Characteristics of Neural Stem Cells Expanded in Lowered Oxygen and the Potential Role of Hypoxia-Inducible Factor-1Alpha

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Key Words

Oxygen/hypoxia, lowered • Neural stem cells • Hypoxia-inducible factor-1 • Differentiation

Abstract

It has recently been reported that hypoxia promotes the survival and proliferation of neural stem cells (NSCs). In the present study, we examine the differentiation ability of neural precursors expanded under lowered oxygen conditions, and the potential role of hypoxia-inducible factor (HIF)-1 α in vitro, which is the key molecule in response to lowered oxygen. The NSCs were cultured in a 3% O₂ environment for 3 days, and differentiated with 1% fetal bovine serum (FBS) for another 5–7 days, and the cell lineage was evaluated by immunohistochemistry, flow cytometry and HPLC. Compared with the normal condition, the NSCs cultured in hypoxia (3% O₂) displayed an increase in the percentage of neurons. Especially the percentage of TH-positive neurons differentiated from NSCs in lowered oxygen increased significantly; the dopamine (DA) content in the medium was higher than under normal conditions. These data indicate that lowered oxygen favors dopaminergic differentiation. We then examined the expression of HIF-1 α during differentiation of NSCs. The levels of HIF-1 α mRNA expression under 3% oxygen did not change as compared with those under normal conditions. However, HIF-1 α protein expression was higher from 3 to 72 h during hypoxia than under normal conditions. Overexpression of HIF-1 α significantly increased the number of

TH-positive cells and the DA content in culture medium under normal conditions. These results suggest that HIF-1 α is involved in the regulation of dopaminergic differentiation of NSCs in lowered oxygen. This study may also offer a new approach to yield DA neurons using a physical factor.

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Introduction

Hypoxia can regulate cellular proliferation and differentiation. In recent years, some reports have described the salutary effects of mild hypoxia on neural progenitors [1, 2] or neurons [3, 4]. However, the effects of hypoxia on stem cell characteristics remain to be fully understood. Morrison et al. [1] and Studer et al. [2] reported that oxygen lowered to more physiological levels (3%) provides marked trophic and proliferative effects on neural precursors and significantly changes developmental kinetics and outcome compared with traditional culture conditions (20%) [5]. Under hypoxic conditions, the differentiation of embryonic stem (ES) cells as well as precursor cells is inhibited [6–8]. Yun et al. [7, 8] demonstrate that, under hypoxic conditions, pre-adipocytes treated with adipogenic hormones maintain their precursor phenotype and can fully commit to adipogenic differentiation upon returning to normoxia. They have also recently demonstrated that hypoxia reversibly arrests pre-adipocytes in an undifferentiated state [9]. We have also found

that hypoxia is capable of inducing the dopamine (DA) phenotype of bone marrow-derived mesenchymal stem cells and P19 cells [10, 11]. These observations indicate that hypoxia plays a critical role in the maintenance and multi-lineage ability of the stem cell phenotype.

The cellular response to hypoxia is manifested by activation of the hypoxia-inducible factor-1 (HIF-1), a transcription factor of the basic helix loop-helix *Per*, *AhR* and *Sim* (bHLH-PAS) family [12]. HIF-1 consists of the O₂-regulated HIF-1 α subunit and the O₂-independent HIF-1 β subunit. Under hypoxic conditions, HIF-1 α is no longer hydroxylated and thus becomes stabilized. HIF-1 α activation results in increased expression of several key targeted genes such as erythropoietin, vascular endothelial growth factor, tyrosine hydroxylase (TH) and OCT4, which function as regulators of cellular proliferation and differentiation [13, 21, 22]. But the exact role of HIF-1 α in maintaining stem cells remains to be discovered.

In the present study, we investigated the effects of 3% oxygen on the characteristics of neural stem cells (NSCs) expanded under hypoxic conditions. To elucidate the role of HIF-1 α in the fate of hypoxia-induced NSCs, we examined the expression of HIF-1 α during differentiation induced by hypoxia, and the effect of HIF-1 α overexpression on the differentiation of NSCs.

Materials and Methods

Isolation and Culture of NSCs

Mesencephalon cells were dissected from Wistar rats on embryonic day 13.5 (E13.5) and mechanically dissociated. They were grown in DMEM/F12 (1:1) medium containing 2 mM L-glutamine, 5 IU penicillin, 5 μ g/ml streptomycin (BioWhittaker), 1% N2 (Life Technologies), 1% B27 (Life Technologies), 20 ng/ml EGF (Sigma) and 20 ng/ml bFGF (Invitrogen).

Condition of Hypoxia

For decreased oxygen cultures, an incubator (Thermo model 3110), which is adjustable for different oxygen concentrations, was used. The incubator was flushed with air containing 5% CO₂ and balanced with N₂. The actual concentrations inside the incubator were 3 or 10% oxygen measured with a microelectrode (Animus Corp., Malvern, Pa., USA). The time of hypoxia was calculated from the measurement indicating the desired oxygen concentration.

Differentiation and Immunohistochemistry

For differentiation of NSCs, the 3rd to 5th generations of NSCs were grown in the proliferation medium and divided into 2 groups. Then these NSCs were expanded either under hypoxia (3 and 10% O₂) or normal (20% O₂) conditions for the differentiation study, and the neural spheres were dissociated with 0.25% trypsinase after 3 days of culture. The cells were counted and planted on 35-mm dishes coated with polylysine at the same den-

sity (5 $\times$ 10⁴/ml), and differentiated by 1% FBS for another 5–7 days under normal conditions. The subtype of NeuN-positive cells was tested by immunocytochemistry.

For immunocytochemistry, the cells were fixed with 4% paraformaldehyde at 4°C for 2 h after 5–7 days of differentiation. Then the cells were pretreated with 5% of NHS to block the nonspecific binding and incubated with a mouse monoclonal antibody against NeuN (Molecular Probe, dilution 1:5,000) for 24 h at 4°C. After washing in 0.1 M phosphate buffer, the cells were then incubated with biotinylated anti-mouse IgG (Vector Laboratories, diluted 1:1,000) at 4°C overnight. NeuN immunoreactivity was visualized as a black nuclear precipitate using a nickel-intensified 3,3'-diaminobenzidine procedure [14]. The numbers of NeuN-positive cells and total cells were counted double-blindly (25 fields per dish) under 200 $\times$ magnification using an electron microscope (Leica DC300). The analyses were repeated 4 times.

Flow Cytometric Analysis

Cells were treated with 0.25% trypsinase and collected in 15-ml 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 (TH, dilution 1:500) in PBS for 1 h, washed with PBST, and resuspended in 100 μ l PBS containing fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Santa Cruz, dilution 1:50). The cells were stained with secondary antibody for 60 min on ice, and assayed in PBS for flow cytometric analysis (FACSibur, BD). The percentage of TH-positive cells was calculated.

High-Performance Liquid Chromatography Analysis

DA release was stimulated in HBSS containing 56 mM KCl for 15 min of incubation as described previously [14]. In brief, at the appropriate medium change, a sample of the culture medium was taken. Protein in the samples was precipitated with 0.4 M HCl, and then the sample was 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 NaPO₄, 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 Alltech (Deerfield, Ill., USA) Absorbosphere HS C18 reverse phase column (10 $\times$ 4.6 mm, 3 μ m) was connected to an electrochemical detector (Coulchem II; ESA Inc., Chelmsford, Mass., USA). Results were normalized against DA standards at varying flow rates and sensitivity.

RNA Extraction and 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 the MgCl₂ concentration and cycle number to determine the linear amplification range. Amplification products were identified by size and confirmed by DNA sequencing. The MgCl₂ concentration was 1.5 mM for HIF-1 α . The program for PCR was as follows: primers for HIF-1 α 5'-TGCTTGGTGCTGATTTGTGA-3', 5'-GGTCAGATGATCAGAGTCCA-3' were used to yield a 209-bp product for 30 cycles at 58°C; and m18 SRNA primer: forward 5'-TTATGGTTCCTTTGGTCGCT-3', reverse 5'-ATGTGGTAGCCGTTTCTCAG-3', was used to yield a 355-bp product for 30 cycles at 56°C.

Fig. 1. Lowered oxygen increases the percentage of NeuN-positive cells. **a** Representative photograph of NeuN-positive cells differentiated from NSCs under normal conditions. **b** Representative photograph of NeuN-positive cells differentiated from NSCs under the 3% oxygen condition. Scale bar = 252 μ m. Black granules are NeuN-positive cells. **c** Diagram showing the percentage of neurons. Each bar represents the mean $\pm$ SD ($n = 4$). * $p < 0.05$, as compared with control.

Protein Extraction and Western Blot

Cells were harvested quickly after either hypoxic or normoxic culture for the desired times and the total protein was extracted with lyses buffer, which contained 100 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2% (v/v) Tween-20, 0.4% NP-40, 20% glycerol, supplemented with protease inhibitors (1 μ g/ml leupeptin and pepstatin, 2 μ g/ml aprotinin and 1 mM PMSF) and phosphatase inhibitors (10 mM NaF and 1 mM Na₃VO₄). Then Western blot analyses were carried out. Extracts were quantified using a protein assay kit (Bio-Rad) and fractionated by 6% polyacrylamide-SDS gel electrophoresis and transferred to a polyvinylpyrrolidone difluoride membrane (Immobilon-P, Millipore, Bedford, Mass., USA). The membrane was blocked with Tris-buffered saline (TBS-T) containing 5% dry milk at room temperature for 2 h. Membranes were incubated with anti-HIF-1 α mouse monoclonal antibody (Chemicon, dilution 1:500) in TBS-T containing 5% nonfat dry milk. Membranes were treated with goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Santa Cruz; dilution 1:1,000) in TBS-T containing 5% nonfat dry milk. Immune complexes were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences).

Construction of Recombinant Adenoviral Vector Ad/HIF-1 α and Adenovirus Infection Assay

The recombinant adenoviral vector overexpressing the human HIF-1 α gene was a kind gift from Prof. Tang Hong (Institute of Microbiology, Chinese Academy of Science). The AdEasy system was used to generate recombinant adenovirus. The complete cDNA of human HIF-1 α was 3,720 bp in length, containing a 2,478 bp open reading frame and a 1,242 bp 5'- and 3'-untranslated region. An open reading frame of 2,478 bp which encoded a sequence of 826 amino acid residues was constructed into the recombinant adenoviral vector. The successfully recombinant adenoviral vector, digested with *PacI* to linearize the plasmid and ethanol precipitated, was used for transfection into HEK293 cells. The recombinant virus produced in HEK293 cells could then be further purified and tittered.

For the adenovirus infection assay, neurospheres were dissociated into single cells before infection with adenovirus. After 2-hour incubation, the virus-containing medium was replaced by fresh complete growth medium. Then, the NSCs were cultured for 72 h, and the expression of HIF-1 α was measured. The modified constructs contained HIF-1 α coupled to green fluorescent protein (GFP) in separate expression cassettes. The infection rate

with the adenovirus was determined by the percentage of GFP-positive cells detected by flow cytometer.

Statistical Analysis

All the experiments were repeated at least three times, unless otherwise indicated. Data are presented as mean $\pm$ SD. Statistical analysis was performed by t test. A statistical probability of $p < 0.05$ was considered significant.

Results

Lowered Oxygen Promotes the Neuronal Differentiation of NSCs

We have reported that lowered oxygen (10% O₂ and 3% O₂) promoted the proliferation of NSCs in vitro [10]; in the present study the differentiation ability of NSCs cultured under the lowered oxygen conditions was investigated. The NSCs were expanded under either lowered or 20% O₂ conditions for 3 days, and differentiated by 1% FBS without EGF and bFGF for another 5–7 days under normal conditions. The cell subtype was tested by immunocytochemistry. We found that the percentage of NeuN-positive cells in hypoxia increased significantly as compared with the normal group. These results show that lowered oxygen (3% O₂) markedly increases the yield of neurons (fig. 1).

Lowered Oxygen Increases the Yield of TH-Positive Neurons and DA Content

We then examined the effect of lowered oxygen on the differentiation of dopaminergic neurons. The neural spheres expanded in either lowered or 20% O₂ and were then divided for differentiation. Precursors in lowered oxygen (3% O₂) resulted in $19.88 \pm 1.44\%$ TH-positive cells, significantly higher than those in 20% O₂ ($10.25 \pm 1.03\%$, $n = 6$, $p < 0.01$; fig. 2a–c). Furthermore, the DA content of the medium in the lowered oxygen (3% O₂) group increased onefold compared with the control group

Fig. 2. Lowered oxygen increased the yield of TH-positive neurons and DA content. **a** Representative photograph of TH and Tuj1 double-labeled cells differentiated from NSCs under normal conditions. **b** Representative photograph of TH and Tuj1 double-labeled cells differentiated from NSCs under the 3% O₂ condition. Scale bar = 252 μm. **c** Diagram showing the number of TH-positive neurons. **d** Lowered oxygen increased the DA yield in supernatant detected by HPLC. Each bar represents the mean ± SD (n = 4). ** p < 0.01 as compared with control.

(fig. 2d). However, there was no significant difference in both TH-positive cells and DA content between the 10% O₂ and control groups. These data suggest that NSCs under 3% oxygen tension increased the yield of TH-positive neurons and DA content.

Effect of Lowered Oxygen on the Expression of HIF-1α during Differentiation of NSCs

We next investigated the expression of HIF-1α during differentiation of NSCs. NSCs were exposed to 20% O₂ or lowered oxygen (3 and 10% O₂) for 3 days, expression of HIF-1α protein increased under the 3% O₂ condition compared with that under 10% O₂ and the control group, and weak expression of HIF-1α protein could be detected under 10% O₂ (fig. 3a, b). Expression of HIF-1α was then assayed by RT-PCR and Western blot, after the NSCs had been exposed to 20% O₂ or lowered oxygen (3% O₂) for 1, 3, 6, 12, 24, 48 and 72 h. The data show that HIF-1α mRNA was constitutively expressed under both the lowered oxygen and normal conditions (fig. 3c, d). There was no difference in the levels of HIF-1α mRNA expression between control (20% O₂) and hypoxia (3% O₂). Lowered

oxygen had no effect on the expression of HIF-1α mRNA during differentiation of NSCs. However, a strong signal was observed in the groups with lowered oxygen (3% O₂) for 3 h which lasted for 3 days, whereas no HIF-1α protein could be detected in the control. This indicates that HIF-1α might be involved in the differentiation of NSCs. We further investigated whether overexpression of HIF-1 protein by Ad/HIF-1α could mimic the effects of lowered oxygen on the differentiation of NSCs under normal conditions.

HIF-1α Is Involved in Differentiation of NSCs into DA Neurons

Infection Rate of Cells Differentiating from NSCs Infected with Ad/HIF-1α

The dissociated neural spheres were infected with Ad/HIF-1α or empty virus (MOI = 50), and cultured under either lowered oxygen (3% O₂) or normal conditions for 3 days. Neurospheres were replanted in the 35-mm dishes and differentiated for another 5 days under normal conditions. The efficiency of infection with adenovirus was calculated with the percentage of GFP-positive cells, and up to 85% GFP-positive cells are shown in figure 4a and b. The expression of HIF-1α protein in neural precursors was assayed by Western blot after infection. Overexpression of HIF-1 in NSCs under either 20 or 3% O₂ conditions was detected after infection with Ad/HIF-1α, as compared with the control, respectively (fig. 4c).

Overexpression of HIF-1α Could Mimic the Effect of Lowered Oxygen on the Differentiation of NSCs under Normal Conditions

We further examined whether overexpression of HIF-1α could enhance the differentiation of NSCs into TH-positive neurons. Under normal conditions, overexpression of HIF-1α could also increase the percentage of TH-positive cells, and increased about onefold as compared with the control. The percentage of TH-positive cells in the Ad/HIF-1α infection under normal conditions had a twofold increase compared with the control. In addition, the percentage of TH-positive cells in Ad/HIF-1α infection under the lowered oxygen condition also had a twofold increase as compared with control without Ad/HIF-1α infection (fig. 5). DA production by these neurons was confirmed by reverse-phase HPLC, which showed significantly increased levels of DA in hypoxia versus 20% O₂ cultures (fig. 6); conditioned medium showed a twofold increase in DA content (n = 5, p < 0.01). These results demonstrate that overexpression of HIF-1α could mimic

Fig. 3. Effects of lowered oxygen on the expression of HIF-1α in NSCs. **a** Representative photograph of the expression of HIF-1α protein detected by Western blot in NSCs at 3% oxygen, 10% oxygen and normal conditions. **b** Semiquantitative analysis of the expression of HIF-1α protein. Lowered oxygen (3% O₂) increased the levels of HIF-1α expression compared with the 10% oxygen and control groups. **c** Representative photograph of the expression of HIF-1α mRNA detected by RT-PCR. Cells were exposed to 20 or 3% O₂ for different periods of time. At the desired times, the cells were collected and RT-PCR analysis was performed on total RNA. **d** Semiquantitative analysis of the expression of HIF-

1α mRNA. There was no difference in the levels of HIF-1α mRNA expression between control (20% O₂) and hypoxia (3% O₂). **e** Representative photograph of the expression of HIF-1α protein detected by Western blot. Cells were exposed to normoxia (C = 20% O₂) or hypoxia (H = 3% O₂) for 1, 3, 6, 12, 24, 48 and 72 h. Whole cell extracts (100 μg) were analyzed by Western blot. A strong signal was observed in the hypoxia (H) groups, whereas no HIF-1α protein could be detected in the control (C). **f** Semiquantitative analysis of the expression of HIF-1α protein. Hypoxia (3%) increased the expression of HIF-1α protein. The expression of HIF-1α was normalized to that of 18S RNA and β-actin (n = 3).

Fig. 4. Infection rate of cells differentiating from NSCs infected with Ad/HIF-1α. **a** Nucleus stained with DAPI. **b** Cells marked by GFP. Up to 85% of NSCs are GFP-positive cells. Scale bar = 200 μm. **c** Expression of HIF-1α protein was detected in neural precursors by Western blot. NSCs infected with Ad/HIF-1α or empty vector were exposed to 20 or 3% O₂ for 3 days. Expression of HIF-1α after infection with Ad/HIF-1α was higher than that in the control group.

Fig. 5. Overexpression of HIF-1α promotes the differentiation of NSCs into TH-positive cells and DA content. **a** Cells double marked with GFP and PE detected by FACS. **b** Diagram of FACS. The percentage of TH-positive cells under normal conditions after infection with Ad/HIF-1α was the same as under hypoxic conditions. Each bar represents the mean ± SD (n = 4). ** p < 0.01 compared with control; ## p < 0.01 compared with hypoxia (3% O₂).

Fig. 6. Overexpression of HIF-1 α increased the DA yield in the supernatant. The content of DA in the supernatant was detected by HPLC. The DA content under normal conditions after infection with Ad/HIF-1 α increased significantly as compared with this in the empty vector group. Each bar represents the mean $\pm$ SD ($n = 4$). ** $p < 0.01$ as compared with control; ## $p < 0.01$ as compared with hypoxia (3% O₂).

the effect of lowered oxygen (3% O₂) on the differentiation of NSCs, and HIF-1 α is involved in the production of DA neurons.

Discussion

In the present study, we identified the differentiation ability of NSCs expanded under lowered oxygen conditions, and found that these NSCs could differentiate into more neurons, especially more TH-positive cells and higher DA yield in the medium. Overexpression of HIF-1 α could mimic the effect of lowered oxygen on the differentiation of NSCs. These results demonstrate that 3% oxygen tension modifies the multi-lineage ability of the NSCs, and HIF-1 α is involved in the production of DA neurons.

The effects of lowered, more physiological O₂ on the proliferation of stem cells are not limited to the CNS and extend to the peripheral nervous system [1, 2, 5] and to non-neuronal tissues [5, 15, 16]. Hypoxia favors the TH-positive cells of several kinds of stem cells, such as P19

cells, MSCs, ES cells [5, 11, 17], this provides a powerful tool for the generation of specific neuron types for clinic application. In particular, lowered O₂ culturing has the practical effect of contributing to more efficient production of dopaminergic neurons for potential transplantation therapies.

Expression of the HIF-1 α mRNA and Protein in NSCs Induced by Hypoxia

It has been widely shown that HIF-1 α is activated in various cells under hypoxic conditions, and HIF-1 α -targeted genes play an important role in maintaining cellular homeostasis in response to hypoxia [12, 13, 18, 19]. However, study on the expression of HIF-1 α mRNA and protein in stem cells is limited. The mechanism of HIF-1 α stabilization is regulated depending on the diversity of cell phenotypes or tissues.

It has been reported that HIF-1 α mRNA was ubiquitously expressed in the tissue and various cells; however, the HIF-1 α protein was soon degraded under normal conditions [20]. HIF-1 α is activated in ES cells under hypoxic conditions (1% O₂), and most of the HIF-1 α target gene expression is reduced in HIF-1 α ^{-/-} ES cells under hypoxia [22]. In our study, the results show that the expression of HIF-1 α mRNA in NSCs did not change 6–72 h after exposure to lowered oxygen (fig. 3c, d). Consistent with this, HIF-1 α mRNA is constantly expressed in muscle cells regardless of the culture conditions [7].

It is known that hypoxia induces the transcription of HIF-1 α mRNA that increases HIF-1 α protein in the presence of continued hypoxia. The activity of HIF-1 α is regulated mainly on the post-transcription level [20]. In normoxia, HIF-1 α is constitutively synthesized and sent to destruction by the ubiquitin-proteasome pathway (half-life <5 min). So HIF-1 α protein is absent or nearly absent in most normoxic cells. Consistent with this, we did not detect the expression of HIF-1 α protein in NSCs in normoxia. With the onset of lowered oxygen, we found that the level of HIF-1 α protein in NSCs was highly expressed. Therefore, these data suggest that the NSCs expanded under hypoxia highly express the HIF-1 α protein, and this may be a critical mechanism by which hypoxia regulates the fate of NSCs.

HIF-1 α Is Involved in DA Differentiation of NSC Induced by Hypoxia

In this study, we show that the differentiation ability of NSCs under hypoxia has been modified, which results in NSCs differentiating into more TH-positive cells. Precursors in hypoxia resulted in $19.88 \pm 1.44\%$ TH-posi-

tive cells, significantly higher than those in 20% O₂ (10.25 ± 1.03%, n = 6, p < 0.01; fig. 2). However, Studer et al. [2] treated NSCs with 3% oxygen and induced 56% dopaminergic phenotype differentiation. The main discrepancy as compared with our study is that they added BMP2 to the differentiation medium, while it is known that BMP2 could promote the formation of neurons. The above results indicate that exposure to hypoxia confined to the differentiation phase does not significantly increase the percentage of neurons expressing TH of NSCs in vitro. This suggests the important effects of hypoxia in promoting dopaminergic differentiation in the proliferation phase but not in the differentiation phase. Because HIF-1α is highly expressed in the NSCs expanded under hypoxic conditions, we investigated the role of HIF-1α in the regulation of the NSCs phenotype. We used Ad/virus HIF-1α to specifically upregulate the expression of HIF-1α protein, and the results showed that NSCs are able to

undergo dopaminergic differentiation under normal conditions when HIF-1α is overexpressed by Ad/virus HIF-1α. Importantly, overexpression of HIF-1α could partially mimic the effects of hypoxia on the DA neuron yield derived from NSCs.

Taken together, we have demonstrated for the first time that the HIF-1-signaling pathway is activated in NSCs by lowered oxygen and plays an important role in hypoxia-induced NSCs differentiation. This indicates that the differentiation ability of NSCs is determined during the expansion of NSC under lowered oxygen.

Acknowledgments

This work was supported by the National Basic Research Program of China (No. 2006CB504100) and a key grant from the Nature and Sciences Foundation of China (No. 30393130).

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