Stem Cell Reports
Report
OPEN ACCESS

# Transcriptional Profiling of Hypoxic Neural Stem Cells Identifies Calcineurin-NFATc4 Signaling as a Major Regulator of Neural Stem Cell Biology

Marta Moreno,[1] Virginia Fernández,[2] Josep M. Monllau,[1] Víctor Borrell,[2] Carles Lerin,[3] and Núria de la Iglesia[1,*]

[1]Clinical and Experimental Neurosciences, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain
[2]Instituto de Neurociencias, Consejo Superior de Investigaciones Científicas and Universidad Miguel Hernández, 03550 Sant Joan d'Alacant, Spain
[3]Endocrinology, Hospital Sant Joan de Déu, 08950 Barcelona, Spain
*Correspondence: niglesia@clinic.ub.es
http://dx.doi.org/10.1016/j.stemcr.2015.06.008


## SUMMARY

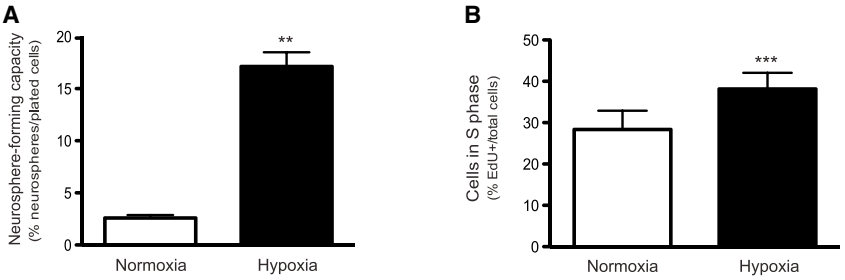
Neural stem cells (NSCs) reside in a hypoxic microenvironment within the brain. However, the crucial transcription factors (TFs) that regulate NSC biology under physiologic hypoxia are poorly understood. Here we have performed gene set enrichment analysis (GSEA) of microarray datasets from hypoxic versus normoxic NSCs with the aim of identifying pathways and TFs that are activated under oxygen concentrations mimicking normal brain tissue microenvironment. Integration of TF target (TFT) and pathway enrichment analysis identified the calcium-regulated TF NFATc4 as a major candidate to regulate hypoxic NSC functions. *Nfatc4* expression was coordinately upregulated by top hypoxia-activated TFs, while NFATc4 target genes were enriched in hypoxic NSCs. Loss-of-function analyses further revealed that the calcineurin-NFATc4 signaling axis acts as a major regulator of NSC self-renewal and proliferation in vitro and in vivo by promoting the expression of TFs, including Id2, that contribute to the maintenance of the NSC state.

## INTRODUCTION

Stem cells, including neural stem cells (NSCs), reside in specialized compartments that contribute to their maintenance. In the embryonic and adult mammalian brains, signals emanating from cells in the niche or the cerebrospinal fluid promote NSC self-renewal and proliferation (Lehtinen et al., 2011; Shen et al., 2004). Therefore, the NSC microenvironment has a strong influence on NSC behavior. Most cells in multicellular organisms live in an environment within tissues where oxygen levels are usually far below atmospheric oxygen concentrations. Physiologic oxygen levels may vary depending on cell type. Within the brain, physiologic oxygen concentrations are at least one order of magnitude lower than in the atmosphere (from 2%–5% in the cortex to 0.4% in pons) (Ereciń-ska and Silver, 2001; Mohyeldin et al., 2010; Panchision, 2009). In the hippocampus, where a specific population of adult NSCs resides, oxygen partial pressure is 3%–4%, a condition that persists from embryonic development. In other words, NSCs reside in a hypoxic environment. Physiologic hypoxia has been shown to promote cell population growth, survival, proliferation, and multipotency (Chen et al., 2007; Pistollato et al., 2007; Studer et al., 2000). However, the molecular mechanisms by which low oxygen levels impact on normal NSC behavior are poorly understood. This is probably due to the fact that most functional studies are performed under non-physiologic experimental conditions (21% oxygen), thereby underestimating crucial factors that are active under physiologic hypoxic conditions.

Hypoxia is known to affect the transcriptional status of the cell by activating important transcription factor (TF) families, such as hypoxia-inducible factor (HIF) or necrosis factor κB (NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B cells), among others (Cummins et al., 2006; Wang and Semenza, 1993). These pivotal factors control a wide range of vital cell functions, such as cell fate, proliferation, and survival in different cell types. As a master regulator of the hypoxic response, HIF is one of the primary candidates to regulate NSC biology at low oxygen levels. Up until now, only a candidate approach-driven study had identified a couple of HIF targets that are, at least in part, involved in mediating the NSC response to hypoxia (Studer et al., 2000). Another study had reported that low oxygen levels repress BMP-dependent SMAD activation, thereby inhibiting gliogenic differentiation of NSC (Pistollato et al., 2007). However, the molecular mechanisms by which physiologic hypoxia controls NSC behavior remained poorly understood.

The NSC response to hypoxia may go beyond the sole upregulation of HIF-dependent gene targets. It also may require the activation of other TFs. Most probably, it may involve the coordinated response of several TFs at the same time. The goal of this study was to identify the crucial TFs that control NSC functions under physiologic oxygen levels. Using gene set enrichment analysis (GSEA), we have shown that physiologically low oxygen levels activate a large number of TFs, well beyond the HIF family of TFs. Furthermore, integration of transcription factor target (TFT) and pathway enrichment analysis allowed us to uncover the calcium-regulated calcineurin-NFATc4 axis as a

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| GENE SET NAME | NES | FDR q-val |
|---|---|---|
| KEGG_GLYCOLYSIS_GLUCONEOGENESIS | 1.95 | 0.027 |
| KEGG_GALACTOSE_METABOLISM | 1.90 | 0.026 |
| KEGG_RIBOSOME | 1.89 | 0.020 |
| KEGG_STARCH_AND_SUCROSE_METABOLISM | 1.80 | 0.044 |
| KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM | 1.79 | 0.040 |
| KEGG_ARGININE_AND_PROLINE_METABOLISM | 1.77 | 0.042 |
| KEGG_PEROXISOME | 1.74 | 0.048 |
| KEGG_ECM_RECEPTOR_INTERACTION | 1.74 | 0.044 |
| KEGG_ARACHIDONIC_ACID_METABOLISM | 1.73 | 0.042 |
| KEGG_PYRUVATE_METABOLISM | 1.72 | 0.041 |
| KEGG_COMPLEMENT_AND_COAGULATION_CASCADES | 1.69 | 0.048 |
| KEGG_GLYCEROPHOSPHOLIPID_METABOLISM | 1.66 | 0.061 |
| KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION | 1.61 | 0.086 |
| KEGG_TASTE_TRANSDUCTION | 1.61 | 0.087 |
| KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM | 1.60 | 0.088 |
| KEGG_ETHER_LIPID_METABOLISM | 1.58 | 0.091 |
| KEGG_OLFACTORY_TRANSDUCTION | 1.57 | 0.100 |
| KEGG_CALCIUM_SIGNALING_PATHWAY | 1.52 | 0.133 |
| KEGG_GLYCEROLIPID_METABOLISM | 1.52 | 0.128 |
| KEGG_DILATED_CARDIOMYOPATHY | 1.51 | 0.139 |

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| GENE SET NAME | NES | FDR q-val |
|---|---|---|
| V$E12_Q6 | 1.81 | 0.109 |
| V$GATA_Q6 | 1.79 | 0.072 |
| V$AP1_Q2_01 | 1.73 | 0.103 |
| YTAAYNGCT_UNKNOWN | 1.72 | 0.086 |
| YATGNWAAT_V$OCT_C | 1.68 | 0.113 |
| V$OCT1_01 | 1.66 | 0.115 |
| GATAAGR_V$GATA_C | 1.65 | 0.113 |
| V$STAT1_01 | 1.61 | 0.145 |
| V$CEBP_Q2 | 1.60 | 0.148 |
| V$AP1_Q4_01 | 1.59 | 0.146 |
| V$OCT1_B | 1.59 | 0.136 |
| V$MYOD_01 | 1.59 | 0.125 |
| V$CP2_02 | 1.59 | 0.120 |
| V$NFKAPPAB_01 | 1.58 | 0.122 |
| V$AML_Q6 | 1.57 | 0.128 |
| V$FXR_Q3 | 1.57 | 0.122 |
| V$MAF_Q6 | 1.56 | 0.120 |
| V$OCT_Q6 | 1.56 | 0.116 |
| V$STAT5A_01 | 1.55 | 0.124 |
| TTCYNRGAA_V$STAT5B_01 | 1.55 | 0.119 |

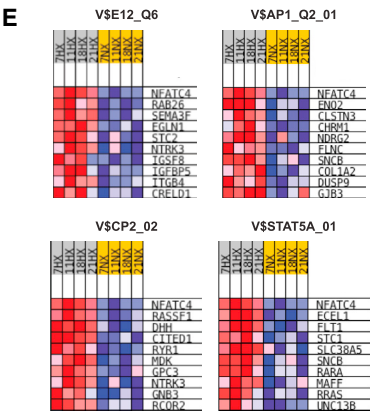


**Figure 1. GSEA of Microarray Datasets from Hypoxic versus Normoxic NSCs Identifies NFATc4 as a Candidate TF to Regulate Hypoxic NSC Functions**

(A) Self-renewal of mouse E13.5 NSCs maintained in 5% oxygen (hypoxia) or 21% oxygen (normoxia) was measured as the efficiency of single cells to give rise to neurospheres at low density. Data are presented as mean ± SEM (n = 4 independent experiments, paired t test, $^{**}p < 0.005$).

(B) Cell proliferation of NSCs maintained in hypoxia or normoxia as in (A) was assessed by EdU incorporation. Data are presented as mean ± SEM (n = 8 independent experiments, paired t test, $^{***}p < 0.001$).

(C) GSEA of microarray datasets using the gene sets of the KEGG pathway database. The top 20 KEGG gene sets that correlate with hypoxic NSCs are shown. GSEA statistical significance: FDR $\leq$ 0.25.

(D) GSEA of microarray datasets using the TFT gene sets. The top 20 TFT gene sets that correlate with hypoxic NSCs are shown. GSEA statistical significance: FDR $\leq$ 0.25.

(E) Enrichment profiles of E12, AP1, CP2, and STAT5A TFT gene sets are shown.

See also Figure S1 and Table S1.

key transcriptional program that controls NSC biology in vitro and in vivo.

## RESULTS AND DISCUSSION

### Physiologic Hypoxia Promotes Neurosphere Formation and Proliferation of Mouse Cortical NSCs

Culturing NSCs at low oxygen levels has been shown to promote cell population growth, survival, proliferation, and multicompetency of NSCs of different origins (Chen et al., 2007; Pistollato et al., 2007; Studer et al., 2000). However, the effect of hypoxia on NSC self-renewal through a neurosphere formation assay had not been tested. We thus measured the neurosphere formation capacity at low density of embryonic day (E) 13.5 mouse cortical NSCs, and we observed that culturing NSCs in hypoxic conditions dramatically increased neurosphere numbers (Figure 1A). Moreover, we confirmed that mouse cortical NSCs displayed increased proliferation at 5% versus 21% oxygen, as measured by EdU incorporation (Figure 1B).

### Transcriptomic Analysis of Hypoxic NSCs

To identify the molecular mechanisms mediating the effect of low oxygen levels on NSC biology, we profiled the global effect of sustained, physiologic hypoxia on NSC gene expression using an unbiased approach by genome-wide microarray analysis. Unsupervised principal component analysis of microarray data showed reasonable separation