Original Paper

Identification and Verification of Candidate Genes Regulating Neural Stem Cells Behavior Under Hypoxia

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Key Words

Neural stem cells • Hypoxia • Differentially expressed genes • Pathways • MicroRNAs

Abstract

Background/Aims: Neural stem cells (NSCs) reside in a hypoxic environment, and hypoxia plays an important role in their development and differentiation. This study aimed to explore the underlying mechanisms by which hypoxia affects NSC behavior. **Methods:** In the current study, we downloaded the gene expression dataset GSE68572 and identified the differentially expressed genes (DEGs) by analyzing high-throughput gene expression in hypoxic and normoxic NSCs. Subsequently, we analyzed these data using a combined bioinformatics approach and predicted the microRNAs (miRNAs) targeting the key gene using miRNA databases. Quantitative real-time PCR (qRT-PCR) was used to validate the expression of the top five DEGs. **Results:** In total, 1347 genes were identified as DEGs. We identified the predominant gene ontology categories and Kyoto Encyclopedia of Genes and Genomes pathways that were significantly over-represented in the hypoxic NSCs. A protein-protein interaction network the identification of miRNAs and their putative targets may offer new diagnostic and therapeutic strategies for liver cancer the top 10 core genes. Vascular endothelial growth factor A (VEGFA) had the highest degree and may be the key gene concerning NSC behavior under hypoxia. Further validation of the top five DEGs by qRT-PCR demonstrated that four DEGs were significantly higher and one DEG was significantly lower in the hypoxic group than in the control group. Seven miRNAs were predicted and proved to target VEGFA. **Conclusion:** This preliminary study can prompt the understanding of the molecular mechanisms by which hypoxia has an impact on NSC behavior and can help to optimize stem cell therapies for central nervous system injuries and diseases.

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Introduction

Neural stem cells (NSCs) are derived from the central nervous system (CNS), including the subventricular zone, dentate gyrus of the hippocampus, and central canal of the spinal cord, and transplantation of NSCs as a therapeutic modality for a number of CNS injuries and diseases has gained significant interest [1-3]. NSCs have the properties of unlimited self-renewal, indefinite ability to proliferate, and multipotency [4, 5]. Transplantation of NSCs can effectively promote regeneration of lost tissue and improve the host micro-environment [6]. However, there are a few challenges for NSC therapy. For instance, in spinal cord injury, the transplanted NSCs are maintained in an undifferentiated state or differentiate along the glial lineage [7, 8], and the transplanted NSCs have a relatively poor survival rate [9]. Therefore, a full understanding of the molecular mechanisms underlying NSC behavior is indispensable to solve these problems and to optimize cell therapies for CNS injuries and diseases.

Oxygen is necessary for most living organisms and is involved in energy production and cellular pathways [10]. Severe oxygen deprivation can lead to cell cycle arrest and cellular apoptosis; however, high concentrations of oxygen can result in the excess production of toxic reactive oxygen species, and thus the cells in the body maintain oxygen homeostasis [11-15]. NSCs have been shown to reside in a hypoxic environment [16]. Hypoxic signaling plays an important role in the differentiation and development of NSCs [17]. Previous studies have identified that hypoxia and hypoxia-inducible factor (HIF)-1 α can regulate the proliferation, differentiation, and neuronal maturation of NSCs [18, 19]. HIF-1 α is an essential transcriptional regulator of metabolism and angiogenesis, and a previous study suggested that HIF-1 α can regulate the fate of NSCs [20]. Furthermore, different levels of oxygen tension play some roles in cell fate through multiple cell signaling pathways [21, 22]. However, most functional studies on NSC behavior are performed under non-physiological experimental conditions (21% O₂) [16]. Moreover, the molecular mechanisms by which hypoxia has an impact on NSC behavior are still not fully clear; therefore, understanding the effects of physiological hypoxic conditions is helpful for controlling NSC behavior.

The goal of the present study was to identify the crucial genes, pathways, and microRNAs (miRNAs) that impact on NSC behavior under hypoxia. Firstly, by comparing the gene expression of hypoxic NSCs with normoxic NSCs in the Gene Expression Omnibus (GEO) database, we identified differentially expressed genes (DEGs) and performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analyses. In combination with protein-protein interaction (PPI) information, we not only identified relevant genes and pathways but also revealed the underlying molecular mechanisms. Furthermore, we predicted the key miRNAs that could regulate the expression of the key genes and pathways. In sum, our analysis provides a better understanding of the effect of hypoxia on NSCs, and the identification of the key genes, pathways, and miRNAs is expected to improve outcomes of NSC transplantation and optimize cell therapies for CNS injuries and diseases.

Materials and Methods

Gene expression microarray data

In this study, the gene expression profiles of GSE68572 were downloaded from GEO (<http://www.ncbi.nlm.nih.gov/geo/>). GSE68572 is based on the Agilent GPL10787 platform (Agilent-028005 SurePrint G3 Mouse GE 8 $\times$ 60K Microarray). The GSE68572 dataset contained eight samples, including four hypoxic NSC samples and four normoxic NSC samples. NSCs were isolated from E13.5 mouse embryos as described previously [23], and were immediately cultured at 37°C, 5% CO₂, and either 5% or atmospheric (approximately 21%) O₂, and these conditions were maintained for two to three passages [16].

Identification of DEGs

The raw data files used for the analysis included TXT files (Agilent platform). The analysis was carried out using GEO2R, which could perform comparisons on original submitter-supplied processed data tables using the GEOquery and limma R packages from the Bioconductor project. Limma provides a summary of the results of the linear model, performs hypothesis tests, and adjusts P-values for multiple testing, and the results include (log) fold changes, standard errors, t-statistics, and P-values. The DEGs between the hypoxic and normoxic NSC samples were selected ($P < 0.05$), and overlapping genes were identified.

GO enrichment and KEGG pathway analysis of the DEGs

After obtaining the DEGs, we submitted the DEG list to the online software Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>) v6.8 to identify overrepresented GO and pathway categories. GO analysis can determine the biological meaning of a large list of genes and categorize the functions of gene products, including biological process, molecular function, and cellular component [24, 25]. KEGG (<http://www.genome.jp/>) is a knowledge base for the systematic analysis of gene function, linking genomic information with higher-level systemic function [26]. Finally, the enriched functions of DEGs were selected via GO and KEGG pathway analyses, and $P < 0.05$ was considered statistically significant.

Construction of the PPI network of DEGs

To illustrate further the molecular mechanism by which hypoxia impacts on NSC behavior, we made use of the Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://www.string-db.org/>) to evaluate the interactive relationships among DEGs. We first submitted the DEG list to STRING, and then, we selected the experimentally validated interactions with a combined score > 0.4 . Subsequently, the PPI network was analyzed using Cytoscape software. Then, we used the plug-in Molecular Complex Detection (MCODE) to screen the modules of the PPI network in Cytoscape. Furthermore, enrichment analyses were performed for DEGs in the corresponding modules. $P < 0.05$ was considered statistically significant.

Isolation and culture of mouse embryonic NSCs

NSCs were taken from the hippocampus of mouse embryos (E13.5). The hippocampus was separated and cut into small pieces, and the pieces were incubated with Accutase (Invitrogen, Carlsbad, CA, USA). After incubation, the pieces were triturated through a 26-gauge needle. Then, the pellets were transferred to growth medium (Dulbecco's modified Eagle's medium: F-12 medium; Invitrogen) supplemented with 2% B27 supplement (Invitrogen), epidermal growth factor (20 ng/mL; Invitrogen), basic fibroblast growth factor (20 ng/mL; Invitrogen), heparin (2.5 µg/mL; Tocris Bioscience, Minneapolis, MN, USA), L-glutamine (1 mM; Invitrogen), leukemia inhibitory factor (10 ng/mL; PeproTech, NJ, USA), and penicillin and streptomycin (both 100 U/mL; Invitrogen). The cells were cultured at 37°C, 5% CO₂ and either 5% or 21% O₂ and maintained in these conditions for 3 passages, and half the medium was exchanged every 2–3 days.

Validation of DEGs with quantitative real-time PCR (qRT-PCR)

The TRIzol reagent (Beijing Solarbio Science & Technology Co., Beijing, China) was utilized to extract total RNA from cells before cDNA was synthesized. The quantity and quality of RNA were determined by NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. SYBR green (Thermo Scientific) was applied for qRT-PCR. The list of the primer sequences is included in Table 1. The mRNA expression levels of DEGs were normalized against GAPDH. After that, the expression levels of DEGs were analyzed with the $\Delta\Delta C_t$ method.

Prediction of miRNAs targeting the core gene

miRNAs are small non-coding RNAs, and they can bind to the 3'-untranslated region of target mRNA to inhibit mRNA expression or the degradation of mRNAs at the posttranscriptional level [27]. The key miRNAs targeting the core gene were predicted using TargetScan (<http://www.targetscan.org/>), miRDB (<http://www.mirdb.org/miRDB/index.html>), and miRanda (<http://www.microrna.org/microrna>). The miRNAs predicted by all three programs were identified as the targets of miRNAs.

Statistical analysis

The collected data were analyzed using GraphPad Prism (version 6.01). The data are presented as the mean $\pm$ standard deviation. Differential expression levels of DEGs were compared by using Student's t

test and two-way analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Identification of DEGs

The gene expression profile GSE68572 was downloaded from the GEO database, and the GEO2R method was used to identify DEGs in hypoxic NSC samples compared with normoxic NSC samples. We used $P < 0.05$, logFC (fold control) > 1.0 or logFC < -1.0 as the criteria, and 1347 genes were identified as DEGs. Among these, 1007 genes (74.76%) were up-regulated, and the remaining 340 genes (25.24%) were down-regulated. Subsequently, the heatmap of the top 50 up-regulated and down-regulated DEGs was obtained to visualize their expression levels in different samples (Fig. 1).

GO term enrichment and KEGG pathway analysis

To categorize these 1347 significant DEGs functionally, they were analyzed using the online software DAVID. The top 30 enriched GO terms of the DEGs are shown in Fig. 2A. GO analysis revealed that the main enriched GO terms were in biological process, such as response to hypoxia, response to decreased oxygen levels, regulation of oxidoreductase activity, and regulation of extracellular signal-regulated kinase 1 (ERK1) and extracellular signal-regulated kinase 2 (ERK2) cascade. Besides the biological process, there were also some enriched GO terms related to molecular function, such as growth factor binding and cargo receptor activity. In addition, the DEGs were also enriched in GO terms related to cellular component, such as receptor complex, proteinaceous extracellular matrix, and collagen trimer. KEGG analysis revealed that the DEGs were enriched in protein digestion and absorption, neuroactive ligand-receptor interaction, glycolysis/gluconeogenesis, cytokine-cytokine receptor interaction, and so on (Fig. 2B).

PPI network of the DEGs and core genes in the PPI network

On the basis of the information in the STRING database, the PPI network contained 663 nodes and 2318 edges. The nodes indicate the DEGs, and the edges indicate the interactions between the DEGs. The top 10 high-degree hub nodes consisted of vascular endothelial growth factor A (VEGFA), HIF-1 α , endothelial PAS domain protein 1 (EPAS1), serine (or cysteine) peptidase inhibitor, clade E, member 1 (SERPINE1), adenylate cyclase 8 (ADCY8), prostaglandin-endoperoxide synthase 2 (PTGS2), collagen, type I, alpha 1 (COL1A1), intercellular adhesion molecule 1 (ICAM1), myogenic differentiation 1 (MYOD1), and collagen, type I, alpha 2 (COL1A2). Among these genes, VEGFA showed the highest node degree of 65. The core genes and their corresponding degree are shown in Table 2. Then, we used MCODE to screen the modules of the PPI network (Fig. 3), and we performed enrichment analysis of the genes involved in the top 3 significant modules. The results showed that the DEGs in modules 1–3 were principally related to neuroactive ligand-receptor interaction, chemokine signaling pathway, calcium signaling pathway, and olfactory transduction (Table 3).

Prediction of miRNAs targeting the core gene

From the results of the PPI network, we found that VEGFA had the highest degree and may be the key gene concerning NSC behavior under hypoxia. The predicted miRNAs targeting VEGFA were obtained based on the miRNA databases (TargetScan, miRDB, and miRanda), and we identified the intersections among the results of the three databases,

Table 1. List of primer sequences used in this study

Gene symbol	Forward (5'-3')	Reverse (5'-3')
VEGFA	CTGCCGTCCGATTGAGACC	CCCCTCCTGTACCACTGTC
HIF1 α	AGCTTCTGTTATGAGGCTCACC	TGACTTGATGTTTCATCGTCCTC
EPAS1	CTGAGGAAGGAGAAATCCCGT	TGTGTCCGAAGGAAGCTGATG
SERPINE1	TTCAGCCCTTGCTTGCCCTC	ACACTTTTACTCCGAAGTCGGT
ADCY8	CTCTACACCATCCAACCGACG	GCACCGAGTCGCTAGACAG
β -actin	AGAGGGAAATCGTGCCTGACA	ACATCTGCTGGAAGTGGACA

Fig. 1. The heat map of the DEGs (top 50 up-regulated and down-regulate genes). Changes in genes expression ($P < 0.05$, $\log_{2}FC > 1.0$ or $\log_{2}FC < -1.0$) are illustrated by a heatmap. Blue indicates a relatively low expression and red indicates a relatively high expression.

namely, miR-361, miR-429, miR-200b, miR-140, miR-205, miR-206, miR-200c, miR-29a, miR-29b, miR-29c, miR-16, miR-15a, miR-15b, and miR-322 (Fig. 4A). Furthermore, the binding sites between VEGFA and the predicted miRNAs are presented in Fig. 4B.

qRT-PCR validation of DEGs

The top five DEGs were chosen for a validation experiment. The results of qRT-PCR showed that four of the up-regulated DEGs (VEGFA, HIF-1 α , EPAS1, and SERPINE1) were increased significantly in the hypoxic group compared with the control group, and one of the down-regulated DEGs (ADCY8) was decreased significantly in the hypoxic group compared with the control group (Fig. 5).

Discussion

NSCs can undergo self-renewal, generate identical cells, and are multipotent for the generation of diverse neural lineages, and they

Fig. 2. GO term enrichment and KEGG pathway analysis. A: Top 30 of GO enrichment. B: Top 30 of KEGG pathway enrichment. The circles represent biological process; the triangles represent cell component; the squares represent molecular function.

Fig. 3. Top 3 modules from the PPI network. A: module 1, B: module 2, C: module 3. The squares represent the DEGs in modules, and the lines indicate interactions between genes.

participate in the repair and regeneration of the CNS [28, 29]. NSCs have been shown to reside in a hypoxic environment [16]. A 2.5–5% O_2 concentration is best for NSCs to proliferate and differentiate into neurons [30]. However, most functional studies on NSC behavior have been performed under non-physiological experimental conditions (21% O_2) [16]. Therefore, understanding the molecular mechanisms by which hypoxia impacts on NSC behavior is essential to control NSC behavior under physiological hypoxic conditions, improve the results of NSC transplantation, and optimize cell therapies for CNS injuries and diseases. In the present study, we downloaded the gene expression profiles of GSE68572 and performed data analysis. A total of 1347 DEGs were screened, consisting of 1007 up-regulated genes and 340 down-regulated genes. Moreover, GO and KEGG pathway analyses were carried out to obtain a better understanding of the biological meaning of the DEGs. Combining the PPI network and miRNA prediction, we identified the potential key genes and miRNAs that could impact on NSC behavior under hypoxia.

GO term analysis showed that the DEGs were mainly involved in the response to hypoxia and response to decreased O_2 levels. The results of these experiments corresponded to what

Table 2. The core genes and their corresponding degree

Gene	Degree	Gene	Degree	Gene	Degree	Gene	Degree
VEGFA	65	PTGS2	38	HIF3A	34	CCL6	32
HIF1A	41	COL1A1	36	FLT1	33	ACTA1	32
EPAS1	41	ICAM1	36	LPAR3	33	CAV1	31
SERPINE1	40	MYOD1	36	COL3A1	32	PDGFRA	31
ADCY8	39	COL1A2	34	PDGFRB	32	PTK2B	31

Table 3. The enriched pathways of modules

Modules	Enriched pathways	P value	False discovery rate	Nodes
1	Neuroactive ligand-receptor interaction	3.3E-6	3.28E-07	AGTR2, DRD4, LPAR3, OPRD1, P2RY14, SSTR2, S1PR5
	Chemokine signaling pathway	1.1E-5	5.08E-07	ADCY8, CCL6, CCR1, CXCL1, GM1987, PPBP
	Cytokine-cytokine receptor interaction	6.2E-2	0.000102	CCR1, GM1987, PPBP
2	Neuroactive ligand-receptor interaction	1.1E-12	1.68E-12	ADORA2A, ADORA2B, CHRM1, CRHR1, GIPR, NTSR2, PTH1R, PTGFR, P2RY6, VIPR1
	Calcium signaling pathway	1.3E-3	0.0018	ADORA2A, ADORA2B, CHRM1, PTGFR
	Alcoholism	2.7E-2	0.0197	ADORA2A, ADORA2B, CRH
3	Olfactory transduction	7.4E-6	9.4E-07	OLFR1436, OLFR370, OLFR380, OLFR429, OLFR1250, OLFR1463, OLFR173

Fig. 4. A: Identification of miRNAs targeting VEGFA using the databases of miRNAs (TargetScan, miRDB and miRanda). B: The putative binding sites of the predicted miRNAs in the 3'-UTR of VEGFA.

Fig. 5. qRT-PCR validation of the top five DEGs. Four up-regulated DEGs (VEGFA, HIF1 α , EPAS1, SERPINE1) and one down-regulated DEG (ADCY8) were validated. (*P<0.05, ***P<0.001)

we expected to find. Moreover, the results also showed that the DEGs were associated with regulation of the ERK1 and ERK2 cascade. A previous study revealed that a novel neurotrophic factor, NF- α 1, can induce the differentiation of NSCs to astrocytes by activating the ERK1/2-Sox9 signaling pathway [31]. Folate is also involved in regulating the proliferation and reduced apoptosis of NSCs accompanied by ERK1/2 phosphorylation [32]. Moreover, several KEGG pathways were over-represented in the DEGs, including protein digestion and absorption, neuroactive ligand-receptor interaction, glycolysis/gluconeogenesis, and cytokine-cytokine receptor interaction. Neuroactive ligand-receptor interaction has been shown to be relevant to stimulate NSC proliferation induced by folic acid [33]. Angiogenesis can promote NSC differentiation in the cerebral cortex by increasing glycolytic activity and providing oxygen [34]. Therefore, GO and KEGG pathway analyses can help us to get a better understanding of the effect of hypoxia on NSCs.

Furthermore, we analyzed the PPI network and found that VEGFA, HIF-1 α , EPAS1, SERPINE1, ADCY8, PTGS2, COL1A1, ICAM1, MYOD1, and COL1A2 were the top 10 core genes, which may be related to NSC behavior under hypoxia. The results of qRT-PCR showed that four of the up-regulated DEGs (VEGFA, HIF-1 α , EPAS1, and SERPINE1) were increased and one of the down-regulated DEGs (ADCY8) was decreased in the hypoxic group compared with the control group. Among these genes, VEGFA showed the highest node degree. VEGFA is primarily involved in angiogenesis and vasculogenesis [35]. The expression of VEGFA and membrane-bound matrix metalloproteinase has been shown to be elevated significantly in

migratory NSCs [36, 37]. In a previous study, miRNA chip analysis was carried out using CNE cells in the presence or absence of hypoxia, and the results showed that VEGF was up-regulated in hypoxia-induced CNE cells compared with control cells [38]. Furthermore, exposure to hypoxia could result in the up-regulation of HIF-1 α and VEGF expression and enhanced neurogenesis in the dentate gyrus and sub-ventricular zone [39]. Compared with a previous study using the same expression profiling approach, our study analyzed the PPI network based on the information in the STRING database in order to evaluate the interactive relationships among DEGs, and this approach is a more efficient method to identify the key genes related to NSC behavior under hypoxia and to generate a better understanding of the underlying mechanisms. Moreover, the results of the current study were in agreement with those of a previous study, which showed that the up-regulation of VEGFA was a response to hypoxia and might affect important NSC functions [16].

To analyze further the potential role of VEGFA, we predicted the miRNAs targeting VEGFA using miRNA databases (TargetScan, miRDB, and miRanda), and we identified the intersections among the results of the three databases, namely, miR-361, miR-429, miR-200b, miR-140, miR-205, miR-206, miR-200c, miR-29a, miR-29b, miR-29c, miR-16, miR-15a, miR-15b, and miR-322. Subsequently, we performed a search in PubMed for these 14 miRNAs and found that 7 of them had been confirmed experimentally to target VEGFA, that is, miR-361 [40, 41], miR-140 [42], miR-15a [43], miR-15b [44], miR-205 [45], miR-206 [46], and miR-200c [47]. Therefore, we speculate that these miRNAs may target VEGFA and play a role in NSC behavior under hypoxia. Some of these miRNAs have been reported in association with NSC behavior. miR-140, a nicotine-sensitive miRNA, has been shown to play a role in the proliferation and maturation of NSCs [48]. The miR-200 family was also demonstrated to play a direct role in neuronal differentiation [49]. Trumbach et al. revealed that the miR-8/miR-200 miRNA family is involved in NSC proliferation, cell cycle exit, cell survival, transition to a neural precursor/neuroblast state, and neuronal differentiation [50]. However, whether these miRNAs influence NSC behavior by targeting VEGFA under hypoxia is unclear, and further experimental studies are needed to confirm these results. Furthermore, HIF-1 α is also a key factor in the maintenance of the adult sub-ventricular zone and regulation of NSC biology under hypoxia [16, 18]. Besides, PTGS2 can promote neural proliferation and differentiation and influences Wnt target gene expression [51]. Using the PPI network, module analysis demonstrated that NSC behavior under hypoxia was associated with neuroactive ligand-receptor interaction, chemokine signaling pathway, calcium signaling pathway, and olfactory transduction.

This study had several limitations. First, the sample sizes for expression profiling were not large, so further studies with larger sample sizes are still needed in order to confirm the results. Second, even though we performed preliminary validation of the results, more in-depth studies are needed in the future. Therefore, we hope that these results can be integrated into future experiments and facilitate further understanding of the molecular mechanisms controlling NSC behavior under hypoxia. Despite these limitations, we believe that this analysis represents a valuable resource and can be considered as a preliminary study for future studies controlling NSC behavior under physiological hypoxic conditions.

Conclusion

The present study identified 1347 DEGs via a comprehensive bioinformatics analysis approach. GO term, KEGG pathway, and PPI network analyses provided a set of related genes and pathways to help elucidate the molecular mechanisms by which hypoxia has an impact on NSC behavior. Validation experiments indicated that VEGFA, HIF-1 α , EPAS1, SERPINE1, and ADCY8 may play critical roles in NSC behavior under hypoxia. Potential miRNAs targeting VEGFA were predicted and can help us to get a better understanding of the underlying molecular network and regulatory mechanisms. These results are expected to contribute to better outcomes of NSC transplantation and optimization of cell therapies

for CNS injuries and diseases; however, further in-depth studies are needed to facilitate a deeper understanding.

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Disclosure Statement

No conflict of interests exists.

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