28, 30].

To the best of our knowledge, this is the first study to show that there was an increase in lineage commitment markers of neurogenesis viz., *NEUROG1*, *DCX* and *ASCL1*, on exposing FNSCs to hypoxic conditions, while observing no change in the astrocytic and oligodendrocytic lineage markers. The mechanism attributed to the increased neurogenesis may be attributed to increase in downstream effectors of the Wnt-signaling pathway, viz. TCF4 and ID2 pointing to the involvement of the Wnt-signaling pathway in mediating this action.

# Declarations


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### *Competing interests*

The authors have no relevant financial or non-financial interests to disclose.

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Final approval of the version to be published: All

### *Data Availability*

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

### *Ethics Approval*

This study was performed in line with the principles of the Declaration of Helsinki. Necessary approval was taken from Institutional Ethics Committee (IEC-340/03.05.2019 dated 7$^{th}$ May, 2019) and Institutional Committee for Stem Cell Research (IC-SCR/96/19(O) dated 8$^{th}$ August, 2019), All India Institute of Medical Sciences, New Delhi, prior to commencement of work.

### *Consent to participate*

Informed consent was taken from mothers undergoing medical termination of pregnancy (MTP).

### *Consent to publish*

Not applicable

# References


1. Kjell J, Fischer-Sternjak J, Thompson AJ, Friess C, Sticco MJ, Salinas F, Cox J, Martinelli DC, Ninkovic J, Franze K, Schiller HB, Götz M (2020) Defining the Adult Neural Stem Cell Niche Proteome Identifies Key Regulators of Adult Neurogenesis. Cell Stem Cell 26(2):277–293e8 PMID: 32032526; PMCID: PMC7005820
2. Bond AM, Ming GL, Song H (2015) Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. Cell Stem Cell 17(4):385–395. doi: 10.1016/j.stem.2015.09.003. PMID: 26431181;

PMCID: PMC4683085

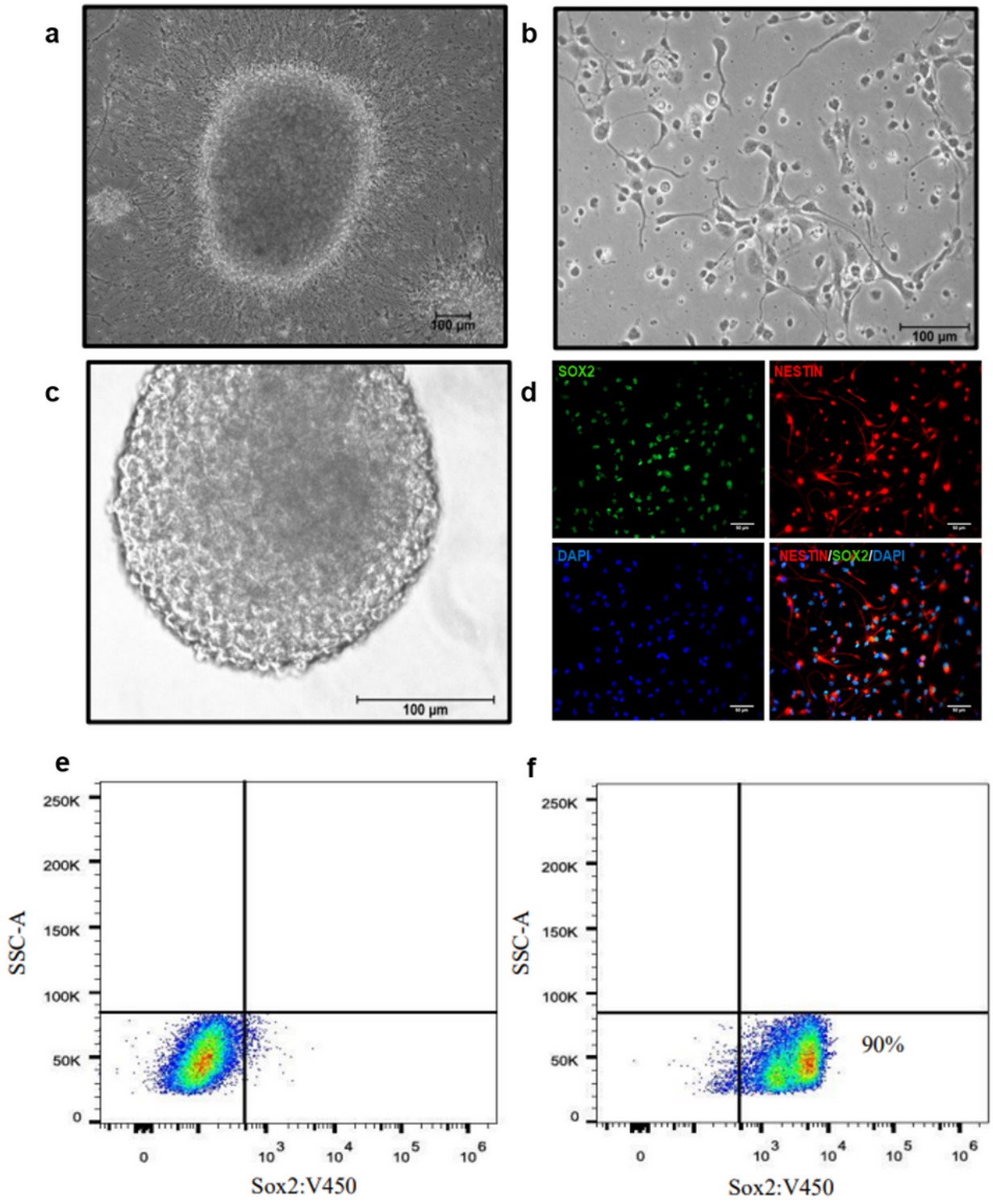
3. Yin X, Li L, Zhang X, Yang Y, Chai Y, Han X, Feng Z (2013) Development of neural stem cells at different sites of fetus brain of different gestational age. Int J Clin Exp Pathol 6(12):2757–2764 PMID: 24294362; PMCID: PMC3843256
4. Večeřa J, Procházková J, Šumberová V, Pánská V, Paculová H, Lánová MK, Mašek J, Bohačiaková D, Andersson ER, Pacherník J (2020) Hypoxia/Hif1α prevents premature neuronal differentiation of neural stem cells through the activation of Hes1. Stem Cell Res 45:101770 Epub 2020 Mar 28. PMID: 32276221
5. Qi C, Zhang J, Chen X, Wan J, Wang J, Zhang P, Liu Y (2017) Hypoxia stimulates neural stem cell proliferation by increasing HIF 1α expression and activating Wnt/β-catenin signaling. Cell Mol Biol (Noisy-le-grand) 63(7):12–19. doi: 10.14715/cmb/2017.63.7.2. PMID: 28838333; PMCID: PMC5614483
6. Giussani DA (2016) The fetal brain sparing response to hypoxia: physiological mechanisms. J Physiol 594(5):1215–1230. doi: 10.1113/JP271099. Epub 2016 Jan 6. PMID: 26496004; PMCID: PMC4721497
7. Martin DS, Khosravi M, Grocott MP, Mythen MG (2010) Concepts in hypoxia reborn. Crit Care 14(4):315. doi: 10.1186/cc9078. Epub 2010 Jul 30. PMID: 20727228; PMCID: PMC2945079
8. Breathnach CS (1991) The stability of the fetal oxygen environment. *Ir J Med Sci* 160(7):189 – 91. doi: 10.1007/BF02957308. PMID: 1757209
9. De Filippis L, Delia D (2011) Hypoxia in the regulation of neural stem cells. *Cell Mol Life Sci* 68(17):2831-44. doi: 10.1007/s00018-011-0723-5. Epub 2011 May 17. PMID: 21584807
10. Sun C, Fu J, Qu Z, Jia L, Li D, Zhen J, Wang W (2021) Chronic Intermittent Hypobaric Hypoxia Restores Hippocampus Function and Rescues Cognitive Impairments in Chronic Epileptic Rats via Wnt/β-catenin Signaling. Front Mol Neurosci 13:617143. doi: 10.3389/fnmol.2020.617143. PMID: 33584201; PMCID: PMC7874094
11. Felfly H, Zambon AC, Xue J, Muotri A, Zhou D, Snyder EY, Haddad GG (2011) Severe Hypoxia: Consequences to Neural Stem Cells and Neurons. J Neurol Res 1(5). 10.4021/jnr70w. doi: 10.4021/jnr70w.. PMID: 24348887; PMCID: PMC3858017
12. Bhagat R, Prajapati B, Narwal S, Agnihotri N, Adlakha YK, Sen J, Mani S, Seth P (2018) Zika virus E protein alters the properties of human fetal neural stem cells by modulating microRNA circuitry. Cell Death Differ 25(10):1837–1854. doi: 10.1038/s41418-018-0163-y. Epub 2018 Jul 26. PMID: 30050059; PMCID: PMC6180120
13. Bernal A, Arranz L (2018) Nestin-expressing progenitor cells: function, identity and therapeutic implications. Cell Mol Life Sci 75(12):2177–2195. doi: 10.1007/s00018-018-2794-z. Epub 2018 Mar 14. PMID: 29541793; PMCID: PMC5948302
14. Zhang Z, Yao L, Yang J, Wang Z, Du G (2018) PI3K/Akt and HIF 1 signaling pathway in hypoxia ischemia (Review). Mol Med Rep 18(4):3547–3554. doi: 10.3892/mmr.2018.9375. Epub 2018 Aug 9. PMID: 30106145; PMCID: PMC6131612

15. Zhang X, Zhu C, Luo Q, Dong J, Liu L, Li M, Zhu H, Ma X, Wang J (2016) Impact of siRNA targeting of β-catenin on differentiation of rat neural stem cells and gene expression of Ngn1 and BMP4 following in vitro hypoxic-ischemic brain damage. Mol Med Rep 14(4):3595–3601. doi: 10.3892/mmr.2016.5667. Epub 2016 Aug 24. PMID: 27573468; PMCID: PMC5042732
16. Pilz GA, Bottes S, Betizeau M, Jörg DJ, Carta S, Simons BD, Helmchen F, Jessberger S (2018) Live imaging of neurogenesis in the adult mouse hippocampus. Science 359(6376):658–662. doi: 10.1126/science.aao5056. PMID: 29439238; PMCID: PMC6986926
17. Shahsavani M, Pronk RJ, Falk R, Lam M, Moslem M, Linker SB, Salma J, Day K, Schuster J, Anderlid BM, Dahl N, Gage FH, Falk A (2018) An in vitro model of lissencephaly: expanding the role of DCX during neurogenesis. Mol Psychiatry 23(7):1674–1684. doi: 10.1038/mp.2017.175. Epub 2017 Sep 19. PMID: 28924182
18. Jaworska J, Zalewska T, Sypecka J, Ziemka-Nalecz M (2019) Effect of the HDAC Inhibitor, Sodium Butyrate, on Neurogenesis in a Rat Model of Neonatal Hypoxia-Ischemia: Potential Mechanism of Action. Mol Neurobiol 56(9):6341–6370. doi: 10.1007/s12035-019-1518-1. Epub 2019 Feb 14. PMID: 30767185; PMCID: PMC6682584
19. Haines B, Mao X, Xie L, Spusta S, Zeng X, Jin K, Greenberg DA (2013) Neuroglobin expression in neurogenesis. Neurosci Lett 549:3–6. doi: 10.1016/j.neulet.2013.04.039. Epub 2013 May 2. PMID: 23643985
20. Kasim M, Benko E, Winkelmann A, Mrowka R, Staudacher JJ, Persson PB, Scholz H, Meier JC, Fähling M (2014) Shutdown of achaete-scute homolog-1 expression by heterogeneous nuclear ribonucleoprotein (hnRNP)-A2/B1 in hypoxia. J Biol Chem 289(39):26973–26988. doi: 10.1074/jbc.M114.579391. Epub 2014 Aug 14. PMID: 25124043; PMCID: PMC4175337
21. Li Y, Wu L, Yu M, Yang F, Wu B, Lu S, Tu M, Xu H (2018) HIF-1α is Critical for the Activation of Notch Signaling in Neurogenesis During Acute Epilepsy. Neuroscience 394:206–219. doi: 10.1016/j.neuroscience.2018.10.037. Epub 2018 Oct 28. PMID: 30394322
22. Varela-Nallar L, Rojas-Abalos M, Abbott AC, Moya EA, Iturriaga R, Inestrosa NC (2014) Chronic hypoxia induces the activation of the Wnt/β-catenin signaling pathway and stimulates hippocampal neurogenesis in wild-type and APPswe-PS1ΔE9 transgenic mice in vivo. Front Cell Neurosci 8:17. doi: 10.3389/fncel.2014.00017. PMID: 24574965; PMCID: PMC3918655
23. Zhang S, Wu M, Peng C, Zhao G, Gu R (2017) GFAP expression in injured astrocytes in rats. Exp Ther Med 14(3):1905–1908. doi: 10.3892/etm.2017.4760. Epub 2017 Jul 10. PMID: 28962102; PMCID: PMC5609138
24. Santilli G, Lamorte G, Carlessi L, Ferrari D, Rota Nodari L, Binda E, Delia D, Vescovi AL, De Filippis L (2010) Mild hypoxia enhances proliferation and multipotency of human neural stem cells. PLoS ONE 5(1):e8575. doi: 10.1371/journal.pone.0008575. PMID: 20052410; PMCID: PMC2797394
25. Shi Z, Wei Z, Li J, Yuan S, Pan B, Cao F, Zhou H, Zhang Y, Wang Y, Sun S, Kong X, Feng S (2018) Identification and Verification of Candidate Genes Regulating Neural Stem Cells Behavior Under

Hypoxia. *Cell Physiol Biochem* 47(1):212–222. doi: 10.1159/000489799. Epub 2018 May 10. PMID: 29763928

26. Moreno M, Fernández V, Monllau JM, Borrell V, Lerin C, de la Iglesia N (2015) Transcriptional Profiling of Hypoxic Neural Stem Cells Identifies Calcineurin-NFATc4 Signaling as a Major Regulator of Neural Stem Cell Biology. Stem Cell Reports 5(2):157–165 Epub 2015 Jul 30. PMID: 26235896; PMCID: PMC4618660
27. Braunschweig L, Meyer AK, Wagenführ L, Storch A (2015) Oxygen regulates proliferation of neural stem cells through Wnt/β-catenin signalling. Mol Cell Neurosci 67:84–92. doi: 10.1016/j.mcn.2015.06.006. Epub 2015 Jun 14. PMID: 26079803
28. Xu D, Li F, Xue G, Hou K, Fang W, Li Y (2020) Effect of Wnt signaling pathway on neurogenesis after cerebral ischemia and its therapeutic potential. Brain Res Bull 164:1–13 Epub 2020 Aug 5. PMID: 32763283
29. Ille F, Sommer L (2005) Wnt signaling: multiple functions in neural development. *Cell Mol Life Sci*. 62(10):1100-8. doi: 10.1007/s00018-005-4552-2. PMID: 15928805
30. Song D, Zhang X, Chen J, Liu X, Xue J, Zhang L, Lan X (2019) Wnt canonical pathway activator TWS119 drives microglial anti-inflammatory activation and facilitates neurological recovery following experimental stroke. J Neuroinflammation 16(1):256. doi: 10.1186/s12974-019-1660-8. PMID: 31810470; PMCID: PMC6896312
31. Kan L, Israsena N, Zhang Z, Hu M, Zhao LR, Jalali A, Sahni V, Kessler JA (2004) Sox1 acts through multiple independent pathways to promote neurogenesis. Dev Biol. 2004 May 15;269(2):580 – 94. doi: 10.1016/j.ydbio.2004.02.005. PMID: 15110721
32. Shin DH, Kim GH, Lee JS, Joo IS, Suh-Kim H, Kim SS, Hong JM (2016) Comparison of MSC-Neurogenin1 administration modality in MCAO rat model. Transl Neurosci 7(1):164–172. doi: 10.1515/tnsci-2016-0024. PMID: 28270935; PMCID: PMC5338457

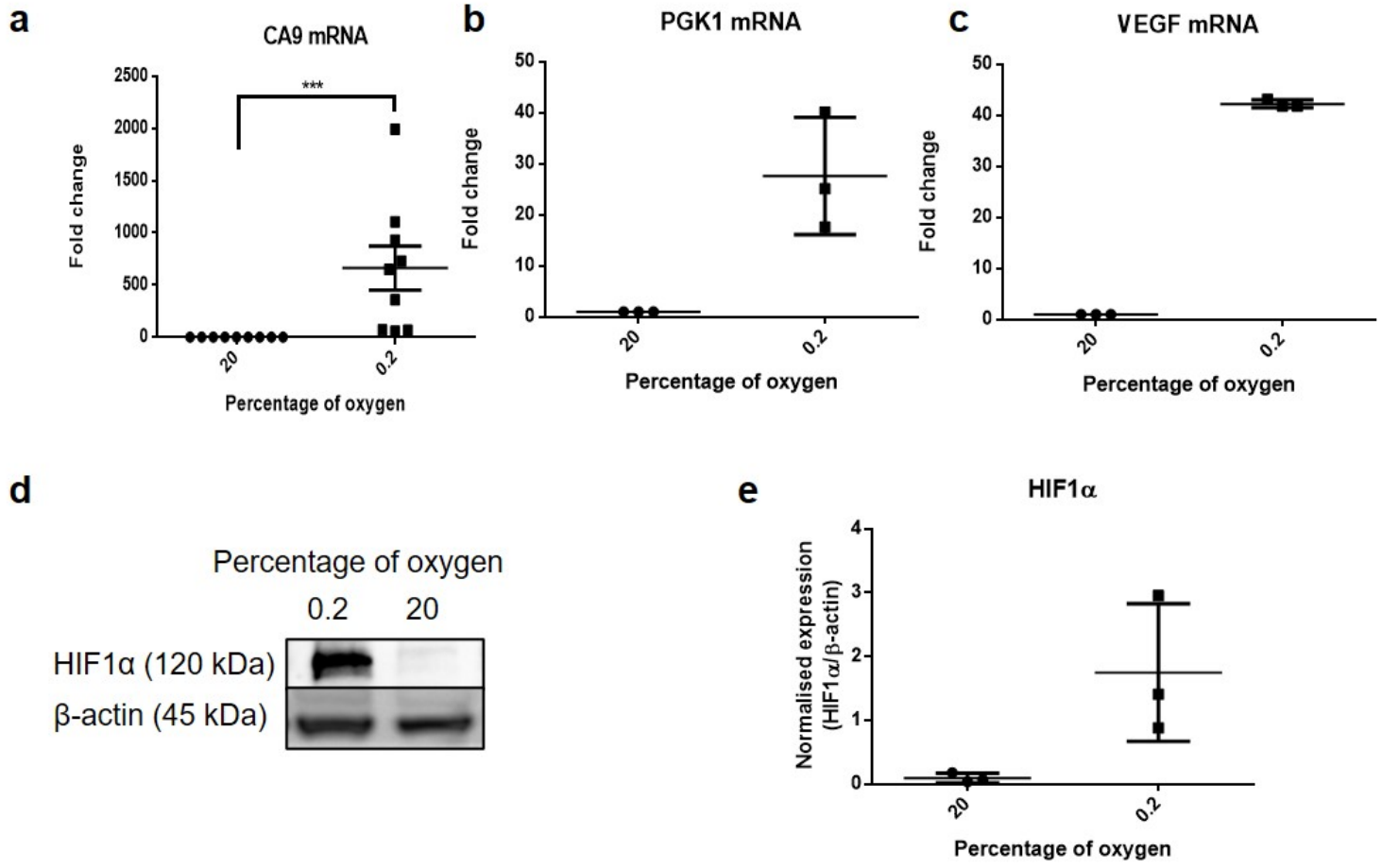
# Figures




## Figure 1

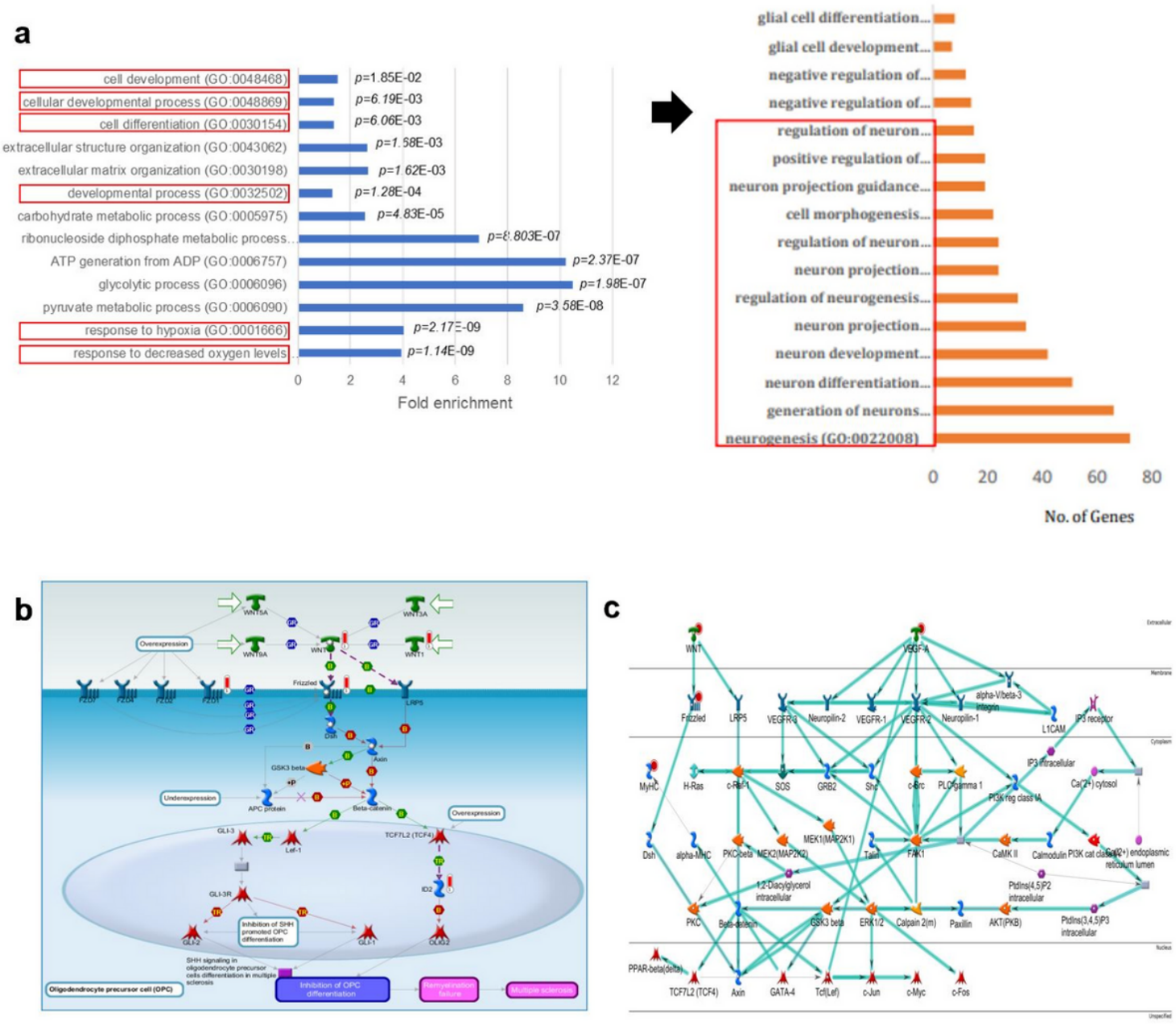
Isolation and characterization of Human FNSCs. (a) Human FNSCs radiating out of the core of the tissue isolated from subventricular zone of aborted fetal brain. (b) FNSCs in monolayer displaying unipolar morphology (c) Human FNSCs forming neurospheres (d) Immunofluorescence image of FNSCs stained for neural stem cell markers Sox2 (green) and Nestin (red). Nuclei are stained with DAPI (blue). (e) Dot

plot of unstained human FNSCs in flow cytometry (f) Human FNSCs stained for Sox2 (antibody conjugated with V450).



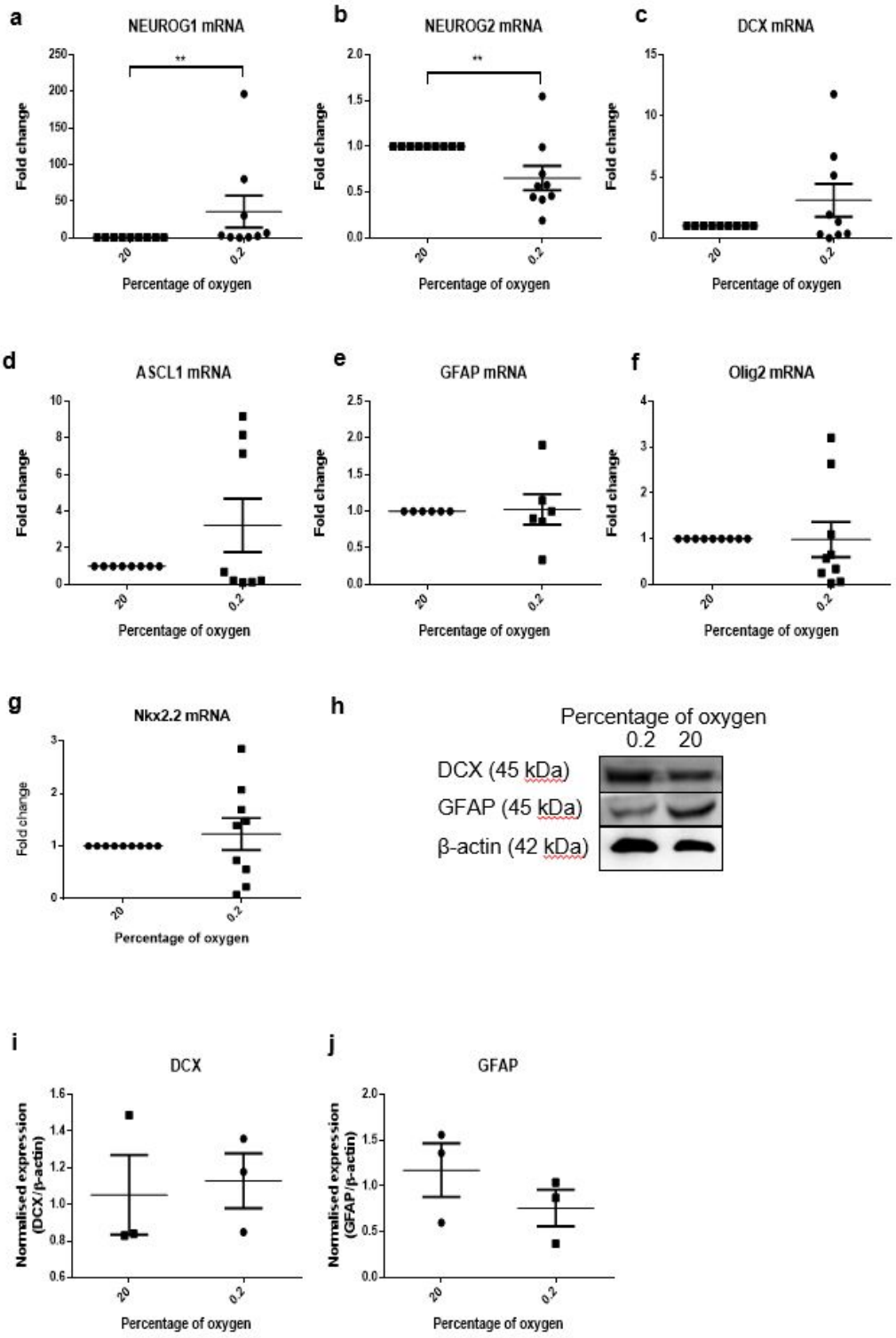

## Figure 2

Exposure of human FNSCs to hypoxia. qPCR analysis of (a) carbonic anhydrase XI (CA9)(n=9) (b) phosphoglycerate kinase 1 (PGK1) and (c) vascular endothelial growth factor (VEGF) (n=3) gene expression in normoxic (control) and hypoxic FNSCs. 18S rRNA was used as reference gene to measure fold change. (D) Representative western blot images and (E) densitometric quantification (n=3) of hypoxia inducible factor 1-α (HIF1-α) expression in hypoxic vs normoxic FNSCs. Data are represented as Mean±SEM. *** $p<0.001$




## Figure 3

Gene ontology and pathway analysis of human FNSCs exposed to hypoxia (0.2% oxygen) (a) Gene Ontology analysis of human FNSCs exposed to hypoxia analyzed in gene ontology consortium (b) Pathway map of genes enriched in neurogenesis after exposure to hypoxia. (c) Network map of genes enriched in neurogenesis




## Figure 4

Expression of lineage commitment markers in FNSCs exposed to hypoxia. qPCR analysis of gene expression of neural cell fate markers (a) Neurogenin 1 (NEUROG1), (b) Neurogenin 2 (NEUROG2), (c) Doublecortin (DCX) and (d) Achaete-scute homolog 1 (ASCL1); astrocytic cell fate marker (e) glial fibrillary acidic protein (GFAP); oligodendrocyte lineage markers (f) Oligodendrocyte transcription factor 2 (Olig2) and (g) NK2 homeobox 2 (Nkx2.2) in FNSCs exposed to hypoxia as compared to controls (n=6-9).

18S rRNA was used as reference gene. (h) Representative western blots and densitometric quantification (n=3) of (i) doublecortin (DCX) and (j) glial fibrillary acidic protein (GFAP) protein expression in FNSCs exposed to hypoxia as compared to controls. Data are represented as Mean ± SEM. **p<0.01

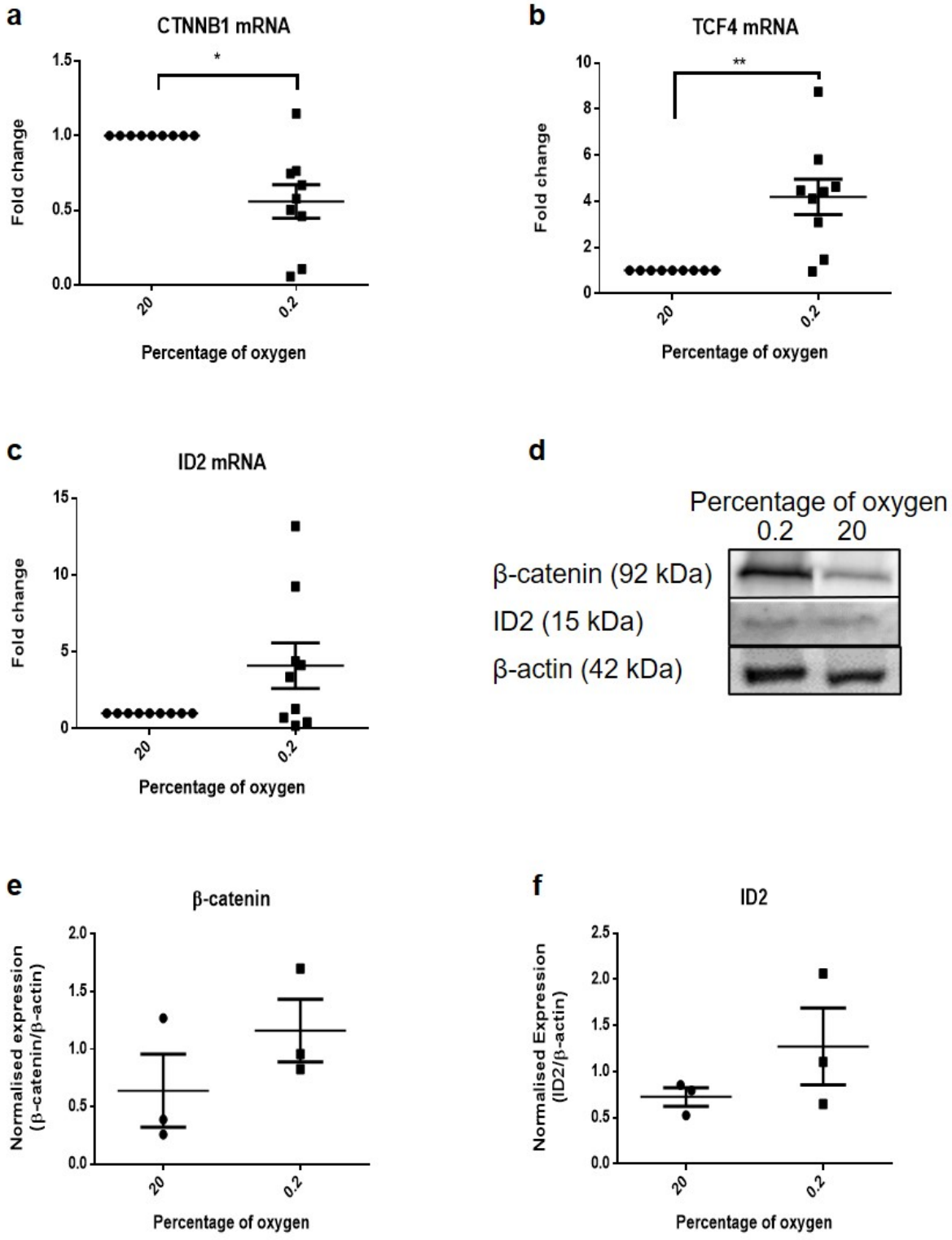



Figure 5

Expression of critical regulators of Wnt signaling pathway in FNSCs exposed to hypoxia. qPCR analysis of gene expression of Wnt signaling pathway members (a) β-catenin (CTNNB1), (b) Transcription factor 4 (TCF4) and (c) inhibitor of DNA binding 2 (ID2) in FNSCs exposed to hypoxia as compared to controls. 18S rRNA was used as reference gene. (d) Representative western blots and densitometric quantification of (e) β-catenin and (f) ID2 protein expression in FNSCs exposed to hypoxia as compared to controls. Data are represented as Mean ± SEM. *p<0.05 **p<0.01

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryinformation.pdf