

Genetically modified human umbilical cord blood cells expressing vascular endothelial growth factor and fibroblast growth factor 2 differentiate into glial cells after transplantation into amyotrophic lateral sclerosis transgenic mice

Albert A Rizvanov^{1,2,3,4}, Daria S Guseva^{5,6}, Ilnur I Salafutdinov^{1,4}, Nezhdana V Kudryashova¹, Farid V Bashirov⁶, Andrey P Kiyasov^{4,7}, Mehmet E Yalvaç³, Ilnaz M Gazizov⁷, Maxim S Kaligin⁷, Fikrettin Sahin³, Marat A Mukhamedyarov⁸, András Palotás⁹ and Rustem R Islamov⁶

¹Department of Genetics, Faculty of Biology and Soil Sciences, Kazan Federal University, ul. Kremlevskaya 18, R-420008 Kazan;

²Core Research Laboratory, Kazan State Medical University, ul. Butlerova 49, R-420012 Kazan, Russia; ³Department of Genetics and BioEngineering, College of Engineering and Architecture, Yeditepe University, 26 Ağustos Campus, Kayisdagi cad., Kayisdagi, 34755 Istanbul, Turkey; ⁴Republic Clinical Hospital, ul. Orenburg tract 138, R-420064 Kazan, Russia; ⁵Laboratory of Cellular Neurophysiology, Institute for Neurophysiology, Hannover Medical School, D-30625 Hannover, Carl-Neuberg Str. 1, Germany; ⁶Department of Histology, Cytology and Embryology; ⁷Department of Anatomy; ⁸Department of Physiology, Kazan State Medical University, ul. Butlerova 49, R-420012 Kazan, Russia; ⁹Asklepios-Med (private practice and research center), H-6722 Szeged, Kossuth Lajos sgt. 23, Hungary
Corresponding authors: András Palotás, Asklepios-Med, H-6722 Szeged, Kossuth Lajos sgt. 23, Hungary. Email: palotas@asklepios-med.eu and Rustem R Islamov, Department of Histology, Cytology and Embryology, Kazan State Medical University, ul. Butlerova 49, R-420012 Kazan, Russia. Email: islamru@yahoo.com

Abstract

Current therapy of a number of neuropsychiatric maladies has only symptomatic modality. Effective treatment of these neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), may benefit from combined gene/stem-cell approaches. In this report, mononuclear fraction of human umbilical cord blood cells (hUCBCs) were transfected by electroporation with dual plasmid constructs, simultaneously expressing vascular endothelial growth factor 165 (VEGF₁₆₅) and human fibroblast growth factor 2 (FGF₂) (pBud-VEGF-FGF₂). These genetically modified hUCBCs were injected retro-orbitally into presymptomatic ALS transgenic animal models (^{G93A} mice). Lumbar spinal cords of rodents were processed for immunofluorescent staining with antibodies against human nuclear antigen (HNA), oligodendrocyte-specific protein, S100, iba1, neuronal β_3 -tubulin and CD34. Co-localization of HNA and S100 was found in the spinal cord of mice after transplantation of genetically modified hUCBCs over-expressing VEGF-FGF₂. Double staining in control animals treated with unmodified hUCBCs, however, revealed HNA⁺ cells expressing iba1 and CD34. Neuron-specific β_3 -tubulin or oligodendrocyte-specific protein were not expressed in hUCBCs in either control or experimental mice. These results demonstrate that genetically naïve hUCBCs may differentiate into endothelial (CD34⁺) and microglial (iba1⁺) cells; however when over-expressing VEGF-FGF₂, hUCBCs transform into astrocytes (S100⁺). Autocrine regulation of VEGF and FGF₂ on hUCBCs, signal molecules from dying motor neurons in spinal cord, as well as self-differentiating potential may provide a unique microenvironment for the transformation of hUCBCs into astrocytes that eventually serve as a source of growth factors to enhance the survive potential of surrounding cells in the diseased regions.

Keywords: amyotrophic lateral sclerosis (ALS), gene therapy, human umbilical cord blood (hUCB), stem cell

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Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a neurodegenerative disease characterized

by gradual loss of motor neurons in the brain and spinal cord leading to muscle paralysis throughout the body. The etiology and pathogenic mechanisms of cell death in most forms of ALS are largely unknown. Due to the lack of

effective therapy, patients die from respiratory failure within 3–5 y after the onset of paralytic symptoms. Major therapeutic strategies are aimed at inhibition of excitotoxicity and oxidative damage, which are key factors in motor neuronal damage. Stem cell therapy is a potential treatment option in degenerating diseases by which lost parenchymal cells might be replaced. It is unreasonable to expect, however, that stem cells *per se* could substitute dying neural cells in rigid, highly organized neural networks.

Glial cells may be very effective in increasing survivability of affected neurons by providing a physical scaffolding support and by secreting neurotrophic factors. Transplantation of predifferentiated stem cells with a potential to become glia seems to be a promising strategy for ALS treatment. Predifferentiated glial cells can be obtained from various stem cell sources, such as embryonic or neural stem cells. A more easily accessible alternative is the human umbilical cord blood (hUCB), which contains several types of stem cells, including hematopoietic (CD31, CD34, CD59, CD90, *oct4*, *nanog*, *oct4*, *sox2*, *sca1*, *fgf4*) and mesenchymal stem cells (CD34, CD90, CD105, *sca1*), as well as endothelial progenitor cells (CD34, *gata2*, *flk1*). The rationale for using hUCB cells (hUCBCs) for cellular therapy is based not only on their potential to differentiate into various cell types, such as erythrocytes, leukocytes, megakaryocytes, myoblasts of heart and skeletal muscle tissue, hepatocytes, vascular endothelial cells, neurons, oligodendrocytes, astrocytes, etc. (for a review see reference¹), but also on their availability, acceptability, potential for long-term storage, compatibility with both auto- and allogenic transplantation in humans, and their low immunogenicity. Moreover, transplantation of hUCBCs is not subjected to legislative regulations, esthetics and religious prohibition.

Cellular therapy has recently been complemented with the genetic modification of stem cells. Over-expression of cell-adhesion molecules, growth factors and other biologically active molecules may lead to addressed homing of transplanted stem cells that ultimately express therapeutic genes and also differentiate into parenchymal cells at the site of degeneration. In line with this notion, here we generated hUCBCs over-expressing either human vascular endothelial growth factor (VEGF) or human fibroblast growth factor 2 (FGF₂), or simultaneously both, for the possible treatment of ALS.

The most widely used animal model of ALS is a ^{G93A} substitutional transgenic mouse expressing human mutant copper/zinc super-oxide dismutase-1 (SOD₁) gene. These rodents are unique in that over-expression of SOD₁ results in an adult-onset phenotype closely recapitulating the human disease at a clinical as well as histopathological level.² The ^{G93A} model has the following four stages:³ (i) premuscle weakness stage, during which muscle strength is maintained at the normal level; (ii) rapid declining stage, where muscle strength sharply (usually by >50% within a period of two weeks) declined; (iii) slow declining stage (SD), which lasts for 4–11 weeks; and (iv) a paralytic stage. In this study, SD stage mice were used, immediately prior to the onset of paralysis of the limbs. The effect of gene modification with plasmid expression constructs, encoding both FGF₂ and VEGF, on the differentiation of

hUCBCs after transplantation into ^{G93A} mice was studied. Phenotyping of transplanted hUCBCs was evaluated by double immunofluorescent staining.

Materials and methods

Generation of plasmid constructs

Generation of recombinant plasmid pcDNA-VEGF, expressing human VEGF₁₆₅, was described previously.⁴ The open reading frame (ORF) of human FGF₂ was polymerase chain reaction (PCR) amplified using specific primers with engineered restriction sites *KpnI* and *XhoI* (forward primer: CGCAGGtACCATGGCAGCCG; reverse primer: CACtCgAgTCAGCTCTTAGCAGACATTGGAAG). The PCR product was cloned into pcDNA3.1+ plasmid using *KpnI* and *XhoI* restriction enzymes. Dual cassette expression vectors were generated using plasmid pBudCE4.1 (Invitrogen, Carlsbad, CA, USA) as a backbone. First, plasmids pBud-EGFP and pBud-VEGF were generated by subcloning enhanced green fluorescent protein (EGFP) and VEGF ORFs from plasmids pcDNA-EGFP and pcDNA-VEGF into pBudCE4.1 using *KpnI* and *XhoI* restriction enzymes. Then plasmids pBud-EGFP-FGF₂, pBud-VEGF-FGF₂ and pBud-VEGF-EGFP were generated by subcloning VEGF, FGF₂ and EGFP ORFs from plasmids pcDNA-VEGF, pcDNA-FGF₂ and pcDNA-EGFP into plasmids pBud-EGFP and pBud-VEGF using *HindIII* and *XbaI* restriction enzymes. Generation of the correct expression plasmids was confirmed by sequencing.

Cell isolation and electroporation

Mononuclear fraction of hUCBCs (hUCB-MC) was isolated by standard technique of sedimentation onto a density barrier (Ficoll, 1.077 g/mL). Isolated cells were transiently transfected by electroporation (Gene Pulser Xcell Total System; Bio-Rad, Hertfordshire, UK) with expression plasmids pBud-EGFP, pBud-EGFP-FGF₂, pBud-VEGF-EGFP or pBud-VEGF-FGF₂ (20 µg DNA, 0.4 cm cuvette, 300 V, 1000 µF). Cells were allowed to recover for 24 h in RPMI-1640 medium (Sigma, Lyon, France) containing 10% fetal bovine serum. Thereafter, 1×10^6 cells were injected retro-orbitally in presymptomatic ^{G93A} mice.

Realtime PCR

Expression level of human VEGF and FGF₂ mRNA in transfected cells was examined by realtime PCR. Total RNA from cell cultures was isolated using a HighPure RNA Isolation Kit (Roche, Hertfordshire, UK) according to the manufacturer's instructions. cDNA synthesis was performed utilizing random hexamer primers and moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) at 37°C for one hour. TaqMan primers and probes (Table 1) were designed using PrimerExpress (Applied Biosystems, Foster City, CA, USA) software. Realtime PCR primers and TaqMan probes, as well as 2.5 × premix for TaqMan realtime PCR, was purchased from Syntol (Moscow, Russia) and used according to the manufacturer's instructions. Amount of RNA was

Table 1 Primers and probes for realtime polymerase chain reaction

Name of primers and probes	Nucleotide sequence
hVEGF-TM49F	TACCTCCACCATGCCAAGTG
hVEGF-TM110R	TGATTCTGCCCTCCTCCTTCT
hVEGF-TMprobe*	TCCCAGGCTGCACCCATGG
hFGF ₂ -TM134F	CCGACGGCCGAGTTGAC
hFGF ₂ -TM203R	TCTTCTGCTTGAAGTTGTAGCTTGA
hFGF ₂ -TMprobe*	CCGGGAGAAGAGCGACCCTCAC
β -Actin-TM-F (human)	GCGAGAAGATGACCCAGGATC
β -Actin-TM-R (human)	CCAGTGGTACGGCCAGAGG
β -Actin-TMprobe (human)*	CCAGCCATGTACGTTGTATCCAGGC

*TaqMan probes contain 5' FAM fluorescent dye and 3' RTQ-1 quencher

normalized using β -actin. Serial dilutions of cDNA synthesized from mRNA of hUCB-MCs, transfected with pBud-VEGF-FGF₂ plasmid, were used to generate a standard curve for relative quantitation of gene expression. Expression level of genes in untransfected control hUCB-MCs was considered as 100%. TaqMan reactions were run in duplicates. Standard error and *t*-test values were obtained using Microsoft Excel 2007, with *P* < 0.05 being regarded as a statistically significant difference.

Animals and treatments

Mice, transgenic for hSOD₁ (B6SJL-TG(SOD₁-G93A)^{dl1}Gur/J), were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred at the animal facility branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (Russian Academy of Science). Mature 21-week-old mice were delivered to Kazan State Medical University, where rodents were housed one/cage, under standard laboratory conditions, with a 12-h light/dark cycle and unlimited access to food and water. Sixteen transgenic female and male mice were randomly assigned into four groups (*n* = 4) based on transplantation of genetically modified hUCBCs expressing EGFP, EGFP-VEGF, EGFP-FGF₂ or VEGF-FGF₂. In experimental groups, 1×10^6 cells were injected retro-orbitally in presymptomatic 24–28-week-old G93A mice. Two weeks after transplantation, mice were euthanized under ketamine–xylazine anesthesia. The animal protocol was approved by the Animal Care and Use Committee of Kazan State Medical University.

Immunofluorescence

Mice were transcardially perfused with cold phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde in PBS (pH: 7.4). Entire spinal cords were removed, postfixed with paraformaldehyde overnight at 4°C, and immersed in 15% sucrose solution in 0.1 mol/L cacodylate buffer (pH: 7.3) for one day at 4°C. Spinal cords were subdivided into cervical, thoracic and lumbar parts. Tissues were frozen for two minutes in 2-methyl-butane (isopentane) precooled to –30°C. For sectioning, the spinal cord segments were attached to a cryostat specimen holder using TissueTek (Sakura Finetek Europe, Zoeterwoude, Netherlands). Serial longitudinal sections of 20- μ m thickness were cut on a cryostat and picked up on

SuperFrostPlus glass slides. The sections were air-dried for at least one hour at room temperature (RT) and mounted in antiquenching medium (for the identification of EGFP-transfected cells throughout the spinal cord) or used for immunofluorescence (for the phenotyping of transplanted cells). To detect transplanted cells in the host tissue and to identify their phenotype, spinal cord cryo-sections were subjected to double immunofluorescent staining. For all sections, antigen retrieval was performed by immersion into 0.01 M of sodium citrate solution (pH: 9.0), heated to 70°C in a water bath for 30 min.⁵ After cooling of the slides to RT, blocking of non-specific binding sites was performed using PBS containing 0.2% Triton X-100, 0.02% sodium-azide and 5% normal goat serum for one hour at RT. Primary antibodies (Table 2) were applied for three days at 4°C following washing in PBS (3 $\times$ 15 min at RT) and incubation with goat anti-mouse IgG conjugated with Cy3 and goat anti-rabbit IgG conjugated with Cy2 fluorescent dyes (dilution 1:200) for two hours at RT. After subsequent washing in PBS, cell nuclei were stained for 20 min at RT with a bis-benzimide solution (Hoechst-33258 dye, 5 μ g/mL in PBS) (Sigma), washed again in PBS, mounted in antiquenching medium and examined with a laser scanning microscopy (Zeiss Axiovert 200 M).

Results

Generation of dual expression cassette plasmids

Various dual expression cassette plasmids were generated using standard genetic engineering techniques utilizing the plasmid backbone pBudCE4.1. Some of the plasmids were constructed to contain a reporter gene *egfp* in order to facilitate their tracking. Efficient expression of EGFP by the resulting vectors pBud-EGFP, pBud-EGFP-VEGF and pBud-EGFP-FGF₂ was confirmed by fluorescent microscopy (data not shown). Finally, a plasmid pBud-VEGF-FGF₂ was constructed, which carries two therapeutic genes, VEGF and FGF₂, under the control of two independent constitutively active promoters, thus resulting in efficient simultaneous and independent expression of recombinant proteins. To confirm expression of VEGF and FGF₂, hUCB-MCs were electroporated with pBud-VEGF-FGF₂ plasmid, followed by realtime PCR analysis of mRNA expression (Figure 1). A significant increase in the mRNA expression level was achieved in transfected hUCB-MCs compared with untransfected control cells. An increase in VEGF mRNA expression over 7×10^4 -fold and FGF₂ mRNA over 1×10^4 times was observed after electroporation with pBud-VEGF-FGF₂ plasmid.

Table 2 Primary antibodies used in immunofluorescent staining

Antibody	Host	Dilution	Source
Human nuclei (HNA)	Mouse	1:20	Chemicon
Oligodendrocyte-specific protein	Rabbit	1:2000	Abcam
CD34	Rabbit	1:100	Abcam
iba1	Rabbit	1:600	Biocare Medical
S100	Rabbit	1:2000	DakoCytomation
Neuronal β_3 -tubulin	Rabbit	1:2000	Covance

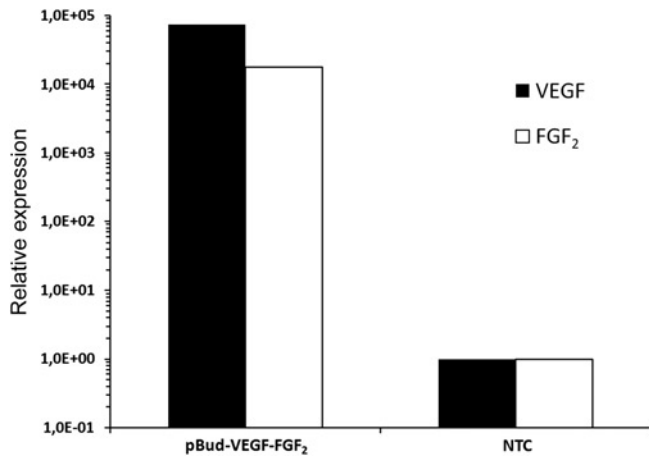


Figure 1 Realtime polymerase chain reaction analysis of human VEGF and FGF₂ mRNA expression levels in human umbilical cord blood mononuclear cells (hUCB-MCs) electroporated with plasmid pBud-VEGF-FGF₂ or non-transfected control (NTC). The level of expression of VEGF and FGF₂ mRNA in NTC was considered 100%. Expression of VEGF mRNA in hUCB-MCs increased over 7×10^4 times and FGF₂ mRNA over 1×10^4 times after electroporation with plasmid pBud-VEGF-FGF₂. VEGF, vascular endothelial growth factor; FGF₂, fibroblast growth factor 2

Identification of hUCBCs in ^G93^A mice spinal cord

To identify transplanted cells in the spinal cords of control and experimental animals, we carried out immunofluorescent staining with an antibody against human nuclear antigen (HNA) as a marker for hUCBCs. To confirm the specific binding of the anti-HNA antibody we also applied standard nuclei staining (see subsection Immunofluorescence). Confocal microscopy has shown co-localization of HNA expression with nuclei staining. HNA+ cells were randomly distributed in the white and gray matter of the spinal cord 14 d after transplantation (Figure 2). Of note, HNA+ cells displayed various nuclear shapes: in particular, both oval and round nuclei of transplanted cells were observed throughout the spinal cord. No HNA+ cells were detected in animals treated with saline used as a negative control in our experiments. As such, these data confirm that retro-orbitally injected hUCBCs survive and migrate through the white and gray

matters of the spinal cord in presymptomatic 24–28-week-old ^G93^A mice.

Expression of plasmid vectors in hUCBCs after transplantation

The transcriptional activity of plasmid vectors was evaluated by examining the expression of reporter EGFP plasmid vector in the transplanted cells. To visualize EGFP expression, an appropriate filter set and laser-491 were used in the confocal microscope. EGFP-expressing cells were observed in the spinal cords of mice transplanted with hUCBCs transfected with plasmids encoding EGFP alone, or in combination with FGF₂ or VEGF (Figure 3). These EGFP-expressing cells are roughly 10 times smaller when compared with neurons. These cells were infrequently also distributed both in the white and gray matter of the spinal cord. Even though the expression of EGFP in cells was detected 14 d after transplantation, EGFP expression may not always be detectable after two weeks due to the elimination of plasmid vectors.

Phenotyping gene-modified hUCBCs in the spinal cord 14 d after transplantation

In order to evaluate which cell types the transplanted cells had differentiated into after 14 d, phenotyping of HNA+ cells with markers for neurons, glial, microglial and epithelial cells was performed. To identify hUCBC phenotype, spinal cord sections were subjected to double immunofluorescent staining. Anti-HNA antibody was used in combination with one of the following markers: neuron-specific β_3 -tubulin, oligodendrocyte-specific protein, S100, iba1 and CD34. In the control group of mice subjected to transplantation with genetically modified hUCBCs expressing EGFP, double staining revealed HNA+ cells expressing microglial marker *iba1* (Figure 4) and the endothelial cell marker CD34 (Figure 5). The iba1+ hUCBCs possess a small oval cell body with numerous processes of small diameter: a characteristic feature of microglial cells. Expression of the astrocyte marker S100 by HNA+ cells was found in the spinal cord of mice after transplantation

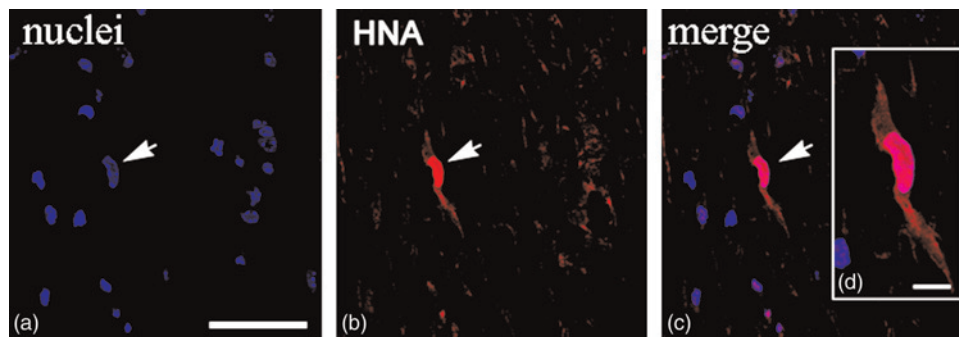


Figure 2 Spinal cord of presymptomatic 24-week-old ^G93^A mice injected retro-orbitally with enhanced green fluorescent protein-transfected human umbilical cord blood cells (hUCBCs). Fourteen days after transplantation, hUCBCs were identified with antibodies against human nuclei antigen (HNA). (a) Nuclei staining (blue); (b) HNA labeling (red); (c) merged image representing HNA-positive hUCBC; and (d) merged photo with higher magnification. Arrows on panels a–c indicate HNA-positive nuclei. Scale bars: (a–c) 50 μ m and (d) 10 μ m (A color version of this figure is available in the online journal)

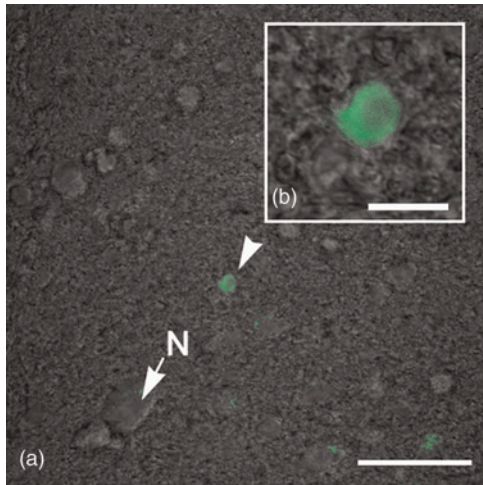


Figure 3 Enhanced green fluorescent protein (EGFP)-expressing human umbilical cord blood cells (hUCBCs) in the spinal cord of $G93^A$ mice 14 d after transplantation. EGFP expression is indicated by arrow-head (green): EGFP-transfected cell. Neuron is indicated by arrow (N). Scale bars: (a) 50 μ m; (b) 10 μ m (A color version of this figure is available in the online journal)

with the genetically modified hUCBCs over-expressing VEGF-FGF₂ (Figure 6). S100+ cells displayed astrocyte-like morphology with many branching processes. Neither the neuronal β_3 -tubulin nor the oligodendrocyte-specific protein were expressed in the hUCBCs in any control or experimental rodents.

Discussion

Recent advances in biotechnology have provided the potential to use combined gene and stem cell therapy in various neurodegenerative disorders, such as Alzheimer's, Parkinson's, Huntington's, Lou Gehrig's disease, as well as several others caused by multiple genetic and environmental factors resulting in neuronal cell death. Cells for transplantation in basic and clinical trials may be obtained from embryonic, fetal or adult tissues that can be predifferentiated into neurons or glia.^{6–10} However, the use of embryonic or fetal organs is highly restricted based on ethical and religious controversies. Stem cells prepared for

transplantation must have predictable and reproducible characteristics, but at the same time the high risk of tumor transformation or immunological conflict must also be taken into consideration. Alternative sources of stem and progenitor cells are mesenchymal stem cells of the bone marrow,^{11,12} hematopoietic and mesenchymal stem cells from hUCB,^{13,14} stem cells of tooth soft tissues,¹⁵ and olfactory ensheathing cells.¹⁶ Gene modification of stem cells may significantly enhance their therapeutic effect by addressed homing, increased survivability, secretion of growth and trophic factors and directed terminal differentiation. Therapeutic growth factors transiently over-expressed by transplanted cells may have autocrine, paracrine and endocrine regulatory mechanisms. Autocrine effects may increase cell survivability, enable control of addressed homing and migration, and modulation of terminal differentiation. Paracrine and endocrine regulation of the transplanted cells may have trophic and cytoprotective effects on the affected parenchymal cells at the site of degeneration by delivering growth and trophic factors to the damaged tissue in order to support cell survival and tissue regeneration.^{17,18}

In our previous investigations we have demonstrated that genetically modified (VEGF) hUCBCs migrate in the spinal cord parenchyma, proliferate and differentiate into endothelial cells to form new blood vessels in ALS mice. Several similar data have shown that hUCBCs, genetically modified with human VEGF, can induce angiogenesis in limb ischemia rat models,¹⁹ as well as in myocardial infarction in mice.²⁰ In the present study we generated hUCBCs over-expressing human VEGF, human FGF₂, or simultaneously both factors. Our results demonstrate that hUCBCs injected retro-orbitally in presymptomatic 24-week-old $G93^A$ mice can undergo guided differentiation into microglial, astrocytic or endothelial cells, but not neurons, based on their pretransplantation gene-modification. Thus, EGFP-transfected cells might be transformed into microglia-like (iba1+) or endothelial-like (CD34+) cells; however VEGF-FGF₂ transfection stimulates them to differentiate into astrocyte-like (S100+) cells.

In the central nervous system, VEGF is produced by neurons, glia and neural stem cells,^{21–23} and has neurotrophic effects. Axotomy increases expression of VEGF and

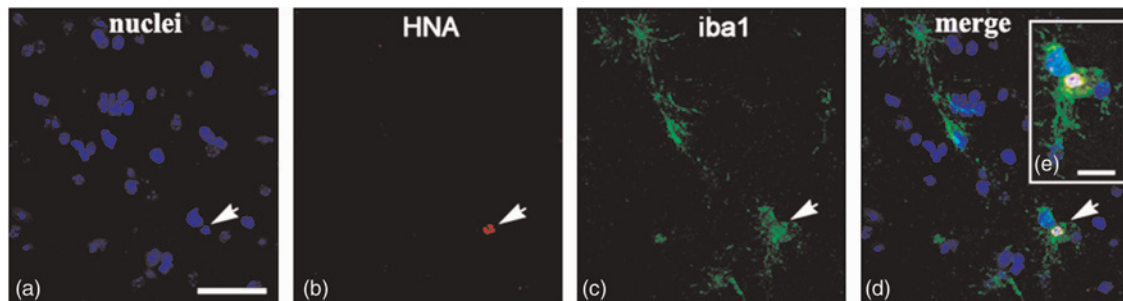


Figure 4 Enhanced green fluorescent protein (EGFP)-transfected human umbilical cord blood cells (hUCBCs) in the spinal cord of $G93^A$ mice 14 d after transplantation. Co-localization of human nuclei antigen (HNA) and *iba1* in hUCBCs is shown. The *iba1*+ cells with ramified processes are depicted, which are characteristic features of microglial cells. Arrows on panels a–d indicate HNA+ nucleus in *iba1*+ cells. (a) Nuclei staining (blue); (b) immunostaining with HNA (red); (c) microglial marker (anti-*iba1* antibody, green); (d) merged picture representing HNA+/*iba1*+ cell; (e) higher magnification of HNA+/*iba1*+ cell that is marked by arrow on panel d. Scale bars: (a) 50 μ m and (e) 10 μ m (A color version of this figure is available in the online journal)

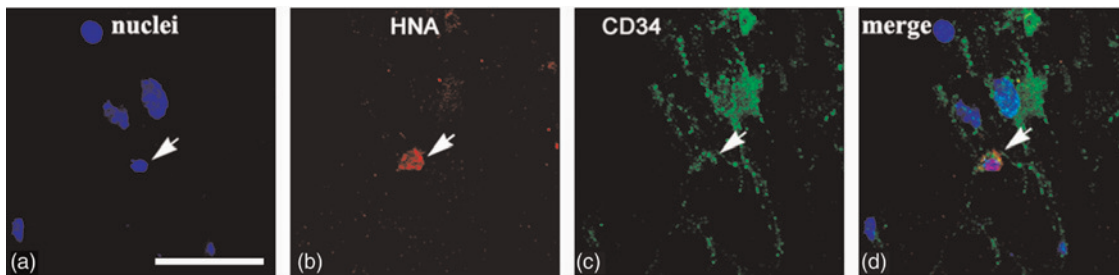


Figure 5 Enhanced green fluorescent protein (EGFP)-transfected human umbilical cord blood cells (hUCBCs) in the spinal cord of $G93^A$ mice 14 d after transplantation. Co-localization of human nuclei antigen (HNA) and CD34 (marker for endothelial cells) is shown. Arrows on panels a–d depict HNA + /CD34+ cells. Scale bar: (a–d) 25 μ m (A color version of this figure is available in the online journal)

its nuclear receptor *flt-1* in lumbar spinal cord motor neurons.²⁴ Recent studies have also shown links between VEGF and ALS.²⁵ Reduced expression of VEGF results in ALS-like progressive degeneration of motor neurons.^{26,27} Injection of VEGF-expressing lentiviral vector into various muscles delayed onset and slowed progression of ALS in $G93^A$ mice.²⁸ The rationale for FGF₂ gene modification of hUCBCs is motivated by its involvement in the differentiation of various cell types via specialized signaling systems. Moreover, FGF₂ is a growth factor with a significant neuroprotective effect. Following neurotrauma, the number of FGF₂-positive cells in the spinal cord is increased up to 15 times,²⁹ suggesting that FGF₂ is an important factor in molecular and cellular mechanisms of neuroregeneration. The positive influence of FGF₂ is partially due to its ability to block caspase-3 gene expression and cell apoptosis at the stage of secondary degeneration,³⁰ which supports regeneration of neurites and restoration of spinal cord function. FGF₂ is strongly implicated in neuro- and gliogenesis from stem cells. Up-regulation of neurogenic gene expression with FGF₂ promotes the migration and maturation of neurons derived from transplanted neural stem cells.³¹ FGF₂ and its receptors contribute to the creation of an important microenvironmental niche that promotes neurogenesis in the adult and aged brain.³² Exposure to FGF₂ selectively induces astrocyte precursors to proliferate, forming clusters of vimentin-positive cells, which, within two weeks, differentiate into astrocytes.³³ FGF₂ promotes conversion of preoligodendrocyte progenitor cells³⁴ and induces oligodendrocyte progenitors in the dorsal

forebrain.³⁵ Neural stem cells cultured with FGF₂/epithelial growth factor (EGF) generate neurospheres, which include cells expressing glial markers.³⁶

Various stem and progenitor cells are present in hUCB-MCs. Culturing or transplantation of hUCBCs results in their differentiation into various cell types; they may give rise to any blood cells, macrophages, endothelial or several mesenchymal cells, etc.³⁷ Gene modification may result in directed differentiation of hUCBCs into any needed cells. Indeed, we have previously demonstrated differentiation of gene-modified hUCBCs into endothelial cells in $G93^A$ mice. Plasmid L₁ cell-adhesion molecule (L₁CAM) and VEGF vectors resulted in addressed homing and transformation of transplanted cells into endothelial cells. In the present investigation we have documented differentiation of hUCBCs into astrocytes: 14 d after transplantation into $G93^A$ mice, hUCBCs over-expressing VEGF and FGF₂ turned into S100+ cells with elongated bodies and extended processes. Self-differentiating potential, modeling the effects of VEGF and FGF₂ on hUCBCs via auto-crine regulation and signal molecules from dying motor neurons in spinal cord possibly created the unique condition and microenvironment for differentiation of hUCBCs into astrocytes. In terms of gene-stem cell therapy of ALS, astrocytic enrichment is known to have neuroprotective effects in motor neuron diseases as it has been demonstrated that astrocyte dysfunction occurs in human ALS and $G93^A$ mice and transplantation of lineage-restricted astrocyte precursors provides therapeutic effect in animal models of ALS.³⁸ Apart from the beneficial effects of the

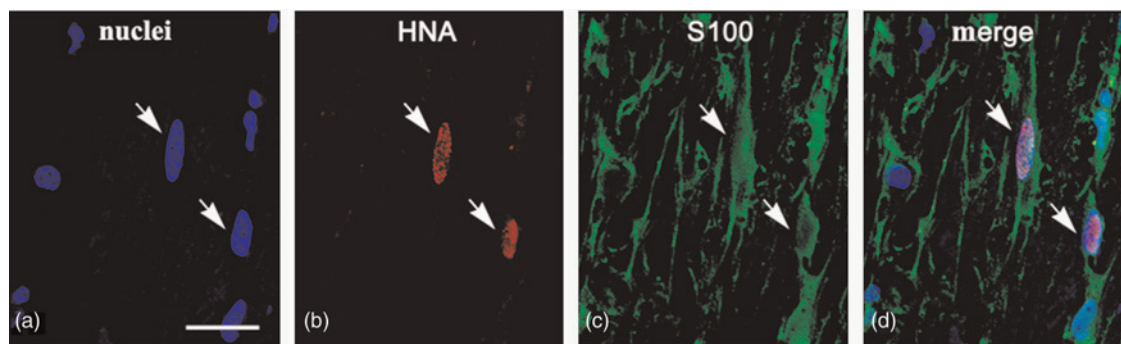


Figure 6 VEGF-FGF₂-transfected human umbilical cord blood cells (hUCBCs) in the spinal cord of $G93^A$ mice 14 d after transplantation. Co-localization of human nuclei antigen (HNA) and S100 is presented. HNA + /S100+ cells have numerous projections: a common feature of astrocytes. Arrows on panels a–d show HNA + /S100+ cells. Scale bar: a–d 25 μ m (A color version of this figure is available in the online journal)

elevated number of astrocytes *per se*, our gene-modified newly formed astrocytes also enriched the local concentration of VEGF and FGF₂ at the site of neurodegeneration, which may further increase survivability of spinal motor neurons. Although our findings have a clinically exploitable potential, further studies are required to address survival of motor neurons, as well as functional benefits of hUCBC transplantation.

Author contributions: All authors contributed to the conception and design, or experiments, analysis and interpretation of the study data, and participated in drafting of the manuscript or revising it critically for important intellectual content. All authors have read and given final approval of the manuscript. AAR and DSG contributed equally to this work.

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