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ORIGINAL PAPER

The Role of Hypoxia in the Differentiation of P19 Embryonal Carcinoma Cells into Dopaminergic Neurons

Li-Ying Wu · Yue Wang · Bing Jin · Tong Zhao · Hai-Tao Wu · Yan Wu · Ming Fan · Xiao-Min Wang · Ling-Ling Zhu

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Abstract Nervous system development at early stage is in hypoxic environment. Very little is known about the role of hypoxia in neuronal development. P19 embryonal carcinoma (EC) cells are a widely used model for studying early neuronal development. In this study we investigated the roles of hypoxia in differentiation of dopaminergic neurons derived from P19 EC cells. Results demonstrate that hypoxia increases the percentage of differentiated neurons, especially neurons of dopaminergic phenotype. To investigate the potential mechanism involved in hypoxia promoted differentiation of dopaminergic neurons, we measured the expression of hypoxia-inducible factor 1α (HIF-1α), based on its characteristic response to hypoxia. The result shows that HIF-1α mRNA level in P19 EC cells increases after hypoxia treatment. It is known that HIF-1α regulates the expression of tyrosine hydroxylase (TH) gene through binding to its promoter. Therefore, we propose that the underlying mechanism for hypoxia promoted differentiation of dopaminergic neurons was mediated by HIF-1α up-regulation under hypoxia.

Keywords Hypoxia · Dopaminergic neurons · P19 EC cells · TH · HIF-1α

Yue Wang—Co-first author.

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L.-Y. Wu · B. Jin · T. Zhao · H.-T. Wu · Y. Wu · M. Fan · L.-L. Zhu (✉)
Department of Brain Protection, Institute of Basic Medical Sciences, Beijing 100850, People's Republic of China
e-mail: linglingzhu@hotmail.com

M. Fan
e-mail: waters97@163.com

Y. Wang · X.-M. Wang (✉)
Neuroscience Research Institute, Peking University, Beijing, China
e-mail: xmwang@ccmu.cdu.cn

M. Fan · X.-M. Wang
Department of Neurobiology, Capital University of Medicine, Beijing, China

Introduction

Oxygen (O_2) is very essential to maintain all kinds of life forms through its role in energy metabolism. Hypoxia or low-O_2 is also important in embryogenesis [1], in which, the natural progression of organogenesis is under hypoxia due to the limited O_2 diffusion in the embryo shortly after gastrulation [2]. Additionally, it has been reported that the nervous system develops in a hypoxic environment. It has been demonstrated that low oxygen could enhance proliferation, survival and characteristic differentiation of dopaminergic precursor cells and promote sympathoadrenal differentiation in isolated neural crest stem cells [3, 4]. Moreover, we have previously demonstrated that hypoxia promoted the proliferation and dopaminergic differentiation of neuronal stem cells isolated from E12 to 14.5 nervous system [5, 6]. These studies were focused on relatively later stage of neurodevelopment. Very little is known about how hypoxia affects the differentiation of neurons derived from embryonal cells during earlier neural development.

P19 embryonal carcinoma (EC) cell is a pluripotent stem cell line of mouse teratocarcinomas which is a well-documented system to explore the molecular mechanism of early events in neural fate determination and differentiation in vitro [7–10]. Treatment of P19 EC cells with retinoic acid (RA) induces the development of neurons, astroglia, microglia, oligodendroglia, and fibroblast-like cells

normally derived from the neuroectoderm [11–14]. In addition, neurons derived from P19 EC cells share the properties of mature neurons in the mammalian nervous system, for example, containing functional synapses, and expressing a number of neurotransmitters including glutamate, gamma-aminobutyric acid (GABA), somatostatin, neuropeptide Y, enkaphalin and catecholamines [13, 15]. Thus, the neuron-differentiational process of P19 EC cells in vitro emulates early neuronal development. Therefore, P19 EC cells as a stable and easily manipulatable system are extensively used to investigate early neurodevelopmental events [8–15].

In this study, we examined the role of hypoxia in differentiation of dopaminergic neurons using this P19 EC cell model system. Our results showed that hypoxia increased the percentage of neurons in differentiated P19 EC cells, and the number of dopaminergic neurons was remarkably increased after exposure to hypoxia. In order to investigate the potential mechanism, we measured the expression of hypoxia-inducible factor 1α (HIF-1α) mRNA level by RT-PCR, due to its peculiar role in hypoxic response [16, 17]. The result showed that hypoxia increased the levels of HIF-1α. HIF-1α is the main component of HIF-1, which mediates the transcription of genes whose cis-acting elements contain cognate hypoxia response elements (HRE) [18]. Additionally, it is reported that tyrosine hydroxylase (TH) gene just contains HRE and it can be activated by HIF-1 [19, 20]. TH is the key enzyme of dopamine (DA) synthesis and usually served as a marker for mature dopaminergic neurons [21–23]. Our results demonstrated that TH mRNA and protein levels were up-regulated after P19 EC cells were exposed to hypoxia. The induction of TH results in an increased DA level [16, 17, 19]. Thus, we propose that the increased level of HIF-1α gene under hypoxia indirectly promotes differentiation of dopaminergic neurons.

Experimental Procedure

Cell Culture

P19 mouse EC cells were obtained from American Type Culture Collection (ATCC number: CRL-1825). P19 EC cells were cultured using a slight modification of procedures described by Finley et al. [24]. Briefly, cells were maintained in α-minimum essential medium (αMEM, Gibco) supplemented with 25 mM $NaHCO_3$, 10% fetal bovine serum(FBS, Hyclone) and antibiotics (100 units/ml penicillin G and 100 mg/ml streptomycin sulfate) at 37°C in a 5% CO_2 humidified atmosphere. To induce cell differentiation, P19 EC cells were grown in medium with 1 μM all-trans retinoic acid (RA, Sigma) in nonadhesive bacteriological-grade Petri dishes (Nunc)for 4 days, and after aggregation cells were plated on adhesive tissue culture dishes (Corning) pre-coated with poly-L-lysine (Sigma) at the seeding density of $1 \times 10^5/cm^2$ in Dulbecco's modified Eagle's medium (DMEM, Gibco) without RA and supplemented with 1% N2 and 2% B27 (Life Technologies) for 4–7 days. On day cytosine 2 arabinoside (Ara-C, Sigma) was added at a final concentration of 5 μg/ml to inhibit division of non-neuronal cells for 2 days. The neuronal differentiation efficiency by RA is more than 70% using this method [7]. Experimental cells were tested with the same protocol except at RA induction stage: control cells were exposed to normoxia, while treatment cells were exposed to hypoxia.

Hypoxic Incubation

For hypoxia treatment, cultures were treated with a modified protocol according to previously described methods [6, 25, 26]. Briefly, petri dishes (Nunc) were placed into a gas-tight incubator chamber that was flushed with a gas mixture of 3% O_2/5% CO_2/92% N_2. The gas mixture was delivered for 10 min daily at a rate of 200 ml/min, and then cells were immediately returned to normoxia conditions.

Immunocytochemistry

Cultured cells were fixed for 20 min at room temperature in 4% paraformaldehyde and 0.025% glutaraldehyde in phosphate buffered saline (PBS), and washed three times with PBS (5 min each). Cells were then incubated with 3% normal horse serum containing 0.3% Triton X-100 for 60 min at room temperature (RT) to block nonspecific binding. The cultures were subsequently incubated with primary antibody in blocking solution (1% normal horse serum, 1% BSA, 0.1% Triton X-100 in PBS) at 4°C overnight. The following antibody concentrations were used: rabbit polyclonal anti-NSE antibody (Chemicon) at 1:2000 dilution, mouse monoclonal anti-NeuN antibody (Chemicon) at 1:1000 dilution, mouse monoclonal anti-MAP-2 antibody (Chemicon) at 1:1000 dilution, mouse monoclonal anti-TH antibody (Chemicon) at 1:2000 dilution. Then cultures were washed three times with PBS in 0.1% Triton X-100 (PBST, 5 min each) and incubated with biotin-conjugated secondary antibody (Vector, 1:1000) for 2 h at room temperature (RT). After washing with PBST for 3 times, cultures were incubated with avidin-biotinylated horseradish peroxidase (HRP) complex (Vector, ABC kit, 1:1000) for 90 min at room temperature, then detected with 0.02% DAB, 0.68% ammonium nickelous sulfate and 0.03% H_2O_2. Processing was stopped with PBS. Immunoreactivity was visualized with a Nikon microscope, and images were captured with a color video camera.

Electrophysiology

Mature and functional neurons were identified by whole-cell patch clamp technique. The recording was performed on P19 EC-derived neurons culturing from 5 days to 10 days after differentiation. For current-clamp recordings, the internal patch pipette solution consisted of (in mM): 140 KCl, 10 HEPES, 10 EGTA, 1 ATP, pH-adjusted to 7.2–7.4 with KOH. The external solution consisted of (in mM): 140 NaCl, 5 KCI, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, 10 glucose, pH-adjusted to 7.2–7.4 with NaOH. Experiments were performed on the stage of a Nikon inverted microscope using standard whole-cell attached patch clamp methods [27]. Cells with phase-bright and neuron-like processes were chosen for study. Data acquisition and analysis were performed using Pclamp software (Axon Instruments).

Flow Cytometric Analysis

Cells were treated with 0.25% Trypsin (Invitrogen) for 3 min at 37°C and collected in centrifuge tubes. After centrifugation, the pellet was resuspended in ice-cold 2% paraformaldehyde buffer. Normal goat serum was added to block nonspecific protein interactions. The cell suspension was then incubated with primary antibody: rabbit polyclonal NSE antibody (Chemicon, 1:500), mouse monoclonal NeuN antibody (Chemicon, 1:300), mouse monoclonal MAP-2 antibody (Chemicon, 1:300), mouse monoclonal TH antibody (Chemicon, 1:500). After washed with PBST, and cells were resuspended in 100 μl PBS containing fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Santa Cruz, 1:50). The cells were stained with secondary antibody for 60 min on ice, washed, and resuspended in PBS for flow cytometric analysis (FACSalibur, BD) [25].

Western Blot

Cells were lysed in 20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.1% Triton X-100 with protein inhibitors, homogenized, and incubated on ice for 1 h. After centrifugation, protein concentration was determined by Bio-Rad protein assay. Each cell lysate was separated on a 10% SDS-PAGE, and proteins were transferred electrophoretically to nitrocellulose membranes. The membranes were incubated in TNT 20 [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% v/v Tween 20] with 5% w/v nonfat dried milk for 1 h, washed, and incubated with TH antibody (Chemicon,1:1000) in TNT 20 containing 1% milk overnight at 4°C. After washing with TNT 20, membranes were incubated in biotin-conjugated secondary antibody (Vector Laboratories, 1:500) for 1 h at room temperature, washed, and then placed in avidin-biotinylated HRP complex solution (Vector, ABC kit, 1:1000) for 60 min. Immunoreactive proteins were detected using 0.02% DAB, 0.68% ammonium nickelous sulfate and 0.03% H_2O_2.

Reverse Transcription-PCR

Cultures were washed once with PBS before solubilization in Trizol (Life Technologies) and then stored at −80°C. RNA extraction was performed according to the recommendations of the manufacturer (Life Technologies). PCR conditions were optimized by varying $MgCl_2$ concentration and cycle number to determine linear amplification range. Amplification products were identified by size and confirmed by DNA sequencing. $MgCl_2$ concentration for TH was 2 mM and for HIF-1α was 1.5 mM. Primer sequences, cycle number, and annealing temperatures were as follows: HIF-1α, forward: 5′-GCGTGTGAGGAAACTTCT-3′, reverse: 5′-TGAGG TTGGTTACTGTTG G-3′, 29 cycles, 57°C, 476 bp; TH, forward: 5′-GTCTACTTCGTGTCTGAGA-3′, reverse: 5′-TATTGTGACGGTGATTGGG-3′, 28 circles, 57°C, 500 bp; β-actin, forward: 5′-GACCTGACGGACTACCTC A-3′, reverse: 5′-TAGAAGCACTTGCGGTGC-3′, 28 circles, 57°C, 578 bp; GAPDH, forward: 5′-CTCGTCT CATAGACAAGATGGTGAAG-3′, reverse: 5′-AGACTC CACGACATACTCAGCACC-3′, 28 circles, 58°C, 305 bp. Samples were run on a 2% agarose gel in 1× TAE buffer with 0.5 μg/ml of Ethidium Bromide at 100 volts. The gel was scanned under UV light in multi-image light cabinet (Alpha Innotech, CA).

High-Performance Liquid Chromatograph (HPLC) Analysis

DA content was measured according to previously described method [5]. Briefly, DA release was stimulated by Hank's Buffered Salt Solution (HBSS) containing 56 mM KCl for 15 min. Proteins in samples were precipitated with 0.4 M HCl, and then centrifuged at 2,500*g* for 10 min at 48°C followed by HPLC analysis. A Shimadzu solvent delivery system was used with the following mobile phase: 92% 75 mM $NaPO_4$, 1.7 mM octane sulfonic acid, 0.05 mM EDTA, pH 3.1, and 8% acetonitril, at a flow rate of 0.8% ml/min. An absorbosphere HS C18 reverse-phase column (10 × 4.6 mm, 3 μm) (Deerfield III; Alltech, USA) was connected to an electrochemical detector (Coulochem II; ESA Inc., USA). Results were normalized against DA standards at flow rates and sensitivities.

Statistical Analysis

Data shown were expressed as mean ± SD from data obtained in at least three independent experiments. Student's *t* tests were used to compare the effects of all

treatments. Differences were considered statistically significant as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Results

Hypoxia Promotes Neuronal Differentiation of P19 EC Cells

P19 EC cells differentiated into neuron-like cells after treatment with RA induction. These cells showed a typical neuronal morphology and exhibited characteristic markers of neurons, such as neuron-specific enolase (NSE, Fig. 1a), neuron-specific nuclear antigen (NeuN, Fig. 1b), and microtubule-associated protein 2 (MAP-2, Fig. 1c). In addition, whole-cell patch clamp recordings demonstrated that depolarizing voltage elicited sodium currents and potassium currents in those neuron-like cells (Fig.1d). These results suggested that neurons differentiated from P19 EC cells were neurons with mature morphology and electrophysiology.

Subsequently we investigated the effect of hypoxia on this RA induced neuronal differentiation. The percentage of differentiated neurons was examined by flow cytometer. Results showed that the percentage of neurons in differentiated P19 EC cells increased after exposed to hypoxia at RA induction stage as compared to the normoxic control (Fig. 1e). However, there were no difference in electrophysiological functions being detected between normoxia and hypoxia groups (data not shown). These results indicated that hypoxia affected the number, but not electrophysiological functions of differentiated neurons.

Hypoxia Increases the Percentage of Dopaminergic Neurons in Differentiated P19 EC Cells

We studied the effect of hypoxia on dopaminergic neuronal differentiation from P19 EC cells. Dopaminergic neurons were identified by immunoreactivity of TH. Data showed that in differentiated neuron-like cells, the percentage of TH-positive neurons in normoxia group were 0.72% on average; in contrast, the percentage of the TH-positive neurons in hypoxia group was strikingly elevated to 15.66% (Fig. 2a). It suggested that hypoxia has a significant effect on P19 EC cells to differentiate into dopaminergic neurons. To confirm this phenomenon, flow cytometric analysis of the percentage of TH-positive cells was used to re-examine this effect of hypoxia. In differentiated P19 EC cells, an average of 26.18% of cells were TH-positive in hypoxia group versus 3.78% in normoxia group (Fig. 2b). These results confirmed that hypoxia could promote P19 EC cells to differentiate into dopaminergic neurons.

Hypoxia Increases the Expressions of TH in Differentiated P19 EC Cells

We further examined TH mRNA and protein levels in differentiated P19 EC cells. Compared with normoxia, TH mRNA and protein levels in cells in hypoxia culture at the RA induction stage displayed higher levels (Fig. 2c–d). These results were consistent with the increase in percentage of TH-positive neurons in differentiated P19 EC cells after hypoxia treatment.

Hypoxia Enhances DA Release from Differentiated Dopaminergic Neurons

One important function of dopaminergic neurons is its DA release. Whether these dopaminergic neurons differentiated from P19 EC cells can release their DA, and whether hypoxia enhances this DA release were important to define the maturity of differentiated dopaminergic neurons. Using reverse-phase HPLC, we measured the DA released into the medium from those differentiated P19 EC cells. Data showed that in normoxia culture DA content in the medium was in a relatively lower level, while DA content was increased after cells exposed to hypoxia at RA induction stage (Fig. 2e). This result demonstrated that dopaminergic neurons differentiated from P19 EC cells have DA releasing function, and hypoxia could elevate this release.

Hypoxia Increases the Expression of HIF-1α in P19 EC Aggregations

To investigate the underlying mechanism of hypoxia effect on the differentiation of dopaminergic neurons, we examined HIF-1α mRNA level in P19 EC aggregations at RA induction stage. Compared with normoxia culture, HIF-1α mRNA level was significantly increased in hypoxic culture (Fig. 3).

Discussion

During embryogenesis, the natural progression of organogenesis involves hypoxia or "low O_2 levels"; due to the limited diffusion of oxygen (O_2) in the embryo shortly after gastrulation [2]. The nervous system is relatively earlier in development. The event related to early embryogenesis will inevitably affect neurodevelopment. How this hypoxia enviroment affects neuronal differentiation during early embryogenesis in the nervous system is still unknown. Although some scattered studies were reformed on and showed that hypoxia enhanced dopaminergic differentiation [3–5], since most cells including CNS precursors, neural stem cells, neural crest stem cells were isolated from later stage of embryogenesis, they could only reflect the

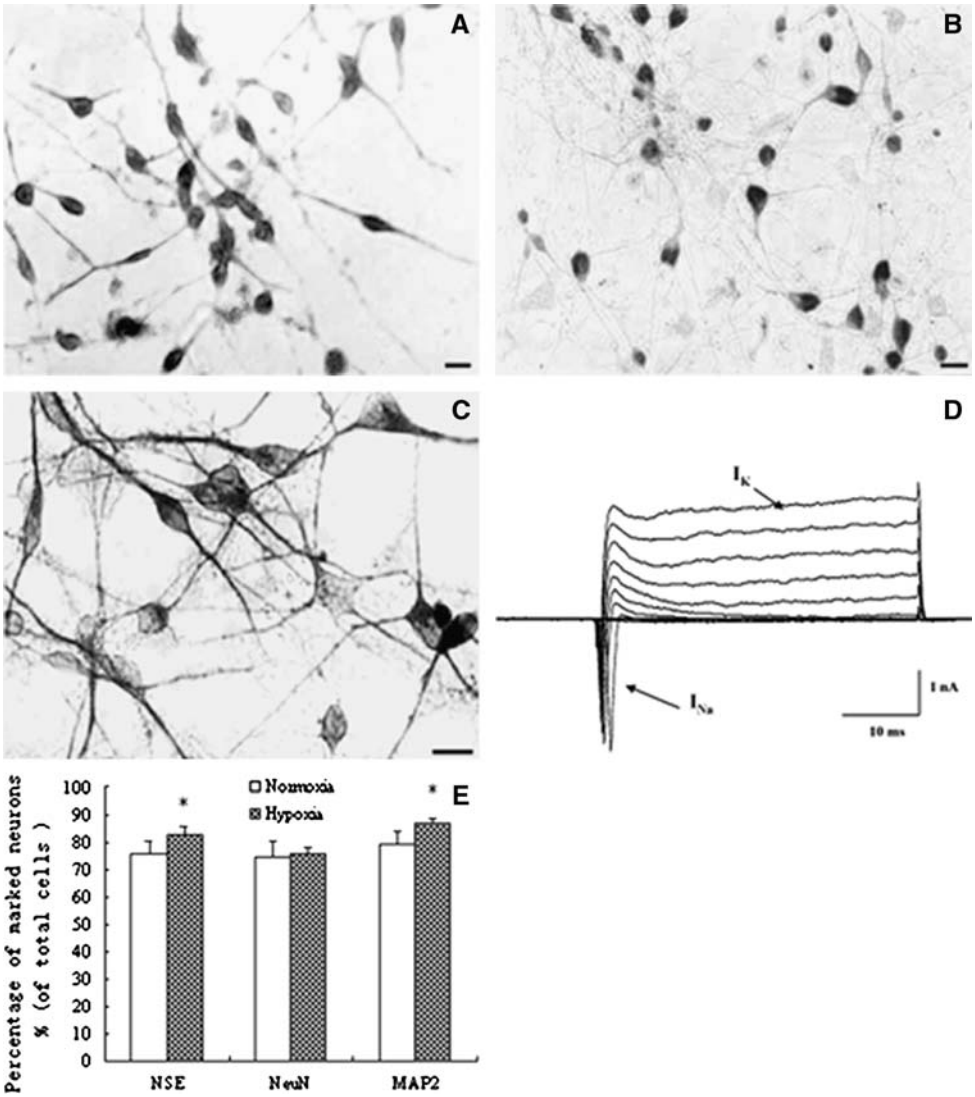

Fig. 1 Identification of neurons derived from P19 EC cells and effects of hypoxia in neuronal differentiation. P19 EC cells differentiated into neuron-like cells with 1 μM RA induction in normoxia culture. (**a**–**c**) NSE, NueN and MAP-2 immunostaining for mature neurons. Scale bar, 30 μm. (**d**) Recordings from whole-cell patch clamp were performed on P19-derived neuron-like cells. Neuron-like cells exhibited electrically evoked sodium and potassium currents when examined with voltage-clamp conditions. I_K, potassium currents; I_{Na}, sodium currents. (**e**) Flow cytometric analysis of percentage of marked neurons after P19 EC cells were exposed to hypoxia. Cells were collected 8 days after plating, and then incubated with respective primary antibody, washed, and resuspended in PBS containing FITC-conjugated secondary antibody. Finally, the cells were resuspended in PBS for flow cytometric analysis. At least 10,000 cells were counted for each sample. Data were expressed in mean ± SD of duplicate measurements from three independent experiments. $^*P < 0.05$ compared with normoxia

physiology at a relatively later developmental stage of nervous system.

In the present study, we investigated the role of hypoxia in an earlier neuronal differentiation stage using P19 EC cell model system. First, we examined the effect of hypoxia on neuronal differentiation. The results showed that hypoxia could increase the percentage of differentiated neurons, while electrophysiologic functions of these differentiated neurons did not change. It suggested that hypoxia did not impede neuronal differentiation; instead, it facilitated neuronal differentiation.

Subsequently we examined the effect of hypoxia on neuronal subtype differentiation. Several neuronal subtypes could be differentiated from P19 EC cells, for example: AChergic neurons, GABAergic neurons, and serotonergic neurons [7]. Hypoxia did not show any significant effects on the differentiation of these neuronal subtypes (data not shown). Interestingly, the number of dopaminergic neurons differentiated from P19 EC cells remarkably increased after hypoxia treatment. We verified the role of hypoxia in differentiation of dopaminergic neurons by FACS. The data confirmed that the percentage of dopaminergic neurons in hypoxia group was at least 7-fold higher than in normoxia group. Therefore, we confirmed that dopaminergic neuron was one of earlier phenotypes among all neuronal subtypes differentiated from P19 EC cells and required hypoxia for its induction.

We measured the change of TH mRNA and protein levels to confirm the characteristics of these dopaminergic neurons. TH is the key enzyme for DA synthesis and usually served as a marker for mature dopaminergic neurons [22, 23]. In addition, TH is a hypoxia-inducible gene, which has been shown in hypoxic PC12 cells [28, 29]. In our study, both TH mRNA and protein levels were

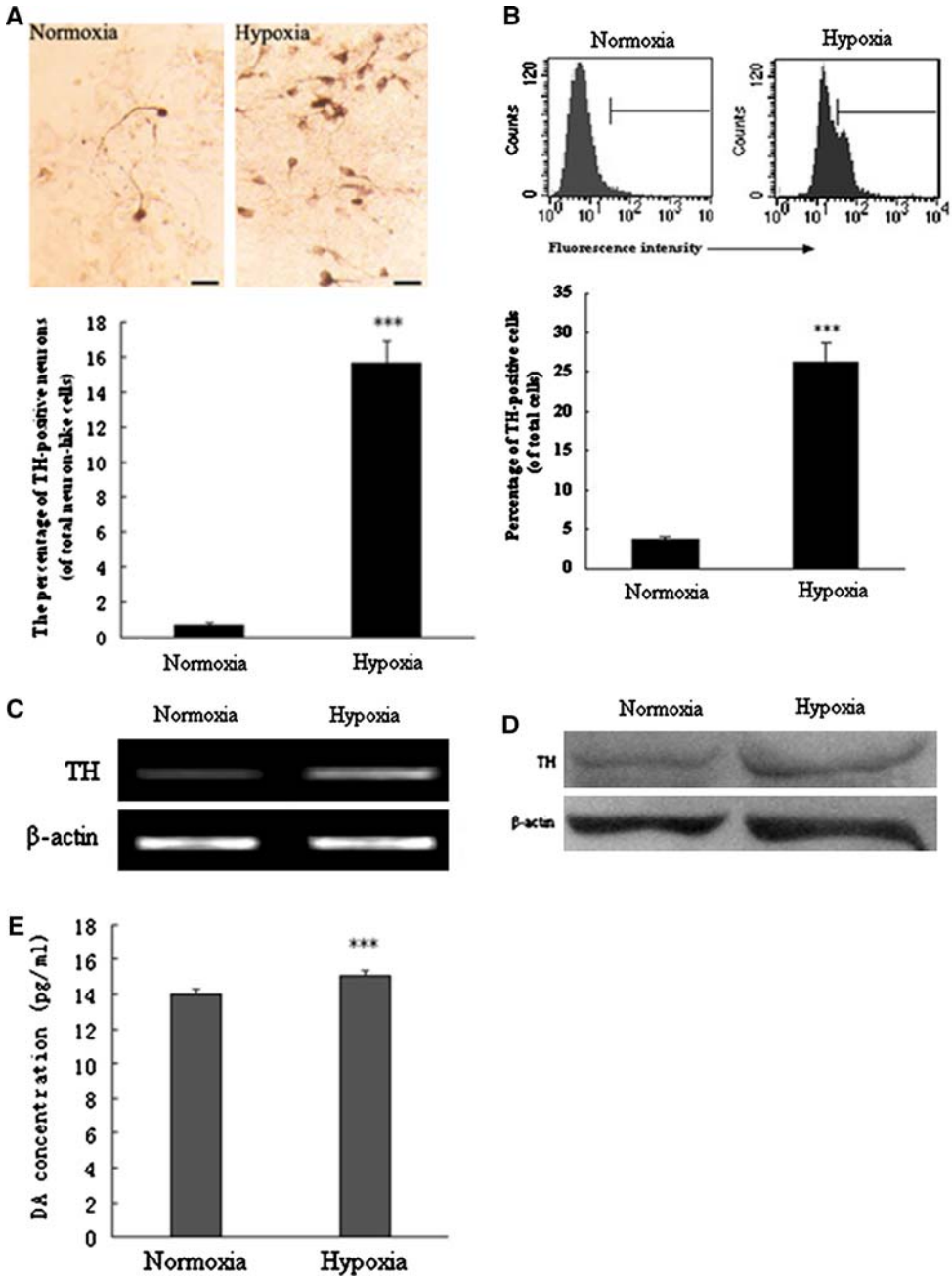

Fig. 2 Effects of hypoxia on differentiation of dopaminergic neurons. (**a**) Immunostaining of TH. Cells were stained for the dopaminergic neuronal marker TH at day 8 post differentiation. The percentage of TH-positive neurons showed a remarkable increase in hypoxia as compared to normoxia. At least 1,500 neuron-like cells were counted in each experiment. Data were presented as mean ± SD of triplicate measurements from 3 independent experiments. ***$P < 0.001$ compared with normoxia. Scale bar, 20 μm. (**b**) Flow cytometric analysis of percentage of TH-positive cells. Cells were collected and incubated in primary antibody, then resuspended in PBS containing FITC-conjugated secondary antibody, washed, and resuspended in PBS for flow cytometric analysis. At least 10,000 cells were counted for each sample. Data are expressed in mean ± SD of triplicate measurements from 3 separate experiments. ***$P < 0.001$ compared with normoxia. The bar indicates the range of TH-positive cells. (**c**) Reverse transcription-PCR analysis of TH mRNA levels at day 8 post differentiation. A significant increase in expression level of TH was measured in hypoxia group. (**d**) Western blot showing expression of TH protein. Cultures were harvested at day 8 post differentiation. Western blot analysis revealed significantly higher TH proteins in samples from hypoxia versus normoxia group. E, DA content in the culture medium was detected by HPLC. The DA content under hypoxia showed a significant increase as compared with normoxia. Data were expressed as mean ± SD of duplicate measurements from 3 independent experiments. ***$P < 0.01$ compared with normoxia

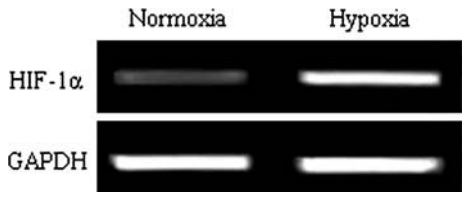

Fig. 3 Effects of hypoxia on HIF-1α mRNA levels. The level of HIF-1α mRNA was estimated by RT-PCR in cultures after 4 days of RA induction. A significant increase was detected in the hypoxia group

increased after exposure to hypoxia. These results further confirmed that dopaminergic neurons were differentiated in an early stage of embryogenesis and required hypoxia.

DA release is one most important function of mature dopaminergic neurons. Whether this dopaminergic characteristics could also be affected by hypoxia is not clear. We measured DA concentration in differentiated P19 EC cells medium using reverse-phase HPLC. The data showed that DA content in hypoxia was higher than normoxia group. The increase in DA content in culture medium might be released from the increase in dopaminergic neurons differentiated from P19 EC cells under hypoxia.

In order to investigate the underlying mechanism of hypoxia-induced dopaminergic neuronal differentiation, we tested the HIF-1α mRNA level in the similar model. HIF-1α is one of subunits of HIF-1, which is a heterodimer composed of HIF-1α and HIF-1β [also called the aryl hydrocarbon nuclear translocator (Arnt)]. HIF-1α is the hypoxically responsive component of this complex, while HIF-1β is expressed constitutively [17, 30]. Under normoxic condition, HIF-1α is rapidly ubiquitinated and degraded through a proteasomal pathway [31, 32]. Under hypoxic conditions, oxygen becomes rate limiting for prolyl hydroxylation, resulting in decreased ubiquitination of HIF-1α and thus HIF-1α is stabilized. Stabilized HIF-1α in hypoxic conditions is translocated to the nucleus to bind with HIF-1β. The heterodimer then binds to hypoxia-responsive element (HRE) in the promoter regions of hypoxia-induced genes and promotes the transcriptions of these genes [30, 31, 33, 34]. In the present study, HIF-1α expression in mRNA level was detected with RT-PCR. HIF-1α mRNA level was significantly increased in hypoxia. This matches well with the increase in TH expression and the enhancement in dopaminergic characteristics in this P19 EC model under hypoxia.

HIF-1α is known to be an important factor regulating TH gene expression through binding to its promoter [16, 17, 19]. Previous studies reported that increased transcription of the TH gene during hypoxia was regulated by a region of the proximal promoter, and this region of the gene contained a number of *cis*-acting regulatory elements including HIF-1 [35, 36]. Although there is no direct relationship being identified between HIF-1 and TH in this study, we can easily interpret that hypoxia increases both HIF-1α and TH expressions to promote the differentiation of dopaminergic neurons from P19 EC cells.

Taken together, these results suggest that hypoxia is essential in differentiation of dopaminergic neurons in early neurodevelopment. Hypoxia exerts this effect through HIF-1α to facilitate TH expression. Therefore, hypoxia in early neurodevelopment contributes to DAergic neuronal differentiation in early embryogenesis.

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