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EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AFTER TRANSFECTION OF HUMAN NEURAL STEM CELLS WITH THE LENTIVIRAL VECTOR ENCODING THE *VEGF165* GENE

We examined the expression of vascular endothelial growth factor (VEGF) and characteristics of human neural stem cells after transfection with the lentiviral vector encoding the *VEGF 165* gene. The latter gene was amplified from the human breast cancer cell line MCF-7 using RT-PCR; then the target gene was cloned into the pCDH–CMV–MCS-EF1-copGFP, a lentiviral expressing plasmid. After transformation, enzyme digestion led to a correct length of the *VEGF165* gene, and DNA sequencing analysis confirmed that the *VEGF165* gene sequence was exactly the same as that reported by the GeneBank. Then, the recombinant lentivirus produced by 293T cells and packaging plasmids were transfected into fourth-passage human neural stem cells (NSCs). One week after transfection with pCDH-*VEGF165*, NSCs expressed VEGF stably, and their proliferation ability significantly increased. Furthermore, human NSCs kept their characteristics and multiple differentiation activity after transfection. Our results indicate that human NSCs can express vascular endothelial growth factor highly and stably via transfection with the lentiviral vector encoding the *VEGF165* gene, which may be useful for future research on function recovery after stroke.

INTRODUCTION

Intracerebral hemorrhage (ICH) is the most dangerous stroke type, as the mortality approaches 50%, and neurological disability in survivors is common. Medical therapysurgery against ICH, such as mechanical removal of a hematoma, prevention of edema formation by drugs, and reduction of the intracranial pressure, shows only limited effectiveness [1]. Recent progress in stem cell biology provides a new way in therapeutic strategies, to replace lost neural cells by transplantation of neural stem cells (NSCs) in the CNS injury [2-4]. However, the low survival rate of grafted NSCs in stroke animals in previous model studies is a grave concern; less than 50% of grafted NSCs survived in ICH mice at two weeks post-transplantation [5].

A prospective way to promote differentiation and survival of transplanted NSCs is to modulate the microenvironment in the injured brain following ICH, and this might be accomplished by supplying additional neurotrophic growth factors, such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and or vascular endothelial growth factor (VEGF). These factors are known to play key roles in proliferation, differentiation, and survival of NSCs. VEGF is one of such growth factors, which could be used in combination with transplanted NSCs to improve the therapeutic efficiency of cellular transplantation. VEGF is an angiogenic growth factor acting as a

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potent mitogen and survival factor specific with respect to endothelial cells [6, 7]; its neuroprotective effect against ischemic injury has been demonstrated [8-12]. Considering evidence of functional recovery in stroke animal models following brain transplantation of human NSCs [13-16] and VEGF treatment [8-12], we hoped to construct a lentiviral vector carrying the human *VEGF165* gene and to transfect it into human NSCs to produce human NSCs with a VEGF-secreting combination. This was expected to provide the advantages in NSC transplantation and gene therapy; the groundwork for VEGF165 genetically modified NSCs transplantation should promote neuroangiogenesis and revascularization of ICH.

METHODS

Cell Lines and Vector. Human NSCs were a gift from the Buck Institute for Research on Aging, Novato (USA). The lentiviral vector pCDH-CMV-MCS-EF1-copGFP containing the *GFP* gene was a gift from the Fudan University, Shanghai Cancer Center (China). The DH5α cell line, breast cancer line MCF-7, and 293T cells were provided by the Surgery Laboratory of the First Affiliated Hospital (Wenzhou Medical University, Wenzhou, China).

Construction and Verification of the Lentiviral Vector pCDH-*VEGF165*. The respective RNA was extracted from MCF-7 cells using Trizol reagent; the human *VEGF165* gene was amplified by the reverse-transcription PCR method. The primers used for the human *VEGF165* gene (GeneBank accession number: AF486837) were the following: 5′-CATAGAAGAT TCT AGAATGAACTTTC TGCTGTCTTGGGTGC AT-3′ (forward) and 5′-ATTTAAATTCGA ATTTCA CCG CCTCGGCTTGTCACATCTGCA-3′ (reverse); those containing the Xbal and ECOR I restriction sites are underlined. These primers were synthesized by the Jikang Gene Technology Co. Ltd. (China). The PCR reaction was performed with the Ex taq DNA polymerase (Takara Co., Japan) in the three steps: (1) 94°C predenaturation for 5 min, (2) 35 amplification cycles at 94°C predenaturation for 30 sec, 59°C primer annealing for 45 sec, then primer extension at 72°C for 1 min, and (3) 72°C for 5 min. The 576 base pair (bp) fragment was generated. A total of 10 µl of the production was used for analysis on 1% agarose gel containing EB, and then the gel was extracted. The pCDH-CMV-MCS-EF1-copGFP vector and human *VEGF165* gene fragment were digested by Xbal and

ECOR I endonuclease (Fermentas, Canada). The *VEGF165* was cloned into the viral vector at 50°C for 15 min using In-Fusion™ HD Cloning Kits (Takara Co., Japan). The connected product was transformed into the *E. coli* competent cells (DH5α) and cultured in LB media (1% tryptone, 0.5% yeast extract, and 1% NaCl) with 50 µg/ml kanamycin at 37°C overnight. This was followed by the restriction enzyme digestion and sequencing analysis (Sangon Biotec, China). Positive clones were designated as pCDH-*VEGF165*. The latter and packaging plasmids (SBI, USA) underwent endotoxin-free extraction. The 293T cells were cotransfected with the plasmids using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, USA). The above cells transfected with the four plasmids without the *VEGF165* target gene served as an empty vector control. The diluted DNA solution (24 µg backbone, 1.2 µg tat, 1.2 µg rev, 1.2 µg gag/pol, and 1.2 µg vsv-g) was combined with diluted Lipofectamine 2000. The mixture was added to 293T cell cultures and incubated at 37°C with 5% CO_2 for 8 h. The medium was then replaced by fresh medium supplemented with 10% serum. Cells were incubated for additional 48 h. The supernatant was collected and concentrated at the 48th h after transfection. The viral titer (TU/ml) was determined by a limiting dilution method [17].

Lentiviral Vector Transfection of Human NSCs. We used the fourth passage of human NSCs under the log phase of growth for studying, centrifuging, resuspending, and adjusting the density of cells to $1 \cdot 10^6$ ml^{-1}, then adding the lentiviral vector (multiplicity of infection = 60) and polybrene (8 µg/ml, Sigma, USA) to the NSC medium and mixing. We designated the pCDH-*VEGF165* group as group 1, the empty vector group as group 2, and the NSCs control group as group 3. The medium was removed 12 h post-transfection. The NSC medium was added for further incubation. Green fluorescence was visible under a fluorescence microscope (Olympus, Japan). The transfection efficiency was detected by flow cytometry.

RT-PCR. Total RNAs were extracted from each group of cells 7 days post-transfection. The primers used for the human *VEGF165* gene (GeneBank accession number: AF486837) were the following: 5′-CCCACTGAGGAGTCCAACAT-3′ (forward) and 5′-TCTTGTCTTGCTCTATCTTTCTTTG-3′ (reverse); those were synthesized by the Jikang Gene Technology Co., Ltd. (China). One microgram of total RNA was reverse-transcribed into first-strand cDNA using an

oligo-dT primer. Reverse transcription was performed with AMV reverse transcriptase (Takara, Japan) for 1 h at 42°C, inactivated for 10 min at 95°C, and cooled to 4°C. The cDNA was diluted to a final volume of 25 µl, and a 2-µl aliquot was used in a PCR reaction containing 1.0 mM DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol primers, and 2.5 units of Taq polymerase (Takara, Japan). Thirty PCR cycles were carried out, and RT-PCR products were separated electrophoretically on a 2% agarose gel containing ethidium bromide (EB) and visualized under UV light; GAPDH was used as the control.

Western Blot Analysis. The proteins were extracted from each group of cells 7 days post-transfection. Protein samples (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose; the blots were probed with antibodies specific for human VEGF165 (1:1000, Millipore, USA). Secondary antibodies (Zhongshan, China) and a chemiluminescence kit (Amersham, USA) were used for immunodetection; GAPDH served as a loading control. Each group of cells ($1 \cdot 10^6$ cells/ml) was incubated in DMEM with 3% fetal bovine serum for 24 h; supernatants were collected by centrifugation at 12,000 rpm for 10 min. The levels of VEGF165 in culture supernatants were determined by ELISA kits specific for human VEGF165 (R&D System, USA), and the absorbance was measured at 450 nm using a spectrophotometer.

CCK 8 Assay Gene-Modified NSCs Proliferation. NSCs were re-plated to 96 coated well plates with poly-L-ornithine ($1 \cdot 10^4$ cells (100 µl/well), cultured with NSC medium + CCK-8 solution (10 µl/well, Dojindo, Japan) at times of 6, 12, 24, 48, 60, and 72 h post-plated, and incubated for 4 h. The absorbance values at 490 nm were measured by a microplate reader (Bio-Rad, USA) to analyze cell proliferation in each group.

Immunocytochemistry. NSCs (8 weeks post-transfection) were plated on 6-well plates with poly-L-ornithine-coated glass coverslips at $2 \cdot 10^5$ cells/well. After culturing with serum-free medium and bFGF for 4 days, we tested the immunopositivity for stem cell markers, nestin and sox2 (Millipore, USA). In order to evaluate the differentiation capacity of pCDH-*VEGF165*-transfected cells, the latter were also re-plated on 6-well plates with poly-L-ornithine-coated glass coverslips at $2 \cdot 10^5$ cells/well. Individual wells were supplied with a growth medium consisting of Neurobasal® Media containing 10% fetal bovine serum (FBS). The samples were then cultivated for 7 days, and antibodies against GFAP, MAP-2, and O4 (Millipore, USA) were utilized to detect differentiated cells. Cells were counterstained with 4′,6-diamino-2-phenylindole (DAPI, Sigma, USA) to identify the cell nuclei. Following immunostaining, the cells were mounted on glass slides using gelvatol, observed, and photographed under a fluorescent microscope (Zeiss, Germany).

Statistical Analysis. Numerical data were expressed as means ± s.d. and analyzed with SPSS 10.0 software (SPSS, USA) using one-way analysis of variance. Intergroup differences with $P < 0.05$ were considered statistically significant.

RESULTS

Construction and Verification of the Lentiviral Vector pCDH-*VEGF165*. The *VEGF165* gene was amplified successfully by reverse-transcription PCR (RT-PCR) from the human breast cancer cell line MCF-7. The results showed a 576 bp specific band in 1% agarose electrophoresis, which was consistent with the theoretical value (Fig. 1A). Recombinant plasmids (pCDH-*VEGF165*) were digested by double restriction enzyme (Xbal and ECORI), and 1% agarose gel electrophoresis showed a right band (Fig. 1B). The results of gene sequencing (Sangon Biotec, Co., Ltd (Shanghai) are consistent with the human *VEGF165* gene sequence in the GeneBank, without mutation or missing, which indicates that the clone has been completed successfully. Forty-eight hours later, abundant green fluorescence under an inverted fluorescence microscope was observed (Fig. 1C). According to calcium phosphate precipitation, the virus titer is $1.0 \cdot 10^8 \sim 1.0 \cdot 10^9$ TU/ml.

Transfection. Five days after transfection of pCDH-*VEGF165* into human NSCs, abundant expression of green fluorescent protein (GFP) in cells can be observed under an inverted fluorescence microscope (Fig. 2A1-A2). The transfection efficiency estimated by flow cytometry (FCM) was 63% (Fig. 2B1-B3).

***VEGF165* Gene Expression in Transfected NSCs.** Expression of the human *VEGF165* gene in transfected NSCs was detected by RT-PCR, Western blot analysis, and ELISA. As a result, a specific positive expression band was observed in the pCDH-*VEGF165* transfection group. It corresponded to a range of 100-200 bp, and there was no band observed in both empty vector transfection group and NSCs control
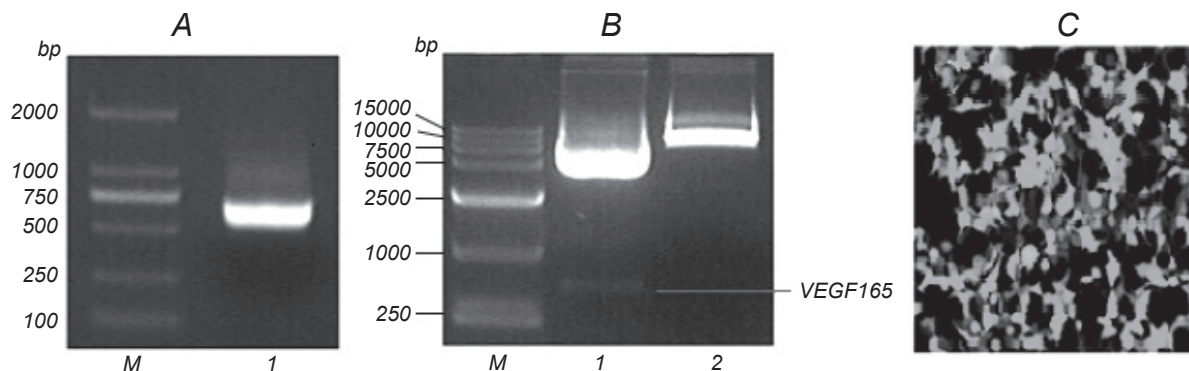
F i g. 1. Construction and verification of the lentiviral vector pCDH-*VEGF165*. A) Human *VEGF165* gene detected by 1% agarose gel electrophoresis. M is DNA marker; 1 is *VEGF165* DNA; B) Identification of pCDH-*VEGF165* by restriction enzyme digestion. M is DNA marker; 1 is pCDH-*VEGF165* digested by Xbal and ECORI, and 2 is pCDH-*VEGF165*. C) GFP expression in pCDH-*VEGF165* transfected 293T cells (×100).

Р и с. 1. Конструювання та верифікація лентивірусного вектора плазміди pCDH–*VEGF165*.

group from RT-PCR (Fig. 3A). Human VEGF165 protein expressed in the pCDH-*VEGF165* transfection group had the molecular mass of 16 kDa; there were no corresponding manifestations in the empty vector transfection group and NSC control group (Fig. 3B). ELISA analyses indicated that human VEGF165 released by pCDH-VEGF165-transfected NSCs in the culture media corresponded to about 440% of the respective indices in the empty vector transfected group and NSC control group: 1947 ± 40.67 pg/10^6 cells·day (mean ± s.e.m.) vs. 443.4 ± 23.69 and 422.6 ± 24.07 pg/10^6 cells·day, respectively ($P < 0.05$) (Fig. 3C).

Cell Proliferation. The cell Counting Kit (CCK8) assay demonstrated that cells in the three groups slowly proliferated during 24 h and began to proliferate more intensely 48 h later. The cell proliferation index

was noticeably higher in the pCDH-VEGF165 group compared to that in the empty vector and control groups ($P < 0.05$). The absorbance values at 490 nm were similar in the empty vector and control groups ($P > 0.05$), suggesting that precisely pCDH-*VEGF165* lentiviral transfection contributed toward enhanced NSCs proliferation (Fig. 4).

Differentiation. When pCDH-*VEGF165* transfected human NSCs were cultured in the serum-free medium with fibroblast growth factor 2 (βFGF) and epidermal growth factor (EGF), the cells proliferated and formed neurospheres. Undifferentiated cells (neurospheres) were suspended in the medium and showed abundant green fluorescence under an inverted fluorescence microscope (Fig. 5A). After the withdrawal of growth factors, the differentiated cells were attached to poly-ornithine-coated culture flasks in the serum-containing
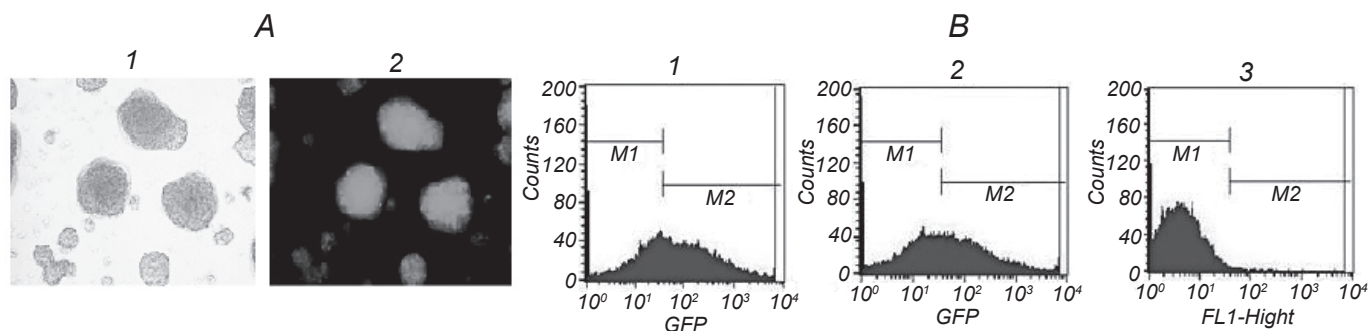

F i g. 2. Transfection of pCDH-*VEGF165* into human NSCs. A) Abundant fluorescence of green fluorescent protein (GFP) expressed in human NSCs 5 days after transfection. A1) Under visible light (x100); A2) in the fluorescence mode (x100). B) Flow cytometric analysis of the transfection efficiency in pCDH-*VEGF165*-transfected human NSCs. The transfection efficiency is about 63% in the pCDH-*VEGF165* group (B1), 54% in the empty vector group (B2), and 0.73% in the NSCs control group (B3).

Р и с. 2. Трансфекція pCDH–*VEGF165* у невральні стовбурові клітини людини.

medium and grew toward the outside from the cell core at the 2nd day (Fig. 5B). These units differentiated into other cells at the 5th day and also carried green fluorescence (Fig. 5C). Immunofluorescence staining showed that pCDH-*VEGF165*-transfected cells still expressed nestin and sox2 proteins (Fig. 5D-E). In order to evaluate the differentiation capacity of the pCDH-*VEGF165*-transfected NSCs, post-mitotic daughter cells in the serum-containing medium were evaluated. Such cells expressed the immunoreactivity to GFAP, MAP-2, and O4; thus, it was possible to identify them as astrocytes, neurons, and oligodendrocytes, respectively (Fig. 5F-H). These facts indicated that the pCDH- *VEGF165*-transfected cells still maintain their stem cell characteristics and keep their ability of differentiation.

DISCUSSION

Neural stem cells show a multipotent capacity to differentiate into neurons and glial cells [18-21]. These cells are able to ameliorate neurological deficits in animal models of Parkinson's disease [22], Huntington's disease [23] and lysosomal storage disease [24] following their transplantation into the brain.

In stroke animal models, intravenously transplanted NSCs migrated selectively to the damaged brain sites caused by ischemia and intracerebral hemorrhage (ICH) [13-16]. These units differentiated into neurons and astrocytes and noticeably promoted functional recovery in these animals. However, a low survival rate of grafted NSCs in rats with ischemia and/or ICH observed in previous studies should be considered an important restriction factor. One significant way to promote differentiation and survival of transplanted NSCs is to modulate the microenvironment in the injured brain following ICH.

VEGF is known to play a key role in proliferation, differentiation, and survival of NSCs. For example, VEGF enhanced the survival level in mouse hippocampal neurons subjected to *in vitro* ischemia [25] and glutamate or N-methyl-D-aspartate (NMDA) excitotoxicity [26, 27]. In a middle cerebral artery (MCA) occlusion-induced ischemia model, VEGF was found to intensify proliferation and increase survival of endothelial cells under conditions of ischemic injury [28]. Moreover, VEGF is also beneficial to the ICH-injured brain indirectly by promoting angiogenesis. The formation of new vessels mediated by VEGF will improve blood supply of the injured region, thereby effectively providing oxygen and nutrients to
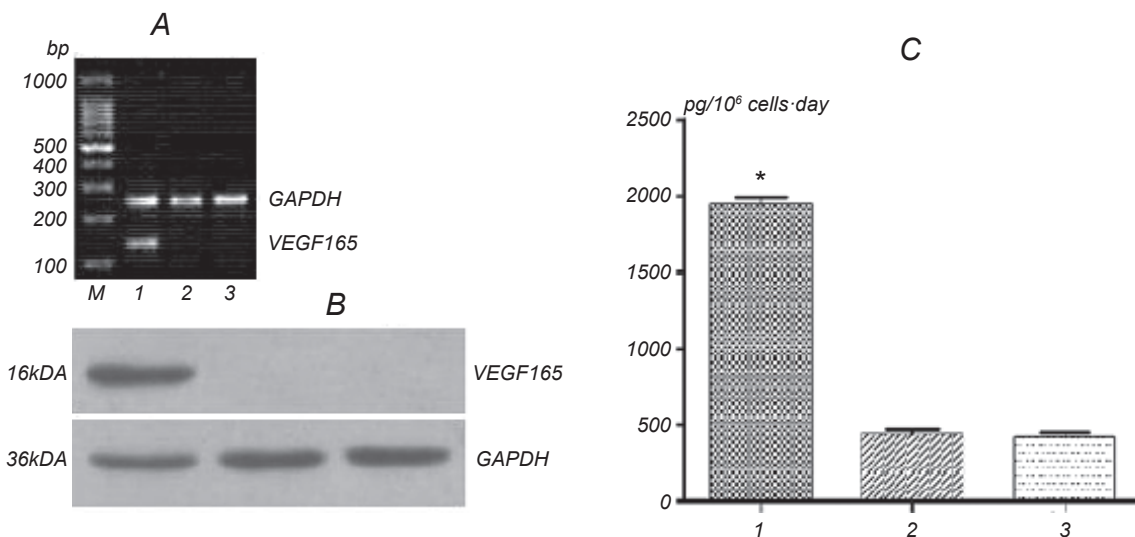

F i g. 3. Expression of the human *VEGF165* gene in pCDH-*VEGF165*-transfected NSCs. A) Human *VEGF165* mRNA expressed in the pCDH-*VEGF165* transfection group (results of RT-PCR; range of 100-200 bp; there was no band observed in the empty vector transfection group and NSC control group). A house-keeping protein, D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as the control. B) Expression of human VEGF165 protein (16 kDa) in the pCDH-*VEGF165* transfection group; there is no expression in the empty vector transfection group and NSC control group (results of Western blot analysis; GAPDH was used as the control). C) Results from the ELISA assay for human VEGF165 indicate that the levels of VEGF165 in the media from pCDH-*VEGF165*-transfected cells (1) are more than four times over those of the empty vector transfection group (2) and NSC control group (3), *$P<0.05$.

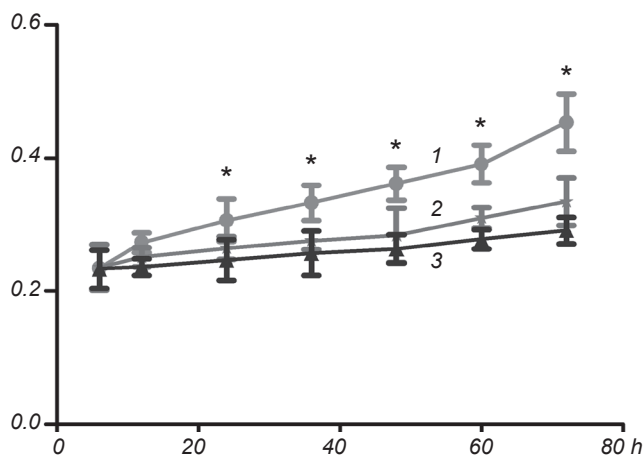Р и с. 3. Експресія людського гена *VEGF165* в невральних стовбурових клітинах, трансфікованих pCDH–*VEGF165*.

into the lentiviral vector. The pCDH-*VEGF165* and packaging plasmids were cotransfected into 293T cells. After 48 h, a high degree of green fluorescence was observed in these cells, indicating that the vector plasmid was really transfected into the latter. The titer of the concentrated virus was $1 \cdot 10^9$ TU/ml. Green fluorescence was noticeable in pCDH-*VEGF165*-transfected NSCs at 3 days and peaked at 7 days. The transfection efficiency was about 63%, as detected by flow cytometry (FCM); positive clones were obtained by FCM selection. RT-PCR and Western blot analysis showed a hybrid band in pCDH-*VEGF165* transfected NSCs, indicating rather intense expression of VEGF protein. An ELISA assay demonstrated that *VEGF165* secretion was over four times higher in the pCDH-*VEGF165* group compared with that in the empty vector and control groups, suggesting that pCDH-*VEGF165* transfection dramatically promoted VEGF165 secretion by NSCs. The CCK8 assay demonstrated that NSC proliferation was noticeably higher in the pCDH-*VEGF165* group compared with that of the empty vector and control groups, suggesting that pCDH-*VEGF165* transfected NSCs produced VEGF protein, and the latter accelerated NSC proliferation. Immunofluoresence staining showed that differentiated cells demonstrated the immunoreactivity to GFAP, MAP-2, and O4, and this indicated that pCDH-*VEGF165*-transfected NSCs still keep their stem cells characteristics and the capacity of differentiating into astrocytes, neurons, and oligodendrocytes, respectively.

Our results provide an experimental basis for the further study of NSC gene manipulation for gene therapy.

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ПОСИЛЕНА ЕКСПРЕСІЯ СУДИННОГО ЕНДОТЕЛІАЛЬНОГО ФАКТОРА РОСТУ ПІСЛЯ ІНФІКУВАННЯ НЕВРАЛЬНИХ СТОВБУРОВИХ КЛІТИН ЛЮДИНИ ЛЕНТИВІРУСНИМ ВЕКТОРОМ, ЩО КОДУЄ ГЕН *VEGF165*

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Р е з ю м е

Ми досліджували експресію судинного ендотеліального фактора росту VEGF та характеристики невральних стовбурових клітин (НСК) людини після їх трансфекції лентивірусним вектором, що кодує ген *VEGF165*. Останній ген ампліфікувався з лінії клітин раку грудей людини MCF-7 з використанням РТ-ПЛР (RC-PCR); цільовий ген клонували в лентівірусну експресуючу плазміду pCDH–CMV–MCS–EF1–copGFP. Після трансформації ензимна обробка дозволяла отримати коректну довжину гена *VEGF165*; аналіз ДНК-послідовностей підтвердив, що послідовність гена *VEGF165* точно відповідала такій послідовності, вказаній Банком генів. Потім рекомбінантним лентівірусом, продукованим клітинами 293Т, та пакуючими плазмідами трансфікували НСК людини четвертого пасажу. Через тиждень після трансфекції pCDH–*VEGF165* у НСК стабільно експресувався *VEGF165*, а їх здатність до проліферації істотно збільшувалася. Крім того, людські НСК зберігали свої характеристики та здатність до багатовекторної диференціації після трансфекції. Отже, наші результати вказують на те, що людські НСК можуть ефективно та стабільно експресувати *VEGF165* після трансфекції лентівірусним вектором із цим геном. Ці дані можуть бути використані в подальших дослідженнях поновлення функцій після інсульту.

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