

## ORIGINAL ARTICLE

# Construction of recombinant adenovirus containing picorna-viral 2A-peptide sequence for the co-expression of neuro-protective growth factors in human umbilical cord blood cells

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**Study design:** Experimental study.

**Objective:** Several neuro-degenerative disorders such as Alzheimer's dementia, Parkinson's disease and amyotrophic lateral sclerosis (ALS) are associated with genetic mutations, and replacing or disrupting defective sequences might offer therapeutic benefits. Single gene delivery has so far failed to achieve significant clinical improvements in humans, leading to the advent of co-expression of multiple therapeutic genes. Co-transfection using two or more individual constructs might inadvertently result in disproportionate delivery of the products into the cells. To prevent this, and in order to rule out interference among the many promoters with varying strength, expressing multiple proteins in equimolar amounts can be achieved by linking open reading frames under the control of only one promoter.

**Setting:** Kazan, Russian Federation.

**Methods:** Here we describe a strategy for adeno-viral co-expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) interconnected through picorna-viral 2A-amino-acid sequence in transfected human umbilical cord blood mono-nuclear cells (hUCB-MCs).

**Results:** Presence of both growth factors, as well as absence of immune response to 2A-antigen, was demonstrated after 28–52 days. Following injection of hUCB-MCs into ALS transgenic mice, co-expression of VEGF and FGF2, as well as viable xeno-transplanted cells, were observed in the spinal cord after 1 month.

**Conclusion:** These results suggest that recombinant adeno-virus containing 2A-sequences could serve as a promising alternative in regenerative medicine for the delivery of therapeutic molecules to treat neurodegenerative diseases, such as ALS.

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## INTRODUCTION

Gene therapy offers potential treatment by introducing functional genes into the cells, thereby making them capable of expressing and producing essential proteins or regulatory RNAs.<sup>1</sup> There are several ways of administering exogenous DNA to the patient: gene therapy of cells outside the body (*ex vivo*), in cell culture (*in vitro*), or directly into the organism (*in vivo*). Gene-cell therapy (*ex vivo* or *in vitro*) assumes isolation and/or cultivation of specific cell types of the patient, introduction of recombinant genes, selection of modified cells and administration to the patient (autologous or allogeneic transplantation). Gene therapy *in vivo* is based on direct administration of cloned and specifically packed DNA sequences into the organism. Efficient delivery of various combinations of therapeutic genes to the target cells remains one of the critical problems in gene therapy. Nowadays, different strategies for protein

co-expression are known: independent internal promoters, internal ribosome entry sites (IRESes), mRNA splicing, bi-directional promoters, and posttranslational proteolysis. However, each of these strategies has its own advantages and disadvantages for application. While employing multiple promoters, interference can occur between promoters leading to transcriptional suppression. IRES elements, identified both in viral and cellular eukaryotic mRNAs, differ in length (up to 1 kb) and efficiency of translational initiation. Significant size of IRESes seems to be a restricted factor for using in vectors with limited packaging capacity such as adeno-associated viruses and retroviruses. Besides, IRES position in vector system significantly affects the amount of synthesized protein: the protein downstream of the IRES is only expressed to ~10% of that upstream. Efficiency of IRESes greatly varies in different cell types.<sup>2–7</sup>

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Picornavirus (foot-and-mouth disease virus (FMDV)) 2A-peptide sequences are now widely used for co-expression of multiple genes. The 2A region of the FMDV encodes a sequence that mediates ribosome skipping and self-cleavage.<sup>8</sup> The advantages of this system are: (a) co-expression of proteins linked by 2A is independent of the cell type, (b) multiple proteins are co-expressed in equimolar amounts from a single open reading frame under the control of only one promoter and, (c) 2A size (54–174 bp) allows the construction of compact expression cassettes compatible with size-restricted vectors. However, it should be noted that (a) 2A-peptides normally remain fused as a C-terminal extension of the upstream polypeptide product, and (b) proline amino-acid residue remains at the N-terminus of the downstream protein. Presence of additional amino-acid residues might have a significant effect on polypeptide folding, and immunogenic properties can be one of the limiting factors. In order to minimize these effects, a number of laboratories have incorporated a furin protease cleavage site between the C-terminus of the upstream protein and N-terminus of the 2A sequence such that the C-terminal extension is 'trimmed' away.<sup>8</sup> However, as furin is primarily localized within the Golgi apparatus, this approach can only be used for secreted proteins.

Therapeutic angiogenesis has been expected to be useful as an alternative method of treatment in patients with ischemic and cardiovascular diseases. However, three large-scale clinical trials using recombinant vascular endothelial growth factor A (VEGF-A) or fibroblast growth factor 2 (FGF2) alone yielded results less significant than anticipated.<sup>9,10</sup> By contrast, combined stimulation with two angiogenic molecules from among VEGF-A, FGF2 and platelet-derived growth factor (PDGF)-BB has been reported to be potent in inducing neovascular formation at least in experimental conditions. However, the mechanisms of synergistic effect are still not well enough understood to be able to design optimal combinations of such agents. VEGF and FGF2 have unique roles in synergistic enhancement of endogenous PDGF-B-PDGFR $\beta$  signaling to promote mature blood vessel formation, in addition to their known mitogenic effects.<sup>11</sup>

Previously, we offered a new approach of human umbilical cord blood mono-nuclear cells (hUCB-MCs) modification with dual-cassette plasmids that were capable of mRNA expression of the genes cloned and protein translation both *in vitro* and *in vivo*. Wild-type hUCBCs after transplantation to amyotrophic lateral sclerosis (ALS) transgenic animals may differentiate into endothelial and microglial cells; however, when overexpressing VEGF-FGF2, hUCBCs expressed intrinsic markers of astrocytes (S100+).<sup>12</sup> We have shown that

UCB-MCs overexpressing VEGF and L<sub>1</sub>CAM had increased homing and differentiation into endothelial cells.<sup>13</sup> Also, we have demonstrated that UCB-MCs transfected with pBud-Sox2-Oct4 plasmid DNA increased cell survivability, migration potential, homing, proliferation and ability to differentiate into endothelial cells, microglial cells and, importantly, into neural cells.<sup>14</sup>

Transient transgene expression is one of the drawbacks of using plasmid vectors. Extrachromosomal plasmid vectors are biologically safe in comparison with integrating vectors. Nevertheless, maintenance of transgene expression seems to be a problem as non-replicative episomic plasmids are compromising long-term transgene expression.<sup>15</sup> Viral method of nucleic acid delivery appeals for development of gene-therapeutic approaches for neurodegenerative diseases as well. Adenoviral vectors can infect a wide range of brain cells, including postmitotic neurons; they possess low pathogenicity and high efficiency of transduction.<sup>16</sup> In order to achieve long-term co-expression of multiple transgenes for ALS gene-cell therapy, we developed different combinations of adenoviruses encoding cDNA of *ncam1*, *vegfl65* and *gdnf* genes for UCB-MC modification and subsequent transplantation to transgenic mice with *sod1* mutation. Significant improvement in behavioral performance (open-field and grip-strength tests), as well as increased lifespan, was observed in animals treated with NCAM-VEGF or NCAM-GDNF co-transduced cells. Active transgene expression was found in the spinal cord of ALS mice 10 weeks after transplantation. Transplanted cells were detectable even 5 months after administration.<sup>17</sup>

Despite promising results on application of mixtures of different viruses, delivery of multiple transgenes still remains a restricting factor for further development of fundamentally innovative approaches for gene-cell therapy purposes. Simultaneous viral modification of target cells significantly depends on viral co-transduction efficiency and cell survivability (because of total high viral titers). Selection of optimal vector for transient expression of several transgenes in one cell is an important aim of regenerative medicine. This paper describes the design of multicistronic adenoviral vector encoding VEGF and basic FGF2 interconnected via FMDV 2A-peptide sequence. Efficacy of recombinant genes co-expression was assessed both *in vitro* and *in vivo* after transplantation of UCB-MCs transduced with adenovirus Ad5-VEGF165-FuP2A-FGF2 to ALS transgenic mice.

## MATERIALS AND METHODS

### Construction of recombinant adenovirus co-expressing VEGF165 and FGF2 genes

Primarily VEGF165-FuP2A-FGF2 cassette was generated according to standard methods of genetic engineering. First, asymmetric PCR amplification with gene-specific primers containing sites for restriction enzymes *EcoRI*, *Sall* and nucleotide sequences of 2A-peptide amino-acid residues was performed. The list of primers is presented in Table 1.

After analysis and purification PCR product, VEGF165-FuP2A-FGF2 was used as a template for additional PCR amplification for further subcloning into pDONR221 vector.<sup>18</sup> PCR amplification of gene fragment VEGF165-FuP2A-FGF2 was carried out in two rounds: the first round is used to amplify cDNA with short flanking attB sites using primers hVEGF—attB1 and hFGF2—attB2, nucleotide sequences are presented in Table 1. Second round of PCR amplification was conducted using adapter primers GW—attB1 and GW—attB2 intended to generate full length flanking attB sites. BP-recombination was performed under standard protocol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), followed by transformation into *Escherichia coli* Top 10 competent cells. Accuracy of PCR amplification and the presence of inserts of interest was confirmed by PCR screening with vector-specific primers and sequencing (Table 1).

**Table 1** List of primers for PCR amplification, cloning and screening

Name of the primer	Nucleotide sequence
hVEGF—F1032— <i>XhoI</i> <sup>a</sup>	CTCgagcACCATGAACCTTCTGCTGTCTTGGGT
VEGF—RFuP2A— <i>EcoRI</i> <sup>a</sup>	GAATTCagcccccgggtttcttcaacatctcctgctgctt cgtggcgcaCgattgCACCGCTCGCTTGT GCCACGAAGGAAGCAGGAGATGTTGAA
hFGF2—F468—P2A <sup>a</sup>	GTCGACTCAGCTCTTAGCAGACATTGGAAG
hFGF2—Stop— <i>Sall</i> <sup>a</sup>	AAAAGcAggCTCACCATgAACTTCTfCTgTCTTgg
hVEGF—attB1 <sup>b</sup>	agaaagctgggtTCAGCTCTTAGCAGACATTGGAAG
hFGF2—attB2 <sup>b</sup>	GGGGACAAGTTTGTACAAAAAAGCAGGCT
GW—attB1 <sup>b</sup>	GGGGACCACCTTGTACAAAAAGCTGGGT
GW—attB2 <sup>b</sup>	GTAAACGACGCGCCAGTG
M13 F(-20) <sup>c</sup>	GGAAACAGCTATGACCATG
M13 R(-27) <sup>c</sup>	

<sup>a</sup>Gene-specific primers.

<sup>b</sup>Primers for Gateway cloning.

<sup>c</sup>Vector-specific primers.

### Creation of expression adenoviral vectors using Gateway cloning technology

To generate adenoviral expression constructs, LR-recombination was carried out from donor plasmids pDONR-VEGF165-FuP2A-FGF2 into destination vector pAd/CMV/V5Dest according to the manufacturer's procedures (Invitrogen). After transformation of resulting genetic constructs into competent *E. coli* cells, positive colonies were screened using gene-specific PCR (Table 1). Correct insert of interest was confirmed by restriction analysis and sequencing. Preparative plasmid DNA isolation was performed using the QIAfilter Plasmid Midiprep Kit according to producer's recommendations (Qiagen, Mainz, Germany).

### Transfection of HEK293A cell line with recombinant plasmid

The 293A cell line is a subclone of the 293 cell line and has a relatively flat morphology suitable for screening adenoviral constructs. HEK293A cell line (Invitrogen) was cultured at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (PanEco, Moscow, Russia) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 1 × antibiotic mixture of penicillin and streptomycin (PanEco, Russia) and 2 mM L-glutamine (PanEco). Transfection of HEK293A cell line with genetic construct pAd-VEGF165-FuP2A-FGF2 was carried out using the transfection reagent TurboFect (Thermo Scientific, Rockford, IL, USA) according to the procedure recommended by the manufacturer. Conformation of recombinant protein expression was performed 48 h after transfection by immunofluorescent analysis.

### Immunofluorescent analysis of recombinant protein expression

For fixing transfected HEK293A cells in the wells of the culture plate after removing the culture medium, prechilled methanol (Tatkhimproduct, Kazan, Russia) was added with further incubation at -20 °C for 10 min. After incubation, cells were washed with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.6). Cell membranes were permeabilized by 0.1% solution of Triton X-100 (Helicon, Moscow, Russia). Incubation with primary antibodies was performed in TBS for 1 h; the cells were then washed with TBS and incubated with secondary antibodies for 1 h (Table 2). Cells were observed using inverted fluorescent microscope AxyObserver Z.1 (Carl Zeiss, Jena, Germany).

### Production of recombinant adenovirus

To produce recombinant adenovirus Ad5-VEGF-FuP2A-FGF2, an adenoviral vector plasmid was linearized with a restriction enzyme *PacI* (Thermo Scientific). Purified linear plasmid was used for genetic modification of HEK293A cell line using transfection reagent TurboFect (Thermo Scientific). The cell line contains a stably integrated copy of the E1 gene that supplies the E1 proteins (E1a and E1b) required to generate recombinant adenovirus.

After transfection, we replaced media every 2–3 days with fresh one until the formation of visible cytopathic regions characterizing with changing of cell

morphology. On day 10 after transfection, cells were scraped and suspensions were collected in sterile 2-ml tubes. After collection, cell suspensions were subjected to several freeze/thaw cycles followed by centrifugation to remove cell debris to prepare a crude viral lysate. Viral stock was stored at -80 °C.

### Amplification and titration of virus

To obtain preparative amounts of adenovirus encoding *vegfl65* and *fgf2* genes, HEK293A cell line was infected with crude viral stocks. After 72 h, cell lysate was collected in 15-ml tubes and subjected to several freeze/thaw cycles according to procedure described above. The supernatant was centrifuged, filtered and stored at -80 °C. Determination of viral titers was performed concerning to Invitrogen suggested plaque assay.

### Immunization of animals and serum collection

All manipulations with animals were performed after approval of local ethic committee of the Kazan Federal University. All animals (species *Mus musculus*, males, age 6 months) had free access to food and water.

Primarily, we collected blood from tail vein of each animal that was used to obtain serum. Experimental animals ( $n=6$ ) were injected  $3 \times 10^9$  viral particles of dialyzed adenovirus Ad5-VEGF165-FuP2A-FGF2 intramuscularly in total volume 50  $\mu$ l. As a positive control, we considered a group of animals ( $n=3$ ) that were injected 25  $\mu$ g of 2A-conjugate (CGDVEENPG conjugated with KLH on C-terminus). The conjugate was synthesized by Almabion company (Voronezh, Russia) based on homological sequences of T2A, P2A, G2A) with complete Freund's adjuvant and sterile phosphate-buffered saline (PBS) (total volume of injection 200  $\mu$ l). After 28 days, we collected serum samples and performed booster immunization for each group. Final serum collection was performed 14 days after booster immunization.

Deoxygenated blood was obtained from venous sinus from experimental animals, which were preliminarily narcotized with chloral hydrate (Acros Organics, Pittsburg, KS, USA). Sera samples were obtained by routine centrifugation (3000 r.p.m., 15 min) of blood samples after 1 h incubation.

### Enzyme-linked immunosorbent assay (ELISA) assay

ELISA assay on VEGF antigen was carried out with the commercial kit (Vector Best, Novosibirsk, Russia) according to the manufacturer's instructions. To assess immunogenicity of multicistronic adenoviral construct *in vivo*, we conducted ELISA assay where 2A-peptide conjugate CG-9 (CGDVEENPG conjugated with KLH on C-terminus, Almabion) served as an antigen. To assess the immune response on adenoviral proteins, we used protein extract of HEK293A cells line showing visible cytopathic effect after adenoviral infection as an antigen. Wells were coated with 10  $\mu$ g of antigen solution in carbonate/bicarbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, 0.2 g l<sup>-1</sup> NaN<sub>3</sub>, pH 9.6). After 12 h, incubation wells were washed twice with PBS-T (PBS containing 0,1% Tween 20). The remaining protein-binding sites were blocked by adding 200  $\mu$ l blocking buffer (PBS containing 3% bovine serum albumin) per well. After washing, wells were incubated with primary antibodies in blocking buffer (serum of the experimental animals, dilution 1:100). As a positive control, we used affinity-purified rabbit polyclonal anti-FMDV 2A-peptide antibody, custom produced for us by GenScript (Piscataway, NJ, USA), dilution 1:3000. Antibody was raised against CGDVEENPG peptide, conjugated to KLH. Incubation of plate at +4 °C for 12–16 h. After incubation with primary antibodies, wells were washed five times with PBS-T. Incubation with secondary antibodies (anti-mouse IgG horseradish peroxidase, A4416, Sigma, Hamburg, Germany) was performed at +37 °C for 1 h. For immunogenicity detection, TMB (3,3',5,5'-tetramethylbenzidine, Vecton, Moscow, Russia) solution was added to each well and incubated for 15 min. Reaction was stopped by adding equal volume of 2 M H<sub>2</sub>SO<sub>4</sub> with further plate read at OD<sub>490</sub>.

### Cell preparation

Isolation of mononuclear cells from umbilical cord blood was performed in 50-ml tubes, according to the previously published procedure.<sup>19</sup> To each tube was added 25 ml of a solution of Ficoll with 1.077 g ml<sup>-1</sup> density (PanEco) gently using an automatic dispenser overlaid with an equal volume of umbilical cord blood mixed with anticoagulant (blood and anticoagulant ratio range 1:1–3:1). Centrifugation was performed at 720 g for 20 min to obtain clear

**Table 2** Antibodies used for immunological studies

Antigen	Host	Dilution	Manufacturer (cat. number)
VEGF <sup>a</sup>	Goat	1:500 <sup>b</sup> 1:1000 <sup>c</sup>	Sigma (V6627)
VEGF <sup>a</sup>	Rabbit	1:100 <sup>b</sup>	Santa Cruz (sc-120)
FGF2 <sup>a</sup>	mouse	1:500 <sup>b</sup> 1:1000 <sup>c</sup>	GenScript (A01718-40)
FGF2 <sup>a</sup>	Goat	1:100 <sup>c</sup>	Santa Cruz (sc-7911)
Human nuclei antigen (HNA) <sup>a</sup>	mouse	1:150 <sup>b</sup>	Millipore (MAB1281)
$\beta$ -Actin (HRP) <sup>a</sup>	mouse	1:2000 <sup>c</sup>	GenScript (A00730-40)
Anti-goat Alexa 488 <sup>d</sup>	donkey	1:1000 <sup>b</sup>	Invitrogen (A-11055)
Anti-mouse Alexa 647 <sup>d</sup>	donkey	1:1000 <sup>b</sup>	Invitrogen (A-31571)
Anti-mouse IgG (HRP) <sup>d</sup>	Goat	1:2000 <sup>c</sup>	Sigma (A4416)
Anti-goat IgG (HRP) <sup>d</sup>	Rabbit	1:2000 <sup>c</sup>	Sigma (A5402)

<sup>a</sup>Primary antibodies.

<sup>b</sup>For immunofluorescent staining.

<sup>c</sup>For western blotting.

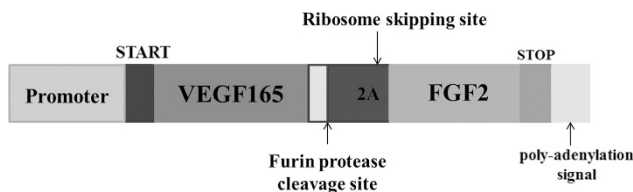
<sup>d</sup>Secondary antibody.

separation of blood into four fractions: erythrocytes, Ficoll, leucocytes, and plasma. Leukocyte fraction was collected into a separate tube, resuspended in DPBS at a ratio of 1:2 and centrifuged at 305 g for 15 min. The resultant cell pellet was resuspended in 10 ml of DPBS and again centrifuged at 305 g for 15 min. To remove erythrocytes, the cell pellet was resuspended in a hypotonic cell lysis buffer (0.168 M NH<sub>4</sub>Cl, 0.1 M KHCO<sub>3</sub>, 1.27 mM EDTA, pH 7.3); at the final stage the cells were washed with a solution of DPBS. After purification, fraction of mononuclear cells from human umbilical cord blood was cultivated in RPMI-1640 medium (PanEco) supplemented with 10 % fetal bovine serum (HyClone) and mixture of antibiotics penicillin and streptomycin (100 U ml<sup>-1</sup>, 100 µg ml<sup>-1</sup>) (PanEco). Immediately after isolation, mononuclear cells were seeded in 10-cm culture dish and transduced with recombinant adenovirus at a multiplicity of infection 10. Cells were incubated for 12–16 h in a humid environment at a temperature of +37 °C with 5% CO<sub>2</sub> content.

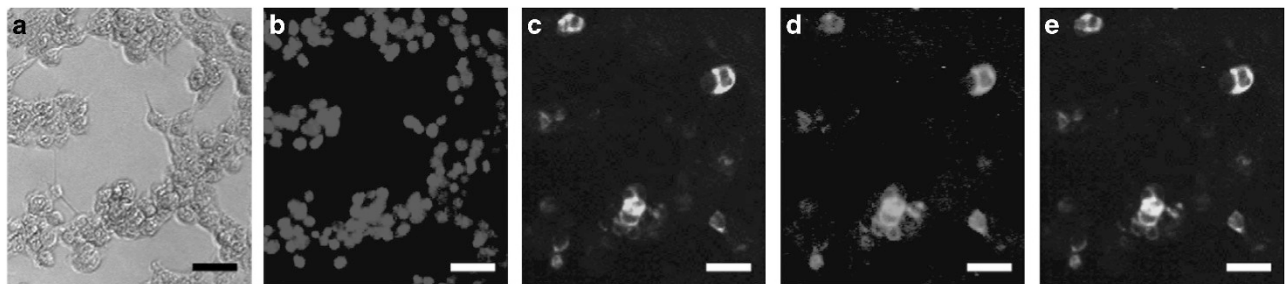
### Animals and treatments

ALS mice, transgenic for mutant human SOD1 gene (B6SJL-TG(SOD1-G93A) dl1Gur/J, Stock No. 002300), were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred at animal facility of Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (Puschino, Russia). Mature 22-week-old male and female mice were delivered to Kazan State Medical University and housed one per cage under standard laboratory conditions. Presymptomatic 27–29-week-old mice were employed in the research. Rats were housed in clear plastic cages (12/12-h light/dark cycle) with food and water available *ad libitum*.

Genetically modified 2 × 10<sup>6</sup> hUCB-MCs were injected retro-orbitally into ALS mice (*n* = 4). Four weeks after transplantation, mice were killed under ketamine–xylazine anesthesia. For histological analysis, euthanized mice were transcardially perfused first with cold PBS and then with cold 4% paraformaldehyde in PBS (pH 7.4). The whole spinal cord was removed, postfixed with paraformaldehyde overnight at 4 °C and then immersed in 30% sucrose solution in PBS (pH 7.4) at 4 °C. Later, lumbar spinal cords were embedded in TBS tissue freezing medium (Triangle Biomedical Science, Durham, NC, USA). Animal treatment protocol was approved by the Kazan State Medical University Animal Care and Use Committee.



**Figure 1** Schematic drawing of expression cassette encoding *vegf* and *fgf2* genes cDNA in a single open reading frame connected by furin cleavage site and 2A peptide sequence. A full color version of this figure is available at the *Spinal Cord* journal online.



**Figure 2** Immunofluorescent assay of HEK293A cells transfected with recombinant plasmid DNA pAd-VEGF165-FuP2A-FGF2. (a) Brightfield channel; (b) DAPI staining; (c) staining with antibodies to VEGF (donkey anti-goat IgG Alexa488 secondary antibodies); (d) staining with antibodies to FGF2 (donkey anti-mouse IgG Alexa 647 secondary antibodies); (e) merge of blue (panel (b)), green (panel (c)) and red (panel (d)) spectra of fluorescence. Scale bar 200 µm. A full color version of this figure is available at the *Spinal Cord* journal online.

### Immunofluorescent staining

Frozen free-floating (20 µm) transverse serial sections of lumbar spinal cord were prepared. To identify transplanted hUCB-MCs and to verify the expression of therapeutic genes, spinal cord sections were subjected to immunofluorescent staining. After blocking of non-specific binding sites with normal donkey serum, the primary antibodies (Table 2) were applied overnight at 4 °C. The subsequent incubation of the sections with proper secondary antibodies (Table 2) for 2 h at room temperature was followed with cell nuclei staining using DAPI (4,6-diamidino-2-phenylindole; 5 mg ml<sup>-1</sup> in PBS, Sigma). Finally, sections were picked up on SuperFrost Plus glass slides (Thermo Scientific), mounted in anti-quenching medium and examined with a laser scanning microscope LSM 780, Carl Zeiss, with ×63 magnification.

To assess the possibility of modified UCB-MCs for differentiation into various cell types, sections were stained with antibodies to Oct6, Aqp4 and HNA antigens according to the protocol described above.

### Statistical analysis

Data are expressed as means ± s.d. We used Student's *t*-test distribution for multiple groups. A value of 0.05 was considered statistically significant. All analyses were performed in a blinded manner with respect to the treatment group. Data were analyzed using MS Excel 2007 software (Microsoft Corporation, Redmond, WA, USA).

## RESULTS

### Design of pAd-VEGF165-FuP2A-FGF2 multicistronic construction

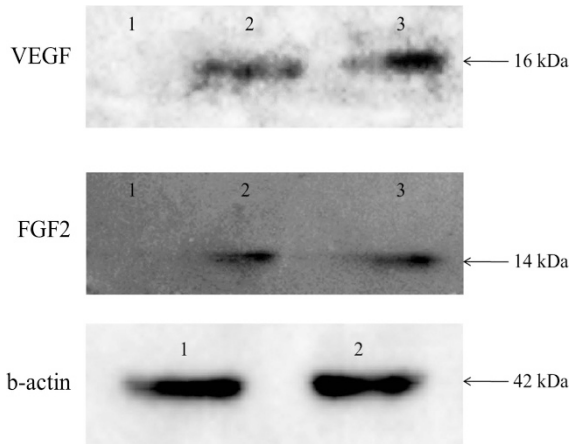
To drive co-expression of both *vegf* and *fgf2* genes from a single mRNA, we first constructed a cassette that contains all regulatory elements (CAP, initiation site, genes interconnected via Fu2A—sequence, stop codon and polyA signaling site). The scheme of expression cassette is presented in Figure 1.

Using gene-specific primers flanked with 2A-peptide sequences, we PCR amplified and cloned VEGF165-FuP2A-FGF2 cassette into pDONR<sub>221</sub> plasmid vector. Then using Gateway recombination, we generated adenoviral expression construct pAd-VEGF165-FuP2A-FGF2. Correct cloning was confirmed using restriction analysis and sequencing (data not shown).

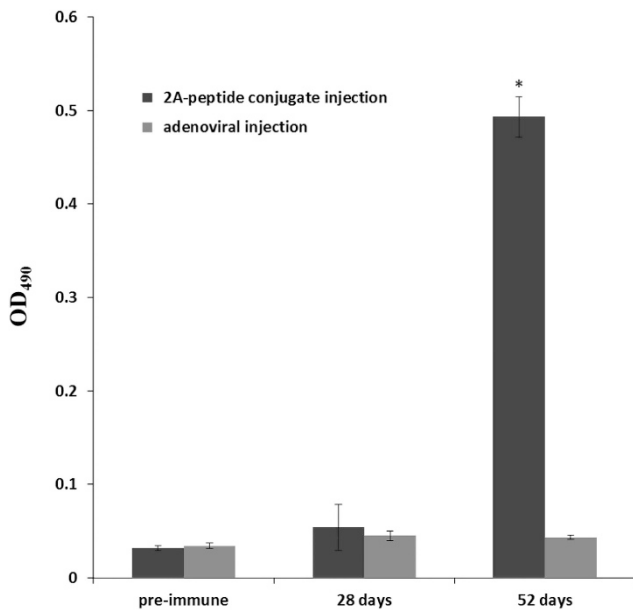
### VEGF165 and FGF2 co-expression *in vitro*

Immunofluorescent assay was conducted 48 h after transfection of HEK293A cell line with recombinant adenoviral plasmid pAd-VEGF165-FuP2A-FGF2. Simultaneous staining with antibodies against VEGF and FGF2 proteins has shown positive reaction confirming co-expression of both growth factors in each transfected cell (Figure 2).

Analysis of cell extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with specific antibody staining has shown expression of monomeric VEGF and FGF2 proteins with the size of



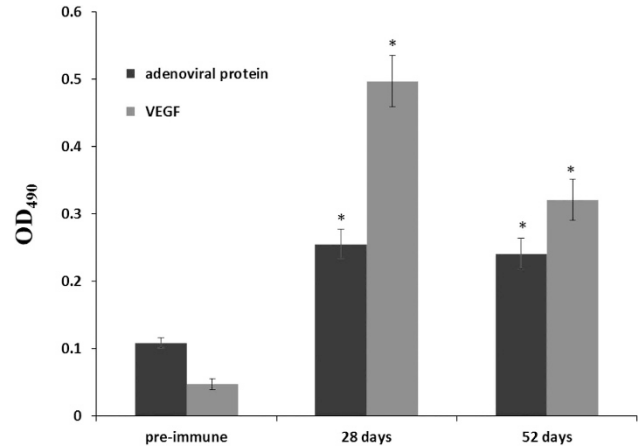
**Figure 3** Western blotting analysis of protein extracts from HEK293A cells transfected with pAd-VEGF165-FuP2A-FGF2 plasmid. Staining against VEGF (goat anti-VEGF monoclonal antibodies, Sigma): 1—non-transfected cells, 2—HEK293A cells transfected with pAd-VEGF165-FuP2A-FGF2, 3—recombinant VEGF; staining against FGF2 (mouse monoclonal antibody to FGF2, GenScript): 1—non-transfected cells, 2—HEK293A cells transfected with pAd-VEGF165-FuP2A-FGF2, 3—recombinant FGF2; staining against b-actin: 1—HEK293A cells transfected with pAd-VEGF165-FuP2A-FGF2, 2—non-transfected HEK293A cells. A full color version of this figure is available at the *Spinal Cord* journal online.



**Figure 4** Direct ELISA assay of immune response to 2A-peptide antigen in mice 28 and 52 days postinfection with adenovirus Ad5-VEGF165-FuP2A-FGF2 ( $n=6$ ) or immunization with 2A-peptide conjugated with KLH ( $n=3$ ). Data present as average  $OD_{490} \pm s.d.$  Asterisk (\*) represents statistically significant differences between mice groups. A full color version of this figure is available at the *Spinal Cord* journal online.

fragments corresponding to recombinant protein. It is worth mentioning that staining with affinity-purified rabbit polyclonal anti-FMDV 2A-peptide antibody (GenScript) has not shown positive reaction (Figure 3).

After confirmation of VEGF and FGF2 co-expression *in vitro* by western blotting and immunofluorescent assay, recombinant



**Figure 5** Direct ELISA assay of immune response to adenoviral and VEGF antigens in mice ( $n=6$ ) after injection of Ad5-VEGF165-FuP2A-FGF2. Data present as average  $OD_{490} \pm s.d.$   $P < 0.05$ . Asterisk (\*) represents statistically significant differences between mice groups. A full color version of this figure is available at the *Spinal Cord* journal online.

adenovirus was obtained. To produce recombinant adenovirus Ad5-VEGF165-FuP2A-FGF2, plasmid pAd-VEGF165-FuP2A-FGF2 was linearized by digestion with *PacI* enzyme, with further transfection of HEK293A cells. After transfection, we replaced media every 2–3 days with fresh one until the formation of visible cytopathic regions characterizing with changing of cell morphology. On day 10 after transfection, cell suspensions were collected in sterile 2-ml tubes. After collection, cell suspensions were conducted by several freeze/thaw cycles followed by centrifugation to prepare a crude viral lysate. Crude viral stock was stored at  $-80^{\circ}\text{C}$ . Preparative amount of adenovirus was obtained by infection of HEK293A cells with primary viral stock. After 4 days postinfection, cell suspensions were collected in sterile 15-ml tubes with further cryolysis. Filtered supernatant was concentrated in CsCl gradient according to standard protocol. Viral titer was determined after dialysis against PBS by measurement of optical density and plaque unit assay. Viral titer is  $3.2 \times 10^9$  pfu  $\text{ml}^{-1}$ .

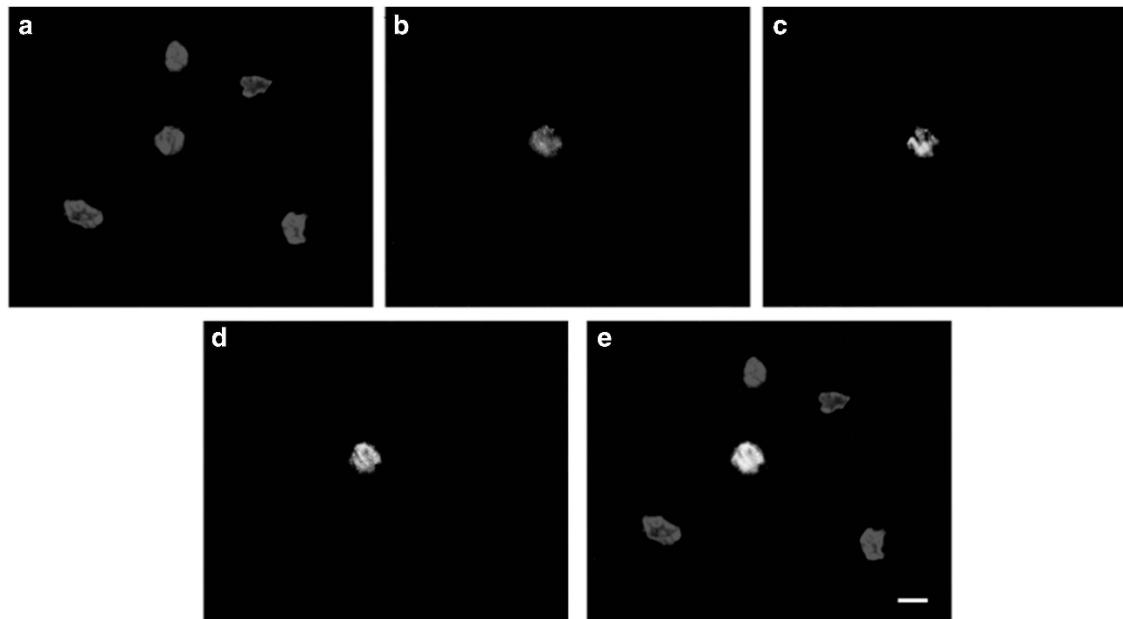
#### Immunogenicity of recombinant Ad5-VEGF165-FuP2A-FGF2 adenovirus

To assess immunogenicity of recombinant adenovirus Ad5-VEGF165-FuP2A-FGF2, we carried out series of *in vivo* experiments on healthy animals. Two groups of animals were injected with dialyzed adenovirus ( $3 \times 10^9$  viral particles per animal) or 2A-peptide/KLH conjugate with complete Freund's adjuvant. After 28 days from first immunization, booster injection was performed with further blood collection after 14 days. Presence of antibodies in sera samples to VEGF, 2A-peptide and adenoviral proteins was detected by direct ELISA. Preimmune sera collected before treatment served as a negative control.

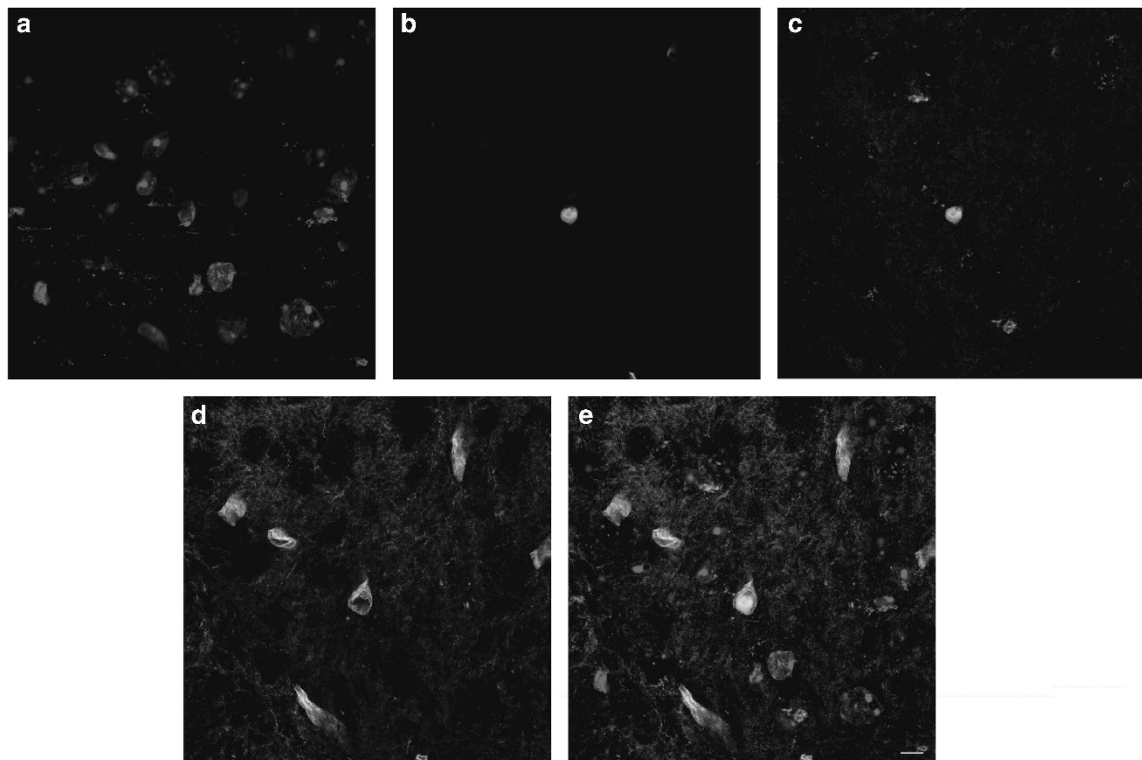
Figure 4 demonstrates robust immune response in 2A-peptide/KLH conjugate-injected animals, whereas no statistically significant immune response was observed in recombinant adenovirus-infected animals. The immune response to VEGF and adenoviral proteins was detected after first immunization (Figure 5).

#### Co-expression of recombinant VEGF and FGF2 in genetically modified hUCB-MCs after transplantation to ALS transgenic mice

Immunofluorescent analysis of lumbar spinal cord was performed 4 weeks after hUCB-MC transplantation. Anti-HNA antibody



**Figure 6** Immunofluorescent analysis of VEGF and FGF2 co-expression in lumbar spinal cords of ALS transgenic mice after transplantation of hUCBCs modified with recombinant adenoviral vector. (a) DAPI staining; (b) staining against human nuclear antigen (donkey anti-mouse IgG Alexa 647 secondary antibodies); (c) staining against VEGF (donkey anti-rabbit IgG Alexa 488 secondary antibodies); (d) staining against FGF2 (donkey anti-goat IgG Alexa 555 secondary antibodies); (e) merge of blue (panel (a)), red ((panel (b)), green ((panel (c)), yellow (panel (d)) spectra of fluorescence. Magnification:  $\times 63$ . Scale bar  $20\ \mu\text{m}$ . A full color version of this figure is available at the *Spinal Cord* journal online.



**Figure 7** Immunofluorescent analysis of lumbar spinal cords of ALS transgenic mice after transplantation of hUCBCs modified with recombinant adenoviral vector. (a) DAPI staining; (b) staining against human nuclear antigen (donkey anti-mouse IgG Alexa 647 secondary antibodies); (c) staining against Oct6 (donkey anti-rabbit IgG Alexa 555 secondary antibodies); (d) staining against Aqp4 (donkey anti-goat IgG Alexa 488 secondary antibodies), (e) merge of blue (panel (a)), red (panel (b)), green (panel (c)), yellow (panel (d)) spectra of fluorescence. Magnification:  $\times 63$ . Scale bar  $20\ \mu\text{m}$ . A full color version of this figure is available at the *Spinal Cord* journal online.

was used to identify grafted hUCB-MCs in the spinal cord. HNA-positive cells were observed in the white matter of spinal cord in all experimental mice. Simultaneous staining of lumbar spinal cord sections using antibodies to HNA, human VEGF and human FGF2 revealed HNA+/VEGF+/FGF2+ hUCB-MCs (Figure 6).

#### Investigation of differentiative potential of genetically modified cells *in vivo*

To assess the possibility of genetically modified UCB-MCs to differentiate into various cell types of neuron origin, quadruple staining of the same sections was performed. Except HNA and DAPI staining, lumbar spinal cords were also analyzed for expression of Oct6 and Aqp4. Simultaneous staining with antibodies to Oct6, Aqp4 and human nuclear antigen revealed positive HNA<sup>+</sup>/Oct6<sup>+</sup>/Aqp4<sup>+</sup> cells in the white matter of lumbar spinal cord (Figure 7).

#### DISCUSSION

Gene therapy is a relatively modern field of regenerative medicine aiming at modifying the genetic apparatus of cells to halt the disease progression. Targeting hereditary diseases with known mutations seems to be most straightforward application for gene therapy. When using different approaches, doctors can either express necessary gene (gain of function) or eliminate pathological genes or mRNA transcripts through genome editing or RNA interference. On the other hand, agnogenic diseases with unknown pathogenesis or genetic origin might require targeting general regenerative aspects of an organism, such as cell death or proliferation.<sup>20</sup>

First step in developing gene therapy drug is verification of efficacy on the cell models (primary and immortalized cell cultures). Although crucial, it does not answer the question about the recombinant vector activity at the level of whole organism. Another important aspect of developing of any genetic vectors before clinical trials is immunogenicity assessment *in vivo*.

Many gene therapy applications would benefit from co-expression of multiple proteins from a single vector. FMDV 2A and '2A-like' sequences (for example, *Thosea asigna* virus 2A, T2A) are used widely for this purpose as multiple proteins can be co-expressed by linking open reading frames to form a single cDNA. But the question of 2A-peptide immunogenicity during co-expression of recombinant proteins from single vectors still remains unclear. 2A-peptide sequences have enabled more complex strategies of co-expression and trans-genesis and have been used in many laboratories with highly successful results. The variations in cleavage activity of the various lengths of 2A-peptides are attributed to the nature of the region immediately upstream of CHYSEL (18–30 amino acid upstream of the cleavage site).<sup>21</sup> Using combination of furin recognition site and 2A-peptide strategy of expression has shown stable co-expression of transgene in different cell lines.<sup>22</sup> Although the 2A system is appropriate for delivering co-expression of particular sets of transgenes, it is not suited in situations where individual transgenes require a different tissue distribution.<sup>23</sup>

Our studies of immunogenic properties of obtained adenoviral construction demonstrated low immunogenicity of 2A peptide sequences when utilizing furin cleavage. We also demonstrated proper posttranslational processing of recombinant proteins. Previously, we demonstrated that co-expression of VEGF and FGF2 using dual cassette expression plasmid in UCB-MCs was effective in promoting differentiation of UCB-MCs into astrocytes after transplantation into ALS transgenic animals.<sup>12</sup> Application of recombinant adenovirus co-expressing VEGF and FGF2 proteins in gene-cell therapy on a

model of ALS demonstrated successful co-expression of transgenes in genetically modified UCB-MCs in the spinal cord of ALS transgenic mice. Stable expression of both transgenes and human nuclear antigen was observed after 30 days posttransplantation. This fact points to survivability and migratory potential of modified hUCB-MCs to the lesion and suggests the expression of different markers intrinsic to various populations of neural cells. The performed experiment deserves close attention in comparison to previously applied strategies because of its efficacy, accuracy and stability with absent immunogenicity. Data obtained serve the initial base for preclinical studies of new approach for ALS gene-cell therapy with adenoviral multicistronic constructions.

Gene-cell therapy using various adenoviral combinations has shown promising results in the treatment of ALS transgenic mice. However, the question of survival of transduced cells and estimation of optional viral load has still remained acute. Using 2A-peptide strategy for co-expression of several genes might be a great opportunity for genetic information economy. Taking into account that package capacity of adenoviral vectors is 8000 bp, it is optional to create a construction containing several therapeutic genes and reporters to assess the efficiency of transduction. Constructed recombinant adenovirus containing 2A-amino-acid sequences has proved to be an attractive alternative for stable co-expression of VEGF and FGF2 genes from single open reading frame. *In vivo* studies have shown stable expression of therapeutic genes and absence of immunogenicity on 2A-antigen in adenoviral trials. Moreover, grafted cells were shown to express specific markers intrinsic to neural cells: Oct6 is a transcription factor and a marker of active nerve regeneration and dedifferentiation of adult Schwann cells.<sup>24</sup> Onset of motoneuron death characterizing amyotrophic lateral sclerosis is closely linked to modified astrocytic and glial environments. Recent studies have shown that AQP4 was overexpressed in the spinal cord, brainstem and cortex of ALS rat. It have been also documented that an increased expression of AQP4 was apparent in the astrocytic endfeet, particularly in those surrounding blood vessels.<sup>25</sup>

Summarizing all of the above approaches of cell-based gene therapy, the recombinant adenoviruses containing furin 2A-sequences is a promising strategy in regenerative medicine for delivery therapeutic molecules to treat, for example, neurodegenerative diseases, such as ALS.

#### DATA ARCHIVING

There were no data to deposit.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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