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Intravenous Transplantation of Human Umbilical Cord Blood Mononuclear Cells Overexpressing Nerve Growth Factor Improves Spatial Memory in APP/PS1 Transgenic Mice with a Model of Alzheimer's Disease

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Abstract
Alzheimer's disease is a progressive incurable neurodegenerative disease manifested by dementia and other cognitive disorders. Gene-cell therapy is one of the most promising trends in the development of treatment for Alzheimer's disease. The study was aimed to evaluate the therapeutic potential of intravenous transplantation of human umbilical cord blood mononuclear cells (UCBMCs) transduced with adenoviral vectors overexpressing nerve growth factor (NGF) for the treatment of Alzheimer's disease in an APP/PS1 transgenic mice model. The transplantation of NGF-expressing UCBMCs was found to improve spatial memory and decrease anxiety in APP/PS1 mice. Grafted cells and their expression of NGF were detected in the cortex and hippocampus of transgenic mice in the period up to 90 days after transplantation. Thus, gene-cell therapy based on the use of NGF-overexpressing UCBMCs is a promising approach for the development of Alzheimer's disease treatments.

Keywords Alzheimer's disease · Nerve growth factor · Stem cells · Gene-cell therapy · APP/PS1 transgenic mice · Umbilical cord blood mononuclear cells

1 Introduction

Alzheimer's disease is a progressive incurable neurodegenerative disease manifested by dementia and other cognitive disorders. Excessive production and accumulation of neurotoxic β-amyloid peptide in nervous and other tissues is thought by most of researchers to be a key factor in Alzheimer's disease pathogenesis [1–3]. To date, the considerable experience in the search and use of various drugs for treatment of Alzheimer's disease is obtained; however, there is still no effective cure available. Thus, the development of novel therapeutic approaches to treat Alzheimer's disease is one of the most important goals for medical science.

The use of umbilical cord blood mononuclear cells (UCBMCs) to deliver various neurotrophic factors into the sites of neurodegeneration is a promising approach in the development of therapy for neurodegenerative diseases [4–8]. A nerve growth factor (NGF), a protein of the family of neurotrophins which is involved in the maintenance of survival, stimulation of growth, and activity of neurons, has a significant therapeutic potential in this regard [9, 10]. NGF was shown to prevent neuronal death in a number of models of neurodegenerative disorders [11–14]. NGF has a positive effect on neuronal survival, synaptic function, and memory in models of Alzheimer's disease [7]. A recombinant NGF gene can be delivered into sites of neurodegeneration using gene-cell constructs.

This study is aimed to transplant UCBMCs transduced with adenoviral vectors expressing NGF to transgenic mice with a model of Alzheimer's disease (APP/PS1 line) with subsequent evaluation of mice behavior and the ability of grafted cells for homing, survival, and expression of a therapeutic gene in the brain.

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2 Materials and Methods

2.1 Construction of Recombinant Adenoviruses Expressing Nerve Growth Factor and Enhanced Green Fluorescent Protein

Plasmid vector pDONR-NGFb was kindly given by Mehmet E. Yalvac (Yeditepe University, Turkey). Plasmid vector pDONR-EGFP was obtained from Addgene (USA). Using the Gateway cloning technology, we have performed reaction of recombination catalyzed with LR clonase enzyme mix II (Life technologies, USA) into adenoviral vector pAd/CMV/V5-Dest. Recombination mixture was further transformed into *Escherichia coli* TOP10. Correct insertion of interest (450 bp for NGF and 700 bp for EGFP) was confirmed by restriction analysis and sequencing. Preparative plasmid DNA isolation was performed using QIAFilter plasmid midiprep kit (QIAGEN, USA) according to producer's recommendations.

2.2 Transfection of HEK293A Cells with Recombinant Plasmids

HEK293A cell line (Invitrogen, USA) was cultured at 37 °C in a humidified atmosphere containing 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS, HyClone, EU), 1% antibiotic mixture of penicillin and streptomycin, and 2 mM L-glutamine. Transfection of HEK293 cell lines with genetic constructs (pAd-EGFP, pAd-NGF) was carried out using the transfection reagent TurboFect (Fermentas Inc., Canada) according to the procedure recommended by the manufacturer. Expression of recombinant genes was confirmed 48 h after transfection by immunofluorescent analysis. Intensity of EGFP expression was assessed by fluorescent microscopy.

Transfected HEK293A cells in the wells of the culture plate after removing the culture medium were fixed with pre-chilled methanol with further incubation at − 20 °C for 10 min. All wash steps were performed with Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.5). Cell membranes were permeabilized by 0.1% solution of Triton X-100 (Helicon, Russia). Incubation with rabbit polyclonal primary antibodies to NGF (Santa Cruz, USA) was performed in TBS for 1 h; the cells were then washed with TBS and incubated with secondary antibodies Alexa Fluor 488 donkey anti-rabbit IgG (Life Technologies, USA) for 1 h. The cell nuclei were stained with the fluorescent dye DAPI (4′,6-diamidino-2-phenylindole; Invitrogen, USA). Results were analyzed using fluorescent microscope Axio Observer Z1 (Carl Zeiss, Germany).

2.3 Production of Recombinant Adenovirus

To produce recombinant adenovirus, Ad5-NGF and Ad5-EGFP HEK293A cells were transfected with purified predominantly linearized plasmid DNA pAd-NGF and pAd-EGFP by digestion with *Pac*I enzyme. After transfection, cultural media was replaced every 2–3 days with fresh one until the formation of visible cytopathic regions characterizing with changing of cell morphology. On the tenth day after transfection, cell suspension was collected in a sterile 2-ml tube. After collection, cell suspension was conducted several freeze/thaw cycles followed by centrifugation to prepare a crude viral lysate. Viral stock was stored at − 80 °C.

To obtain preparative amounts of adenovirus encoding *ngf* and *egfp* genes, HEK293A cell line was infected with derived virus. After 72 h, cell lysate was collected in 15-ml tubes and subjected cell line to several freeze/thaw cycles according to procedure described above. Virus concentration and purification was performed by two-step centrifugation in CsCl density with further dialysis and determination of viral titer by optical density and plaque assay.

2.4 Cell Preparation and In Vitro Analysis

Umbilical cord blood was taken after obtaining informed consent of the pregnant and prenatal screening for contraindications to blood donation. Blood was collected in CPDA-1250 GG plastic containers (Terumo, Japan) and delivered to laboratory. Isolation of nuclei containing red blood cells was performed in 50-ml tubes according to the previously published procedure [15]. After purification, fraction of mononuclear cells from umbilical cord blood was cultivated in RPMI-1640 medium supplemented with 10% FBS and mixture of antibiotics penicillin and streptomycin (100 U/ml, 100 μg/ml) (PanEco, Russia). Immediately after isolation, mononuclear cells were seeded in a 10-cm culture dish and transduced with recombinant adenoviruses Ad5-NGF and Ad5-EGFP with MOI 10. Cells were incubated for 12–16 h in a humid environment at + 37 °C with 5% CO_2 content.

2.5 Western Blot Analysis of Genetically Modified UCBMCs

Expression of *egfp* and *ngf* genes in transduced UCBMCs was assessed by Western blot and fluorescent microscopy. Adenovirus infected cells were lysed in × 1.5 sample buffer (10% glycerol, 50 мМ Tris-HCl (pH 6.8), 2 мМ EDTA, 2% sodium dodecyl sulphate (SDS), 144 мМ 2-mercaptoethanol, 0.0084% bromphenolic blue) and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [16]. Proteins were transferred (55 min, 118 mA) onto PVDF cellulose membranes using Trans-Blot® SD semi-dry electrophoretic transfer cell (BioRad, USA) and blocked for 2 h at room temperature with 5% non-fat dry milk in phosphate-buffered saline (PBS) and 0.5% Tween 20. After three washes, membranes were incubated (18 h, 4 °C) with the rabbit polyclonal anti-NGF antibody (1:300, Santa Cruz, USA), mouse

monoclonal β-actin-HRP antibody (1:6000, GenScript, USA), and mouse monoclonal GFP antibody (1:300, Santa Cruz, USA). Antigen-antibody complexes were identified with goat anti-rabbit IgG-HRP conjugated antibodies (Sigma, Germany) and developed using ECL Western blotting substrate kit (horseradish peroxidase) according to the manufacturer's instructions.

2.6 Xenotransplantation of UCBMCs to Transgenic Mice

Prior to transplantation, the UCBMCs were precipitated by centrifugation and diluted in sterile saline to the concentration of 2×10^7 cells/ml. Xenotransplantation of genetically modified cells in the amount of two million per animal was performed in the retro-orbital venous sinus of mice.

2.7 Experimental Groups of Animals

Transgenic mice with a model of Alzheimer's disease expressing mutant human genes of an amyloid precursor protein and presenilin 1 (genotype B6C3—Tg(APP695)85Dbo Tg(PSENI)85Dbo) were initially purchased from the Jackson Laboratory (USA) and housed in the Pushchino Animal Facility of the Branch of the Institute of Bioorganic Chemistry, RAS (Moscow Region). Transgenic mice were taken from "Collection of SPF laboratory animals for basic biomedical and pharmacological studies of RAS Institute of Bioorganic Chemistry" supported by Program of Bioresource Collections of the Federal Agency of Scientific Organizations of Russia.

Four experimental groups of animals were formed: wild type (WT—control, $n = 25$), transgenic mice (Alz—control, $n = 25$), transgenic mice transplanted with EGFP-expressing UCBMCs (Alz-EGFP, $n = 19$), and transgenic mice transplanted with NGF-expressing UCBMCs (Alz-NGF, $n = 9$). Animals had an age of 6 months old at the moment of cell transplantation (non-treated animals were age-matched with Alz-EGFP and Alz-NGF groups).

2.8 Behavioral Tests

T-maze T-maze setup (Open Science, Russia) was used to study spatial memory in mice. The mice were trained on a rewarded alternation task using a conventional T-maze [17]. Food consumption was limited during an experiment. The learning lasting for 14 days started after the test preparation period of 7 days of duration. T-maze learning was started on day 16 after transplantation. Every day, the mice had six pairs of training trials. The first trial of each pair was a forced trial, in which one of the goal arm doors was closed and the mouse was constrained to selecting the opposite arm where food was placed. On the second, or free-choice trial, both goal arm doors were opened, but only the arm opposite the one selected in the forced trial was baited. The criterion for a mouse having learned the rewarded alternation task was 3 consecutive days of at least five correct responses out of the six free trials. The mice that did not reach the criterion of learning during entire period of training were assigned with criterion value of 14. Latency to make an arm choice for mice was measured in all trials.

Plus Maze Plus-maze setup (Open Science, Russia) was used to study orientation, exploratory behavior, and anxiety in mice. The maze consisted of a central section and four side ones. A mouse was individually placed in the central section and a sequence of its passings from one section to another was recorded. The test was over having achieved 13 passings. The latency of starting the exploration, the time spent in central and side sections, the number of "patrolling" cycles, and the number of boluses (defecations) were calculated. Plus-maze test technique has been described in details previously [18].

Open field Locomotor activity in mice was evaluated using an open-field setup (Open Science, Russia). Setup represents round arena divided by lines into sectors and holes in the floor. Each animal was individually placed in setup and allowed to freely explore for 3 min. The numbers of line crossing (horizontal activity), vertical stands (vertical activity), and lookings into the hole (exploratory activity) were counted. Open-field and plus-maze tests were carried out on days 5–10 and 33–35 after transplantation and averaged.

The results of behavioral tests are presented as the mean value and standard error of the mean. Statistical significance of differences was assessed with Student's t test or Fisher's exact test, differences being significant at $p < 0.05$. The Fisher exact test was used to evaluate intergroup differences between percentages of trained animals and their total number; all other differences were assessed with the Student t test.

2.9 Immunofluorescent Staining of Brain Slices

The identification of UCBMCs in the mice brain was performed on days 7, 48, and 90 after transplantation. Anesthetized animals were transcardially perfused with phosphate-buffered saline (PBS) and a 4% paraformaldehyde solution (4 °C), then the brain was removed, fixed in a 4% paraformaldehyde solution for 24 h, and placed in a 30% sucrose solution in PBS supplemented with 0.02% sodium azide.

To prepare cryostat slices, the tissue was placed into a Neg 50 embedding medium and frozen for 2 min. The sections were placed in PBS, washed in a 0.1% Triton-X100 solution in PBS (PBST), and incubated in a 5% donkey serum solution in PBST for 45 min at room temperature.

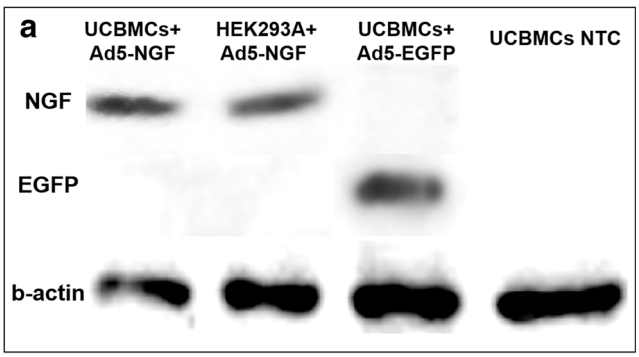
For antigen detection, the slices were incubated with antibodies to a human cell nucleus marker (HNu, Millipore,

1:150) and NGF (Santa Cruz Biotechnology INC, 1:200) for 24 h at 4°C, then washed in PBS and incubated with secondary antibodies (Alexa Fluor 555 donkey anti-mouse and Alexa Fluor 647 donkey anti-rabbit, Molecular Probes, 1:200) for 2 h at room temperature in dark, and washed in PBS. To visualize nuclei, the slices were additionally incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min and washed in PBS. Stained preparations were embedded in a Shandon ImmuMount medium and examined under a confocal scanning microscope LSM 510-Meta (Carl Zeiss).

3 Results

3.1 In Vitro Study of Recombinant Gene Expression

Genetically modified UCBMCs with recombinant adenovirus Ad5-NGF at 5 days post-infection have undergone Western blot analysis which has revealed specific band (14 kDa) evident for N-epitope of NGF (Fig. 1a). We have also detected specific band (20 kDa) intrinsic to EGFP in cell lysates of UCBMCs which had been previously modified with Ad5-EGFP (Fig. 1a). This fact confirms efficiency of transduction of cells with recombinant adenoviruses Ad5-NGF and Ad5-EGFP. Successful EGFP expression in UCBMCs transduced with recombinant Ad5-EGFP was also confirmed 5 days post-infection with fluorescent analysis (Fig. 1b).



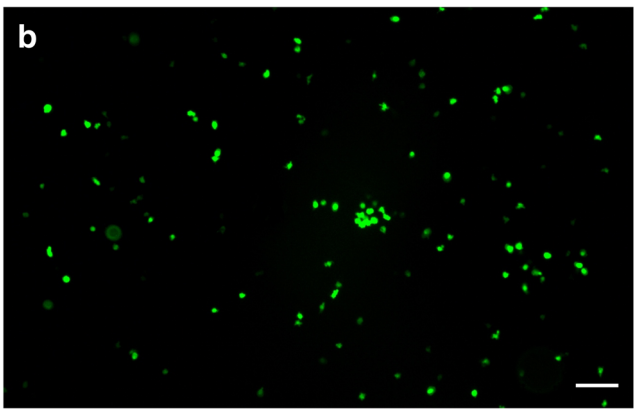


Fig. 1 Analysis of NGF and EGFP expression in genetically modified umbilical cord blood mononuclear cells. **a** Western blot analysis of cell lysates for following preparations: UCBMCs transduced with Ad5-NGF, HEK293A cells transduced with Ad5-NGF, UCBMCs transduced with Ad5-EGFP, and non-transduced UCBMCs (control). Panel represents staining to NGF antigen (14 kDa), EGFP (20 kDa), β-actin (42 kDa). **b** Fluorescent images of UCBMCs transduced with recombinant Ad5-EGFP 5 days post-infection showing EGFP fluorescence. Scale bar represents the value of 100 μm

3.2 Assessment of Spatial Memory of Mice in T-Maze

It was found that wild-type mice learned in T-maze for 10.0 ± 0.5 days, with 35.4 and 75% mice of the total number in the group learned on days 8 and 14, respectively. The latency to make arm choice reduced from 26.3 ± 1.9 to 10.1 ± 1.8 s (by 61.6%) in the process of learning. Transgenic mice with a model of Alzheimer's disease (the Alz group) learned significantly longer as compared to the WT mice—for 13.2 ± 0.3 days. A percentage of trained mice in Alz group was significantly lower as compared to the WT group—7.9 and 18.4% of the animals learned on days 8 and 14, respectively. At day 1 of learning, the latency of arm choice in Alz group was 35.9 ± 3.2 s, then it reduced by 55% down to 16.0 ± 1.9 s by day 14. Thus, the latency of arm choice at first and last training days was significantly more in Alz group as compared to the WT group.

The transplantation of UCBMCs expressing EGFP or NGF significantly improved spatial memory performance in APP/PS1 mice (Fig. 2).

Mice of the Alz-EGFP group learned for 11.8 ± 0.5 days, with 10.5% of them trained on day 8 (no significant difference from the Alz group) and 57.9%—on day 14 (significantly more as compared to the Alz group). In the process of learning, the latency of arm choice reduced from 25.4 ± 2.7 to 9.9 ± 1.6 s in the Alz-EGFP mice (by 61%) that was similar to that in the WT group.

Mice of the Alz-NGF group learned for 11.4 ± 1.0 days, with the percentage of the animals trained on days 8 and 14 being significantly higher as compared to the Alz group (33.3 and 55.6%, respectively) (Fig. 2b, c). The latency of arm choice reduced from 29.3 ± 6.7 to 6.8 ± 1.0 s (by 76%) during learning, demonstrating the most evident dynamics of this parameter among the studied groups of animals (Fig. 2a).

Thus, the transplantation of UCBMCs expressing EGFP or NGF significantly improved spatial memory in transgenic mice with a model of Alzheimer's disease. NGF-expressing UCBMCs had a more pronounced effect on memory evidenced by significantly increased numbers of trained Alz-NGF mice versus Alz and Alz-EGFP animals even on day 8 of testing, as well as by a most evident decrease of the latency of arm choice by Alz-NGF mice as compared to the rest groups of animals.

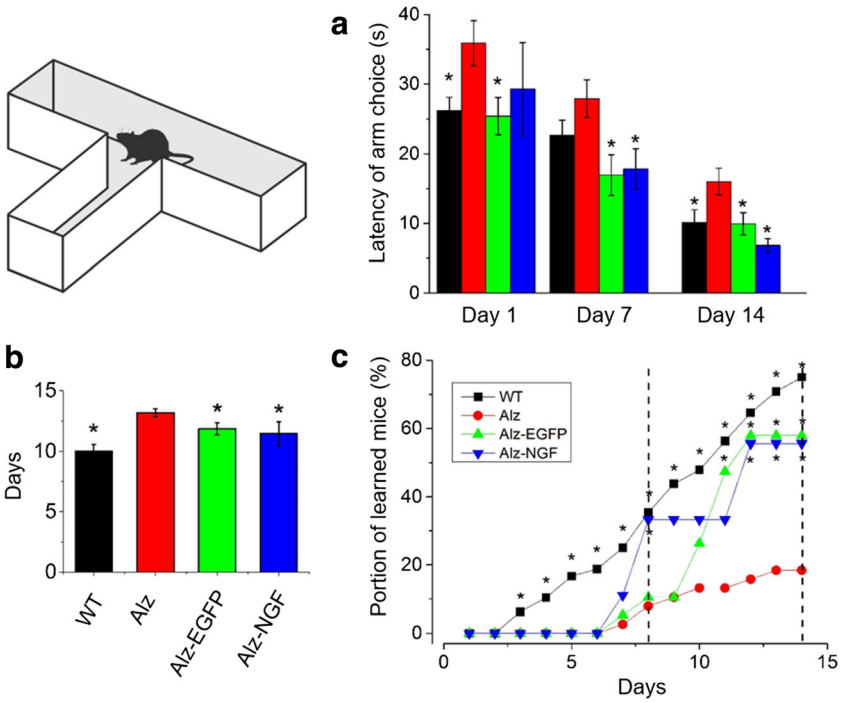


Fig. 2 Effects of transplantation of human umbilical cord blood mononuclear cells overexpressing nerve growth factor on spatial memory parameters in APP/PS1 transgenic mice. The T-maze experiment results are shown for different experimental groups of mice (black marks—WT, red marks—Alz, green marks—Alz-EGFP, blue marks—Alz-NGF). **a** Latencies of arm choice on training days 1, 7, and 14. **b** Duration of learning. **c** Progress of learning for experimental groups of mice (on *x*-axis—a day of training, on *y*-axis—the number of trained animals as a percentage of the total number of animals in the group). An asterisk indicates values that differ significantly from those in the Alz mice group

3.3 Assessment of Orientation and Exploratory Behavior and Anxiety of Mice in Plus Maze

The wild-type mice spent 68.3 ± 6.2 and 90.5 ± 10.7 s in central and side sections of the plus maze, respectively, with the latency of starting exploration being 10.7 ± 1.8 s and the numbers of patrolling cycles and defecation being 1.7 ± 0.1 and 1.4 ± 0.2, respectively. The Alz group mice had insignificantly lower values of the time spent in maze side sections (72.9 ± 7.3 s) and the latency (8.1 ± 2.1 s). There were no significant differences in the plus-maze parameters in Alz-EGFP mice as compared to the Alz ones. The Alz-NGF mice had insignificantly shorter values of the time spent in side sections (60.0 ± 3.3 s) and a significantly fewer number of boluses (0.4 ± 0.1). Thus, UCBMCs transplantation did not impair the behavior activity in APP/PS1 mice in the plus maze. A decreased number of boluses in Alz-NGF mice compared to the rest groups of animals might suggest reduced anxiety levels in Alz-NGF mice.

3.4 Assessment of Locomotor Activity of Mice in the Open Field

Based on the open-field test results, the values of horizontal, vertical, and exploratory activities were 40.7 ± 4.3, 0.7 ± 0.3, and 4.8 ± 0.8, respectively, in the WT group mice. The Alz group mice demonstrated a significantly decreased vertical activity (0.1 ± 0.1). There were no differences in the open-field activity values in Alz-EGFP and Alz-NGF mice as compared to the Alz ones. Thus, the transplantation of UCBMCs did not have any significant effects on the APP/PS1 mice behavior in the open-field test.

3.5 Immunofluorescent Staining of Brain Slices from APP/PS1 Transgenic Mice Treated with UCBMCs Overexpressing NGF

The immunofluorescent staining revealed HNu^+ cells in the cerebral cortex, hippocampus, and connective tissue between the hippocampus and the cortex in the Alz-NGF mice on day 7 after transplantation. The cells were detected both in the brain parenchyma and in the periphery of blood vessels that indicates the ability of cells to migration. Double immunofluorescent staining revealed NGF-expressing UBCMCs in the cerebral cortex and the hippocampus (Fig. 3a). HNu^+ cells and NGF^+ HNu^+ cells were detected in the brains obtained from transgenic mice in 48 and 90 days after transplantation (Fig. 3b).

Thus, grafted cells were detected in the hippocampus and cerebral cortex of APP/PS1 mice at different time points after UCBMC transplantation; their ability to express a recombinant NGF gene for long periods after the transplantation was confirmed.

4 Discussion

NGF belongs to the family of neurotrophins and performs a number of physiological functions in the nervous system including pain signaling, maintaining neuronal survival, and

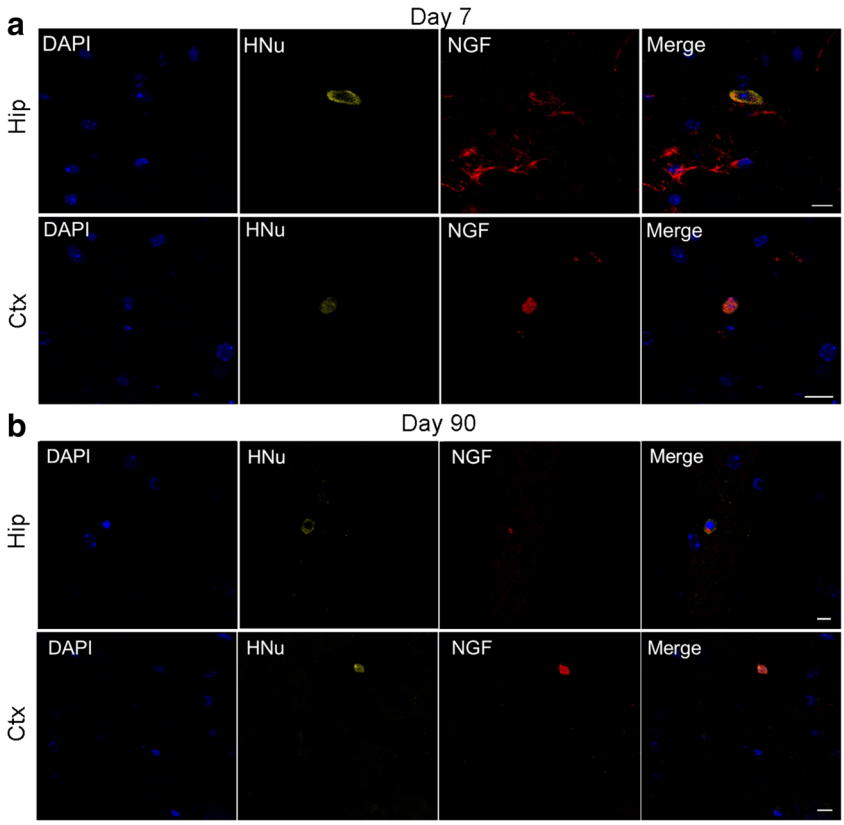


Fig. 3 Visualization of grafted cells in the brains of APP/PS1 transgenic mice. Triple staining of cortical and hippocampal slices of APP/PS1 transgenic mice after transplantation of NGF-expressing UCBMCs (Alz-NGF group) with antibodies to HNu (yellow) and NGF (red). Nuclei are stained with DAPI (blue) **a** 7 days after transplantation and **b** 90 days after transplantation. Scale bar represents the value of 10 μm

stimulating their proliferation [19, 20]. The basal forebrain cholinergic system is the main target of NGF. It has been established that maintenance of the viability of mature neurons of the basal cholinergic system depends upon the availability of NGF [21, 22].

Alzheimer's disease is characterized by degeneration of cholinergic neurons including due to a decreased NGF expression and resulting in the loss of neurons and synapses, the accumulation of β-amyloid peptide, and the formation of neurofibrillary tangles in the hippocampus, impaired synaptic plasticity, and cognitive disorders [23–27].

NGF is often considered for use in the treatment of dementia; however, it is limited by the NGF inability to penetrate via the blood-brain barrier due to the large size of the molecule. Intranasal NGF administration was found to prevent the accumulation of β-amyloid peptide and a hyperphosphorylated tau-protein in brain [28]. The use of gene-cell constructs capable of penetrating via the blood-brain barrier and expressing and/or stimulating the expression of NGF in brain seems to be the most effective strategy. Transplantation of genetically modified fibroblasts or neural stem cells expressing NGF leads to increase of a number of neurons, improvement of cognitive abilities, and increase of NGF level [29, 30]. The NGF delivery with adenoviruses or cell encapsulation resulted in positive changes of cholinergic biomarkers and stable NGF expression in nervous tissue [31, 32]. However, there are still no univocal proofs of clinical efficacy of abovementioned methods for NGF delivery available. Thus, search for effective carriers for NGF delivery remains to be actual. Previously, we demonstrated that UCBMC intravenous transplantation have positive clinical effects on APP/PS1 transgenic mice [8]. Intravenous transplantation is widely used in cellular therapy of neurological and degenerative diseases. The main advantages of UCBMCs for use in gene-cell therapy are safety, availability, low immunogenicity, homing capacity, ability to penetrate via the blood-brain barrier, and ability to migrate into sites of neurodegeneration [5, 6, 33]. It was shown over last years that the use of human UCBMCs for the treatment of neurodegenerative diseases may not require HLA matching or immunosuppression [34, 35]. High survivability level of UCBMCs in brain parenchyma partially may be explained by CNS immune privilege [36]. UCBMCs can be genetically modified with introducing vectors providing the expression of therapeutic or reporter genes. It is established that UCBMCs include hematopoietic, mesenchymal, and embryonic stem cells, side population cells, and endothelial progenitor cells [37]. UCBMCs are shown to secrete cytokines (IL-6 and IL-10), chemokines (IL-8, MCP-1, SDF-1b), growth factors (ANG, HGF, VEGF, PDGF, EGF), and neurotrophic factors (NGF, GDNF, BDNF, neurotrophins 3 and 5) [37–39].

Our study has resulted in two most important findings. First, the intravenous transplantation of NGF-expressing

UCBMCs significantly improved spatial memory in APP/PS1 mice, with the effect being more prominent as compared to the transplantation of UCBMCs expressing the EGFP reporter protein gene. Second, the grafted cells capable of expressing NGF were detected in the cerebral tissue even 90 days after transplantation. This must provide long-term therapeutic effects produced by NGF.

Our data suggest that gene-cell therapy with UCBMCs overexpressing NGF has a number of advantages associated with NGF neuroprotective properties comparing to use of EGFP expressing or native UCBMCs. Gene-cell constructs based on UCBMCs and NGF-overexpressing adenoviral vectors has high therapeutic potential for the treatment of Alzheimer's disease and can be used in further preclinical studies.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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