Transplantation of GDNF-expressing human neural stem cells by lentiviral vector into transgenic SOD1 (G93A) ALS mice

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The Master's Thesis

submitted to the Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Master of Medical Science

Sungju Park

June 2008

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June 2008

ACKNOWLEDGEMENTS

I gratefully acknowledge Professor Kook In Park for editorial assistance and supervision of this thesis. I also thank to Professor Seung-Min Kim and Professor Chul Hoon Kim for the judgment of the thesis. This work is aided by lab members, and I was also helped by Dr. Didier Trono, the provider of lentiviral vectors. I gratefully thank to my parents for physical and spiritual support to my degree.

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ABSTRACT

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive and lethal motor neuronal death. About 2-3% of ALS is caused by mutation in the Cu/Zn superoxide dismutase 1 (SOD1) gene. The mutant SOD1 transgenic rodent is a major model of the disease. Even though there are several discrepancies in human cases, many pathological and clinical investigations were conducted using this model. Neural stem cells have migration, integration, and differentiation capacity. They also have anti-inflammatory effects and could deliver several trophic factors into injured central nervous system (CNS) tissues. Glial cell line-derived neurotrophic factor (GDNF) is a potent trophic factor for motor neurons. In this study, we investigated the effects of intrathecal and/or intraventricular transplantation of human neural stem cells (hNSCs) on the neurobehavior and survival of ALS mice. The hNSCs were engineered to express GDNF by lentiviral vector before transplantation. Donor cells showed engraftment and migration into the injured brain and spinal cord parenchyma. The transplanted GDNF-expressing hNSCs showed trophic effects on motor neurons of the spinal cord. The host motor neurons displayed larger cell bodies and longer cellular processes than those in the vehicle-

injected control group. Donor cells also differentiated into GDNF- and excitatory amino acid transporter 2 (EAAT2)-expressing astrocytes. Transplantation of GDNF-expressing hNSCs showed motor neuronal trophic effects. However, improvement of motor performance in ALS mice was not observed on the whole, except initial short-term improvement after transplantation of hNSCs. Moreover, survival of hNSCs-transplanted ALS mice worsened compared to vehicle-injected mice. This result was largely due to the decreased survival period in female ALS mice in the cell treatment group. The results imply that long-term trophic factor delivery could be harmful, and there is a sexual dimorphism in the rodent ALS model. The inappropriate delivery of trophic factors in the CNS of ALS mice could be another possibility for the low treatment efficacy of hNSCs transplantation. Therefore, investigation of the pathogenetic mechanisms of ALS and appropriate hNSCs transplantation techniques should be developed in the future.

Key words : amyotrophic lateral sclerosis (ALS), human neural stem cells (hNSCs), superoxide dismutase 1 (SOD1), transplantation, glial cell line-derived neurotrophic factor (GDNF), gene therapy

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I. INTRODUCTION

ALS is a late-onset neurodegenerative disease characterized by progressive, lethal motor neuronal death. The disease's hallmark is a selective degeneration and loss of motor neurons in the brain and spinal cord.[1, 2] This leads to spasticity, hyperreflexia (upper motor neurons), generalized weakness, muscle atrophy, and paralysis (lower motor neurons). About half of patients die within 3-5 years of symptom onset (45-60 years). Most cases of ALS are sporadic, however, 10% are inherited and 20% of these cases are caused by mutation in the SOD1 gene on chromosome 21. The mutation of SOD1 induces non-cell-autonomous death of motor neurons by 1 or more unknown toxic properties. Many researchers have tried to identify the disease's etiology and pathology and have made some progress but more studies are needed.

According to some recent research, there are a few plausible pathological mechanisms of mutant SOD1-mediated toxicity. First, mutant SOD1 proteins come into detergent-insoluble aggregates, and then the aggregates interfere with axonal transport, mitochondrial activity, and other cellular machineries.[1, 2] In the end, motor neurons undergo apoptosis. Second, mutant SOD1 proteins secreted from motor neurons activate microglias and astrocytes (also by autonomous activation), and then

the activated microglias and astrocytes secrete several toxic factors, such as TNFα and NO.[2] Additionally, activated astrocytes are unable to regulate synaptic glutamates due to diminished glial glutamate transporter (GLT-1/EAAT2).[1, 2] These non-cell-autonomous toxic factors are responsible for motor neuronal death.[1] In conclusion, motor neuronal death is mediated by not only impairments within motor neurons but also effects of non-neuronal neighbor matters.

To improve ALS, we focused on hNSCs expressing long-term GDNF. GDNF has potent effects on ALS with high affinity for motor neurons, and can prevent their death following various insults.[3, 4] In addition to glutamate uptake, astrocytes play an important role in motor neuron protection by the secretion of neurotrophic factors such as GDNF.[5, 6] There are a number of delivery methods of therapeutically potential molecules to the CNS. Including direct in vivo gene therapy using viruses modified to encode GDNF, injection of encapsulated cells secreting neurotrophic factors and ex vivo gene therapy using transplantation of genetically modified cells exist.[7-10] Direct injection of viral particles might be dangerous because the transport of viruses to other regions of the CNS could produce side effects.[11] Capsules are difficult to administer or remove from the spinal cord. Using modified cells, ex vivo transplantation in which no live virus is injected into the subject has increased safety. What is more, stem cells have migration, integration, and differentiation capacities at injured sites,[12-14] and they have some anti-inflammatory effects.[14, 15] They can deliver the growth factors over a large area. Finally, transplantation could reduce stress on host cells by adding healthy glial cells into diseased environments. Therefore hNSCs might be a suitable source for ex vivo gene therapy.[9, 10]

In previous studies, we performed transplantation and behavioral studies using naive hNSCs and hNSCs expressing various neurotrophic factors, such as BDNF, IGF-1, GDNF, and VEGF, via the adenoviral vector system. They showed some positive effects in terms of motor function in transgenic ALS mice. However, the adenoviral vector system has a transient expression capacity and is more immunogenic than other viral vector systems.[16] Therefore, we need to investigate the effects of neurotrophic factors expressing hNSCs for a long period in ALS mice. In

addition, the route of transplantation was an intrathecal space only into the cisterna magna in previous studies. Symptoms of ALS include abnormalities of upper and lower motor neurons of the whole CNS,[1, 2, 17-19] so to increase the trophic and therapeutic effects of trophic factors expressing hNSCs on upper and lower motor neurons of the whole CNS of ALS mice model, the route of transplantation has been changed to the intrathecal and intraventricular space by injecting cells into both lateral ventricles and cisterna magna of ALS mice model. The lumbar region of the spinal cord in mice is too small to transplant any cells and the transplantation procedure itself can damage the spinal cord.

Because the mice group of GDNF-expressing hNSCs transplantation showed stronger forelimb strength and better motor coordination than those of H-H buffer, naive hNSCs, or other neurotrophic-expressing hNSCs injection, we constructed a lentiviral vector expressing GDNF. Even though there are some limitations in human application, lentiviral vector has low immunogenicity and long-term expression capacity,[16] and can be an effective experimental tool to transfect foreign genes to hNSCs. In addition, many studies were conducted to find therapeutic lentiviruses such as site-specific or non-integrating lentiviruses.[20-22] Because the adult neural stem and progenitor cell niche is altered in SOD1 transgenic mouse model of ALS,[23] intraventricular injection of hNSCs might be useful to support and substitute the impairment of adult neurogenesis in the stem cell niche of the CNS.

In this study, we showed that GDNF-expressing hNSCs via lentiviral vector infection can survive in the brain and spinal cord of transgenic ALS mice model and act as additional source of GDNF. Although there were several positive expectations, there was no behavioral improvement and survival of ALS mice worsened. In these experiments, we also found a differential pattern of disease between male and female transgenic ALS mice.

II. MATERIALS AND METHODS

1. Animals

We used SOD1 transgenic mice (Jackson Laboratories, Bar Harbor, ME, USA) expressing the human mutant Cu/Zn superoxide dismutase gene (G93A; Gly93 → Ala mutation) for the ALS model. The mutation has a dominant gain-of-function effect. Male transgenic mice were bred with background-matched B6C3/F1 wild-type females. The progeny were genotyped by polymerase chain reaction (PCR) at 4-5 weeks after birth as previously described,[24] and maintained as SOD1 G93A heterozygotes for subsequent studies. All transgenic ALS mice received the same care and housing and distributed evenly to the control and treatment groups.

2. Cell culture

Human fetal tissue from cadavers at 13 weeks of gestation was obtained with full patient consent and approval of the research ethics committee of Yonsei University College of Medicine, Seoul, Korea. The methods of acquisition conformed to NIH and Korean government guidelines. The telencephalic brain was dissected, dissociated in trypsin, and seeded into tissue culture-treated 100-mm plates at a density of 600,000 cells/mL of serum-free growth medium. Mitogenic stimulation was achieved by adding fibroblast growth factor-2 (FGF-2, R&D, Minneapolis, MN, USA) and leukemia inhibitory factor (LIF, Sigma, St. Louis, MO, USA). Heparin (Sigma) was added to stabilize FGF-2 activity. All of the cultures were maintained in a humidified incubator at 37°C and 5% CO_2 in air, and half of the growth medium was replenished every 3-4 days. The cells formed neurospheres during the first 2-5 days of growth. The neurospheres were passaged every 7-8 days by dissociation with 0.05% trypsin/EDTA (T/E; GIBCO). The concrete manners of cell culture are described in a previously published report.[25]

3. Lentiviral vector construction

We constructed lentiviral vectors carrying the GDNF gene (we named it “Lenti-GDNF”) and infected hNSCs with the vectors. Lentiviral vectors were produced by calcium precipitate (transient) transfection method using vectors obtained from Trono lab (http://tronolab.epfl.ch/). The backbone of the transfer vector was pWPI (Figure 1). Packaging and envelope plasmid were psPAX2 and pMD2G, and eGFP was a reporter gene. The production was done in accordance with the method given by Trono lab. Briefly, after preparation of the vectors (transfer vector 20 μg, packaging plasmid 15 μg, envelope plasmid 6 μg in 0.5 mL distilled water), we added 0.5 mL of 2X HBS and 50 μl of 2.5 M $CaCl_2$. We incubated the mixture for about 20 minutes, and then added dropwise the mixtures on the cell plate. Six hours later, we replaced the medium. At 48 hours after transfection, we collected, spanned down, and filtered the virus-containing medium. The transfection was conducted to 293FT cell line and produced viral vectors were concentrated by ultracentrifugation under the sucrose cushion. The viral titer was checked as transducing unit (TU) by fluorescent-activated cell sorting (FACS) analysis method given by Trono lab.

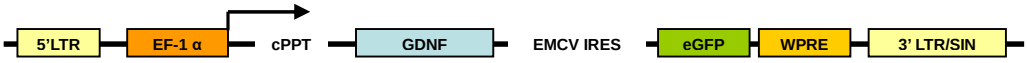

Figure 1. Construction of lentiviral vector bearing GDNF. The lentivirus was produced by second generation packaging system. The vector contains EF1- α promoter and central polypurine tract (cPPT), which support nuclear import of proviral DNA in stem cells. The bicistronic expression of GDNF and GFP was gained from usage of IRES. WPRE is post transcriptional enhancer of woodchuck hepatitis virus. LTR and SIN imply long terminal repeat and self-inactivation vector construct.

4. Viral vector infection

For lentiviral transduction, hNSCs were seeded onto 100 mm cell culture dish. The dish contained serum-free growth medium containing FGF-2, LIF, and heparin. One hour later, Lenti-GDNF was added to the dish with a multiplicity of infection (MOI) of 3. The next day, the medium was replaced with fresh medium. To confirm the safety of lentiviral infection, we took the survival analysis of lentivirus-infected hNSCs and observed the GFP and GDNF expression patterns and cell morphology. Because the virus-infected hNSCs have to be confirmed their viability and the change of GDNF production rate, lentiviral infection to hNSCs was conducted at least 10 weeks prior to transplantation.[5] GDNF expression was checked by PCR and enzyme-linked immunosorbent assay (ELISA) kit. We followed the user manual provided with the ELISA kit (R&D). In addition, the cell types and morphologies of lentivirus-infected hNSCs under proliferation and differentiation conditions were observed and identified by immunocytochemistry.

5. Transplantation

Lentivirus-infected hNSCs had been labeled with bromodeoxyuridine (BrdU) before transplantation for 5 days. The cells were prepared into a suspension of 8×10^4 cells per μl in H-H buffer (1X Hank's balanced salt solution, 10 mM HEPES, pH 7.4; GIBCO). After anesthesia with an intraperitoneal injection of ketamine (50 mg/kg) and rompun (10 mg/kg), the experimental mice were fixed on a Benchmark™ digital stereotaxic instrument (myNeuroLab.com, St. Louis, MO, USA). Cell transplantation was performed into the cisterna magna (10 μl) and/or lateral ventricles (into the both side, each 4 μl) of the transgenic ALS mice using a 26-gauge needle at about 75 days after birth as described previously.[26, 27] The ventricular injection sites were 0.9 mm lateral and 0.1 mm anterior to bregma and 2 mm depth from dura. Cyclosporine (10 mg/kg) was injected intraperitoneally every day from 1 day before transplantation to the day of death or sacrifice.

6. Behavior test

As the main symptom of ALS is impairment of motor function, beginning at 1 week prior to transplantation, we measured body weight and motor performance in ALS model mice using wire-maneuver and the rota-rod (Columbus Instruments, Columbus, OH, USA) test once a week. The maximum time allowed to an animal was 180 seconds in wire-maneuver and 300 seconds in rota-rod test. Along with behavioral tests, survival analysis was also conducted. The mortality of mice was determined by the day of death or inability to right itself within 30 seconds when placed on their side. During the behavior test, we observed whether transplantation could influence normal behavior of mice.

7. Immunochemistry

Using various cell type-specific markers and fluorescent molecules, we performed immunochemistry. To characterize Lenti-GDNF-infected hNSCs in vitro, we performed immunocytochemistry with the following antibodies: anti-hNestin (human-specific Nestin, 1:200; Chemicon, Temecula, CA, USA), anti-GFAP (glial fibrillary acidic protein, 1:1500; Dako, Carpinteria, CA, USA), anti-Tuj1 (neuronal class β-Tubulin III, 1:500; Covance, Berkeley, CA, USA), anti-NeuN (neuronal nuclear protein, 1:40; Chemicon), anti-CNPase (2’3’-cyclic nucleotide 3’-phosphodiesterase, 1: 500; Sternberger, Lutherville, MD, USA), anti-O4 (oligodendrocyte marker O4, 1:200; Chemicon), and anti-PDGFR (platelet-derived growth factor receptor, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, USA).

After transplantation, we conducted histochemical analysis in tissues of the brain and spinal cord of the ALS mice model. We first perfused the experimental mice with chilled 1X phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA; Sigma) in 0.1 M PIPES buffer, and then the brain and spinal cord were removed and post-fixed. These tissues were cut in 16 μm sections on cryostat. The sections were stained with the following antibodies: anti-Tuj1 (1:200; Sigma), anti-

GFAP (1:500; Sternberger, Lutherville, MD, USA), anti-GFP (green fluorescent protein, 1:300; Invitrogen), anti-EAAT1 (excitatory amino acid transporter 1, 1:100; Santa Cruz Biotechnology), anti-EAAT2 (excitatory amino acid transporter 2, 1:100; Santa Cruz Biotechnology), anti-GDNF (1:50; Santa Cruz Biotechnology), anti-NF (neurofilament, 1:2000; Chemicon), and biotin-conjugated anti-HSP27 (heat shock protein 27, 1:40; Stressgen, Ann Arbor, MI, USA). The stained sections were mounted and examined by immunofluorescence microscopy (BX51; OLYMPUS, Tokyo, Japan).

8. Statistics

Because transgenic mice have different characters according to their litter cage, data were analyzed primarily by unpaired t-test. Survival analysis was conducted by Kaplan-Meier method. All data were shown as mean ± s.e.m.

III. RESULTS

1. Characterization of Lenti-GDNF infected hNSCs

Lentiviral vector titer was 1.08 x 10^5 TU/μl and the cells were infected at 3 MOI. Lentiviral vector infection has been confirmed by long-term culture. The effects of lentiviral infection were observed during at least 10 weeks. Two weeks after infection, cell survival and GFP expression levels were stabilized. According to the ELISA data, Lenti-GDNF-infected hNSCs secreted approximately 32 ng of GDNF from 10^6 cells during 48 hours in proliferation condition (Figure 2D). We performed immunocytochemistry for characterization of Lenti-GDNF-infected hNSCs. Transduction of the viral vector was confirmed by GFP and GDNF expression (Figures 2A-C). Most hNSCs that expressed GFP also expressed GFAP (astrocyte marker). A small portion expressed Tuj1 and no cells expressed NeuN (neuronal marker), PDGFR, O4, and CNPase (oligodendrocyte marker). However, most cells also expressed hNestin (neural stem cell marker). All cells looked like neural or radial glia stem cells or glial progenitors. No fully differentiated cells were seen.

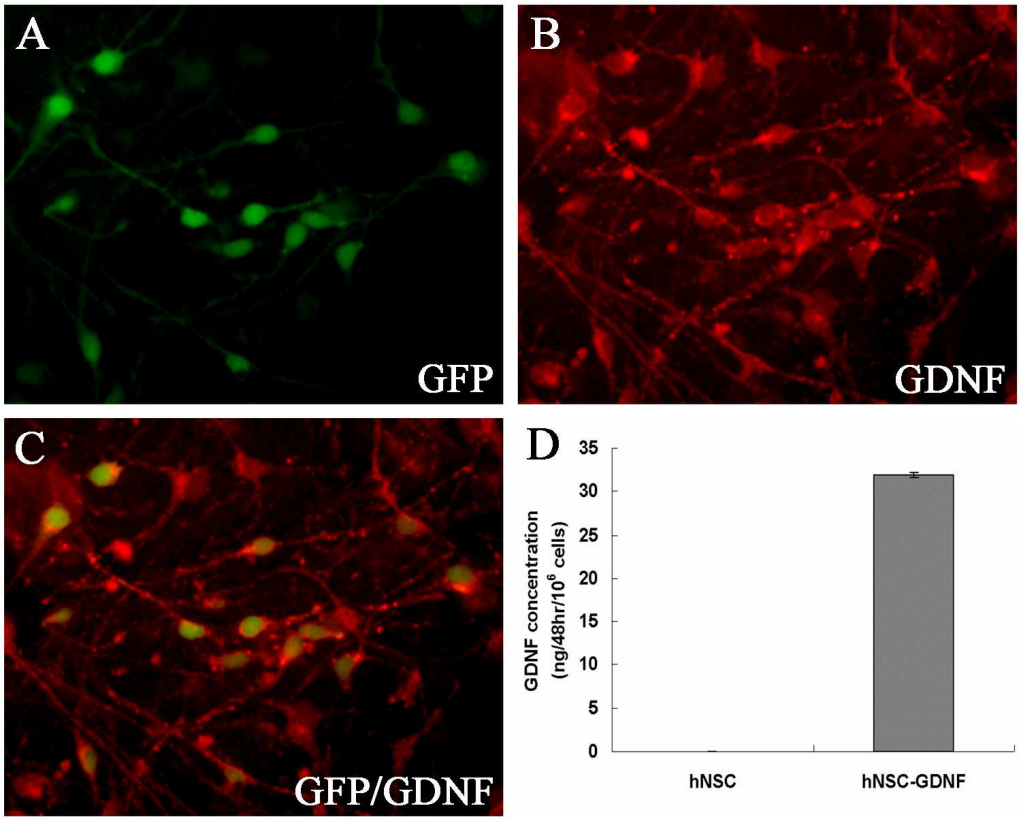

Figure 2. In vitro characterization of Lenti-GDNF-infected hNSCs. (A-C) show that Lenti-GDNF-infected cells expressed GFP and GDNF. (D) ELISA data indicate that the cells secrete about 32 ng per 10^6 cells during 48 hours.

2. Distribution and differentiation patterns of the hNSCs after transplantation

The animals were sacrificed at 1, 2, 3, or 4 weeks after transplantation and at the end point of the disease. In both groups, transplanted cells showed migration and engraftment capacities throughout the brain and spinal cord. One or two weeks after transplantation, we found that transplanted cells were forming neurospheres in the lateral, third, and forth ventricles (Figure 3). Some of the neurospheres were integrating into the brain stem in the forth ventricle (Figures 3B,C). We could find engrafted cells in the spinal cord's white matter (Figure 3D). We also observed more scattered donor cells when we analyzed the mice at 3-4 weeks after transplantation. The transplanted cells could differentiate into the neurons, astrocytes, and oligodendrocytes in SOD1 transgenic ALS mice but consistent with AdGDNF experiment, Lenti-GDNF-infected hNSCs were mainly differentiated into astrocytes in this study (Figures 3,4,5). About 30% of astrocytes that were differentiated from hNSCs expressed EAAT2 (Figure 4). The astrocytes could reduce the glutamate-mediated excitotoxicity by absorbing excess extracellular glutamate.

3. Trophic effects of transplantation of the hNSCs on motor neurons of spinal cord

Most of the transplanted cells differentiated into astrocytes in the spinal cord and some cells expressed EAAT2. The donor cells also expressed GDNF (Figures 5A-C). The motor neurons of the transplantation groups had larger cell body and long processes than the vehicle group (Figures 5D-G). This result means that transplantation of hNSCs could protect dying host motor neurons. In the spinal cord, EAAT2-expressing astrocytes could reduce damage of the motor neuron by taking up additional excitatory glutamates and secreting GDNF, which have motor neuronal trophic effects.

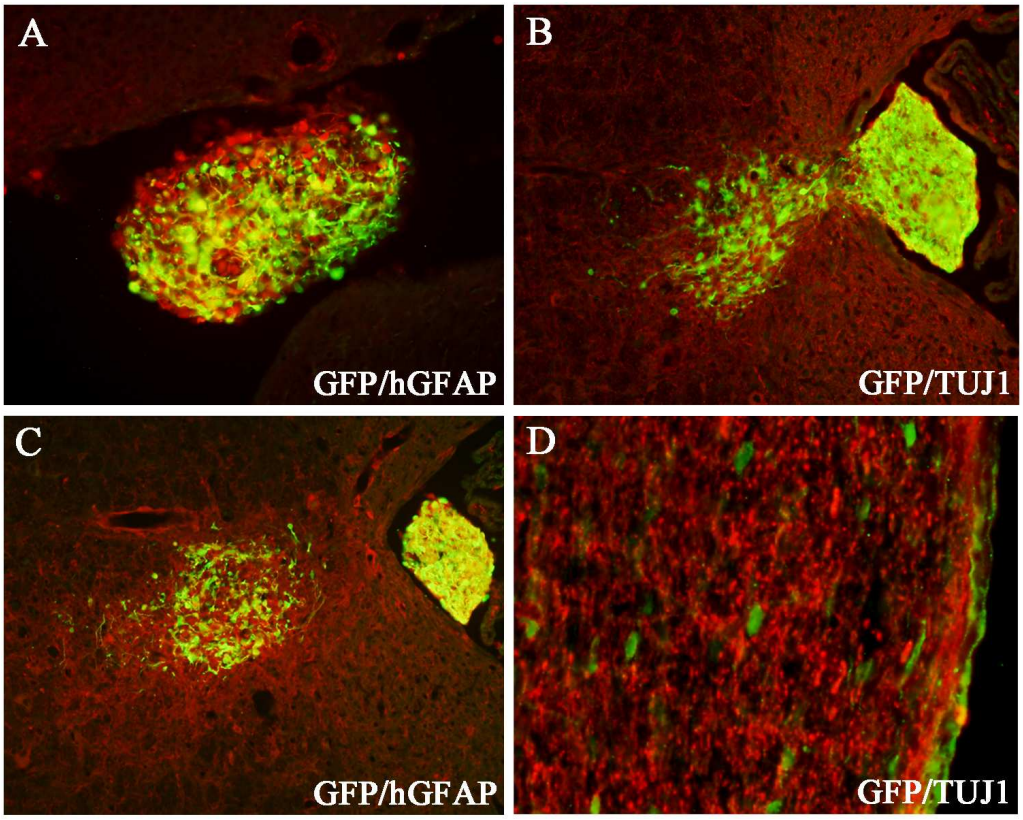

Figure 3. Transplanted hNSCs migrated and engrafted into host tissues. (A) Donor cells in the lateral ventricle at 2 weeks after transplantation. Some of the cells also expressed human GFAP (hGFAP), astrocyte marker. One week after transplantation, extensive engraftment in the forth ventricle (B,C) and GFP-positive donor cells in the cervical spine (D) were found. We could hardly find Tuj1-positive neurons.

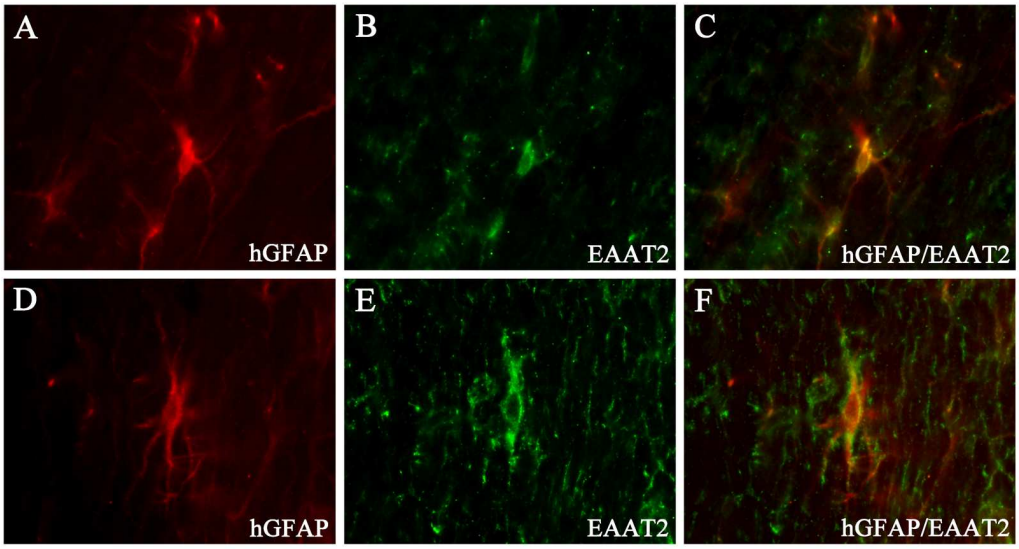

Figure 4. In vivo differentiation patterns of GDNF expressing hNSCs. (A,D) Four weeks after transplantation, most transplanted cells differentiated into astrocytes in the spinal cord. (B,C,E,F) About 30% of these cells also expressed the glutamate transporter EAAT2.

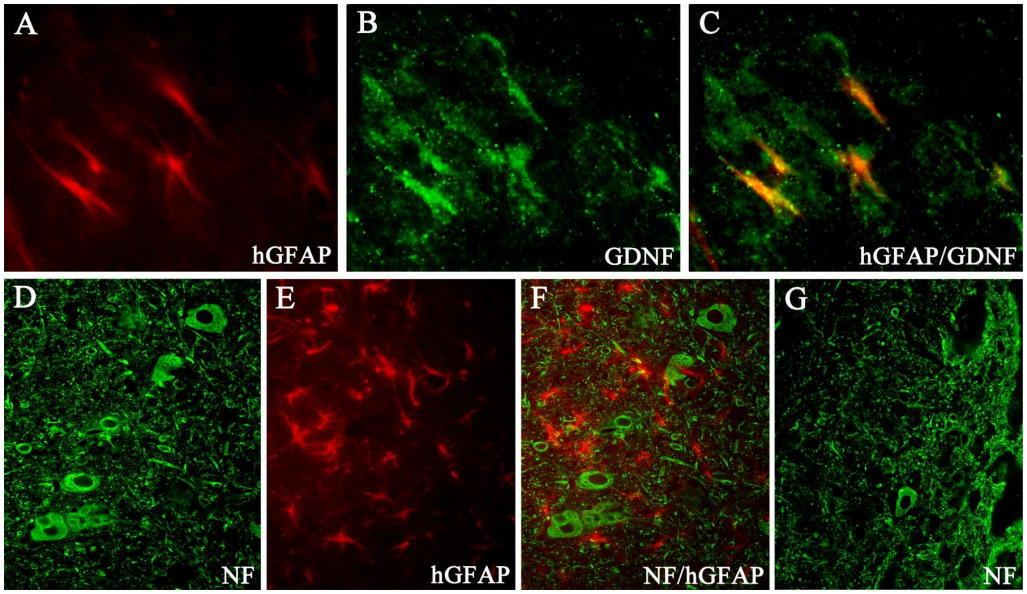

Figure 5. The donor cells have trophic effects on spinal motor neurons. (A-C) For weeks after transplantation, astrocytes derived from hNSCs still expressed GDNF in the spinal cord. (D-G) The motor neurons of cell-treated mice have larger cell bodies compared to vehicle-injected mice. (D-E) The spinal cord of hNSCs-treated mice. (G) The spinal cord of vehicle-injected mice.

4. Effects of transplantation of the hNSCs on motor performance

We performed wire-maneuver and rota-rod test to evaluate the effects of hNSCs transplantation on motor function. Overall, improvements on motor function were not observed. There were no statistical differences in wire-maneuver and rota-rod test but the experimental mice showed short-term improvement in rota-rod test. Their performance was better than the vehicle group until 5 weeks after transplantation. After that, performance became worse than the vehicle group (Figure 6A). The data of both experimental groups (cisterna magna injection group and both lateral ventricle and cisterna magna injection group) were consistent. This tendency was mainly because of the female data (Figures 6B,C). Likewise, short-term improvement of wire-maneuver test was seen (Figure 6D). There was no difference in weight during the experimental period. This result means that trophic effects on motor neuronal cell body were not linked to the neuromuscular junction and/or muscle.

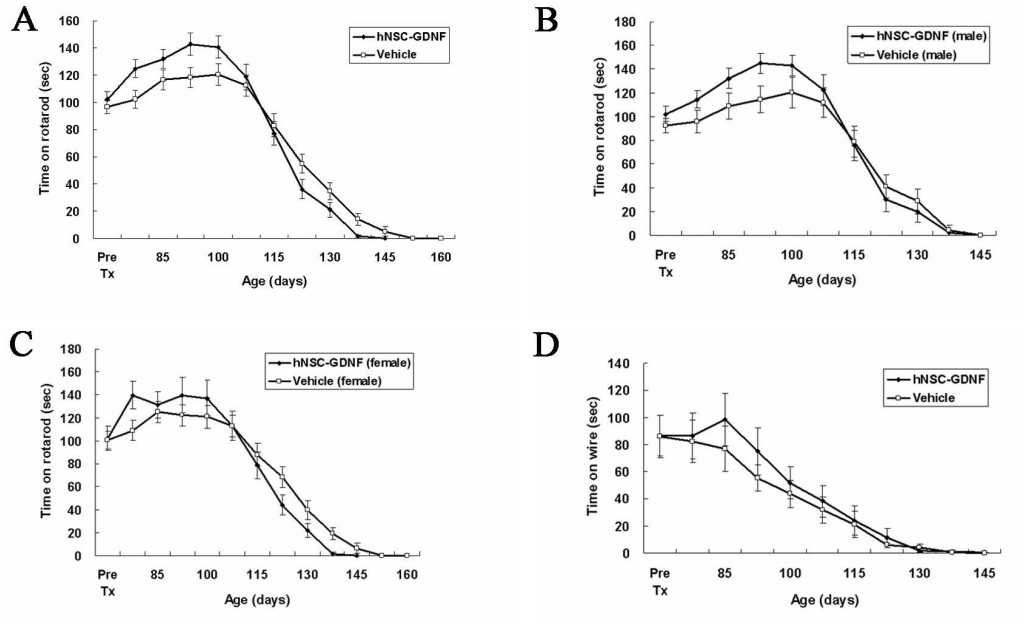

Figure 6. Motor performance of SOD1 transgenic ALS mice. (A) Rota-rod graph shows improvement of motor function during 5 weeks after transplantation and worsened performance in the last several weeks. (B) Rota-rod graph of male transgenic mice. (C) Rota-rod graph of female transgenic mice. (D) Wire-maneuver test graph shows short-term improvement of upper motor performance.

5. Effects of transplantation of the hNSCs on survival of SOD1 TG mice

Transplantation of GDNF-expressing hNSCs did not prolong the survival period of transgenic ALS mice (Figure 7). In case of cisterna magna injection, average survival times of vehicle and hNSCs were 143.33 ± 2.87 and 132.78 ± 2.94 days. When they were injected through both the cisterna magna and lateral ventricles, mean survival times of vehicle and hNSCs were 142.50 ± 2.51 and 135.47 ± 2.17 days. Both data were statistically significant ($p < 0.05$). We could not distinguish between vehicle and hNSCs in disease onset but there were definite differences in disease onset and progression pattern between male and female mice. When we analyzed the survival data of males alone, the mean values of vehicle and hNSCs were 135.18 ± 2.89 and 129.14 ± 2.28 days in the case of cisterna magna injection. In the same manner, the female data were 152.30 ± 3.32 and 138.44 ± 2.94 days. This tendency also was seen in the other experimental group. When hNSCs were injected through both the cisterna magna and lateral ventricles, the average survival time of vehicle and hNSCs were 137.13 ± 3.41 and 133.10 ± 2.70 in males and 147.88 ± 2.66 and 138.86 ± 3.42 in females. The negative result of the survival analysis was due to the worsened survival of female ALS mice (Table 1).

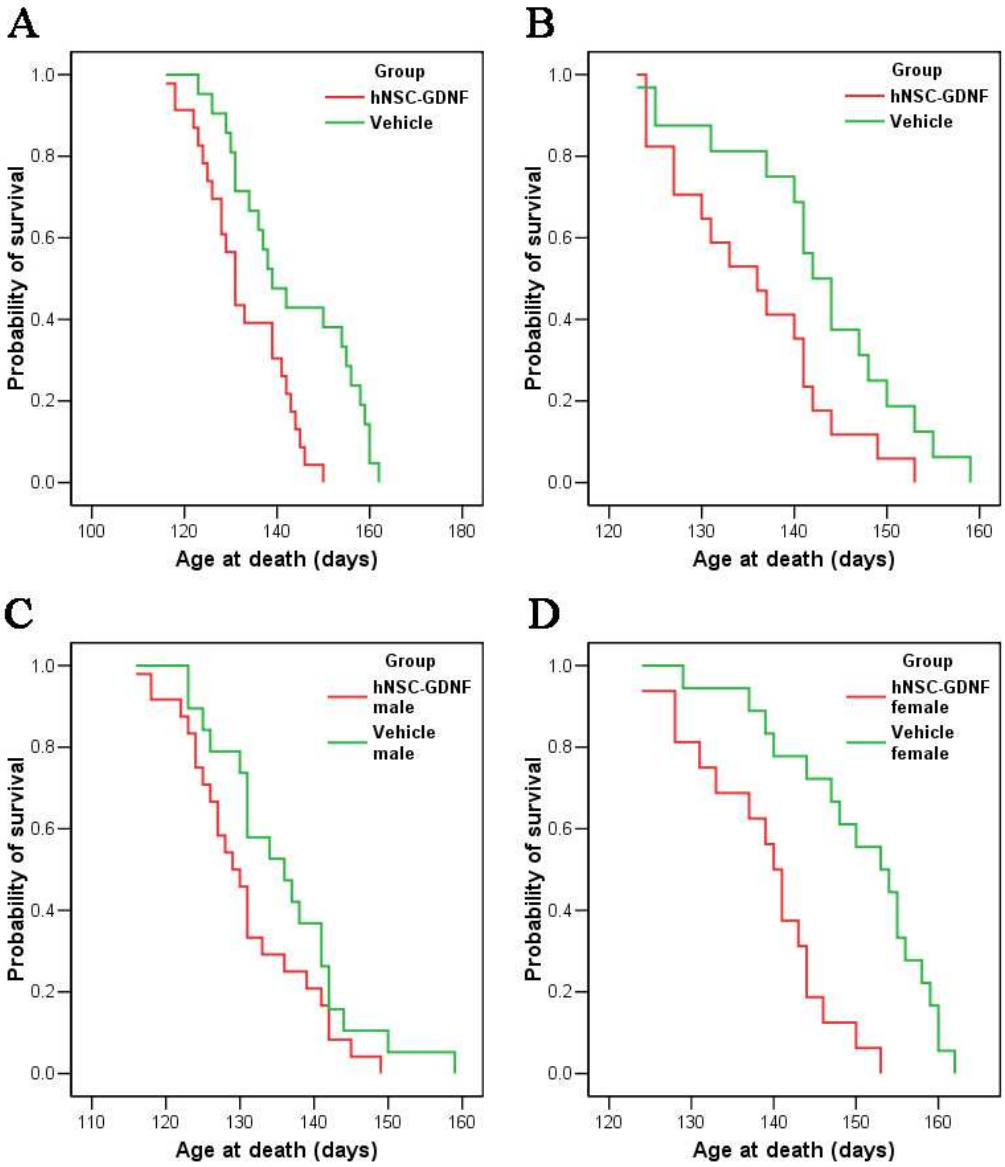

Figure 7. Survival curves. (A) Survival curve of cisterna magna injection group. (B) Survival curve of lateral ventricle and cisterna magna injection group. (C) Survival curve of total male transgenic mice. (D) Survival curve of total female transgenic mice.

Group	CM	CM & LV	Total male	Total female
Cell group mean ± s.e.m.	132.78 ± 2.01 (n = 23)	135.47 ± 2.17 (n = 17)	130.79 ± 1.75 (n = 24)	138.63 ± 2.15 (n = 16)
Vehicle group mean ± s.e.m.	143.33 ± 2.87 (n = 21)	142.50 ± 2.51 (n = 16)	136.00 ± 2.16 (n = 19)	150.33 ± 2.20 (n = 18)
Significance	$P < 0.05$	$P < 0.05$	Not significant	$P < 0.01$

Table 1. Survival data. CM represents cisterna magna injection, CM&LV represent cisterna magna and lateral ventricle injection. Total survival data were 133.93 ± 1.48 days in the cell transplantation group (n = 40) and 142.97 ± 1.93 days in the vehicle group (n = 37) ($P < 0.01$).

IV. DISCUSSION

There have been many studies to improve ALS, a lethal and progressive motor neuron disease. Pathological investigations, drug, gene, and cell therapies have been conducted over many years. Even though there are some limitations, many approaches have been developed to cure the disease, such as aiding function of intracellular organelles,[28-30] blocking astrocyte and microglial activation[31, 32] (anti-inflammatory effects), diminishing glutamate mediated excitotoxicity,[33] and slowing motor neuron degeneration with several trophic factors.[34-36] Because hNSCs have anti-inflammatory effects and the ability to deliver trophic factors as well as the capacity to substitute degenerating motor neurons, if there are appropriate transplantation methods, stem cell therapy would be a powerful tool to treat ALS.

We used lentiviral vector system for gene delivery. The viral vectors were produced by second generation production system. Because the lentivirus could randomly integrate into host chromosomes, they would have a bad influence on stem cell survival. So, to diminish the lethal effects, we infected hNSCs with a low dose of lentivirus and had a long-term culture prior to transplantation (at least 10 weeks). GDNF production rate was consistent with recently published data, which has motor neuronal trophic effects and no side effects.[4] Ex vivo gene therapy using hNSCs could be another source to escape the safety concerns. The cell character was slightly changed according to immunocytochemistry results. Lenti-GDNF-infected cells have stem cell-like characters and could differentiate into neurons and glias but according to the in vitro and in vivo differentiation patterns, Lenti-GDNF-infected cells seem that they have gained glial-specific differentiation capacity.

Transplantation itself had no adverse effects on survival of ALS mice. We compared survival of intact ALS mice with the vehicle injection and naïve hNSCs transplantation groups. We could not find any differences in disease pattern and survival in these mice. The transplanted cells could survive and migrate into the host tissues and differentiate into neurons and glias. Some of the cells were found near host motor neurons and the cells showed protective effects by secreting GDNF and

taking up excess glutamate after differentiation into EAAT2-expressing astrocytes. It is certain that GDNF-expressing hNSCs have motor neuronal trophic effects. However, some of the cells remained in the ventricular region and cerebrospinal fluid (CSF) tract until 4 weeks after transplantation, and their engraftments and integrations were limited. According to previously published data, trophic effects were the highest when the trophic factors were delivered retrogradely.[34-36] Because the target of the trophic effect was not muscle, the neuromuscular junction is considered the main target.[37, 38] What is more, the SOD1 transgenic model has altered axonal transport, the main reason of the effects could be low targeting of the trophic factors.[39] Besides, siRNA-encoding lentivirus, which reduces mutant SOD1 protein, was more effective when the virus injected through muscle rather than the spinal cord.[40, 41]

The negative survival data was due to worsened survival of female ALS mice. The late disease onset and slow progression of female SOD1 mice was known,[42] and it seems that the main reason is the protective role of estrogen.[43] Interestingly, estrogen stimulates expression of GDNF in the developing hypothalamus.[44] Therefore, excessive expression of GDNF could be a crucial reason for the negative survival of female SOD1 mice. We also observed some irritable behavior, such as jumping and squeaking, in female mice that cells were injected through both the cisterna magna and lateral ventricles. Long-term delivery of trophic factors could be harmful. In the Parkinson's disease patient, side effects of central delivery of GDNF were reported.[45, 46] As we observed in the AdVEGF-infected hNSCs transplantation, a low dose of trophic factor delivery would be better than a high dose.[47] It seems that the modulation of expression level of trophic factors is important. The precise mechanisms of the negative effects need to be studied. In vivo ELISA tests and other trophic factor experiments could be helpful to explain this result.

There have been several experiments using stem cells for the treatment of ALS. When human umbilical cord blood cells (hUBCs) or bone marrow cells were systemically injected into ALS mice, they showed improvement of motor performance and survival.[48, 49] Transplantation of hNSCs derived from human embryonic spinal cord had beneficial effects on disease progression and survival

when the cells were injected into the lumbar spinal cord with combined immune suppressants.[50] But when GDNF-expressing human neural progenitor cells (hNPCs) were transplanted into the lumbar spinal cord, there was no benefit to survival and behavior,[5] even though there were trophic effects on motor neurons.[4] Intrathecal injection of hNSCs derived from hUBCs or human mesenchymal stem cells (hMSCs) also have no effects.[51] The systemic injection of cells is not controllable and could cause unexpected side effects. What is more, systemic injection has a great disadvantage with respect to clinical trials because they need a great amount of cells. Transplantation into the lumbar spinal cord is very difficult and invasive to the mouse.

Even though intrathecal and/or ventricular injection of the cells have several advantages, such as wide distribution of the administered cells, easy application, low dose, and non-invasive method, the experiments had no beneficial effects. Because of the low nutrient level of CSF, survival rate of transplanted cells might be low.[51] Furthermore, disruption of the blood-brain barrier (BBB) and blood-spinal cord barrier (BSCB) of the G93A SOD1 transgenic mice was reported,[52] and the donor cells could have suffered strong immune rejection. High doses of immune suppressants would be required when the viability of the cells is affected by immune rejection.

Because we cannot treat ALS before disease onset in human cases, present transplantation timing that is conducted before the disease onset is another problem for the therapy.

V. CONCLUSION

In conclusion, to overcome the present problems in stem cell application, we need to develop new approaches. Transplanted hNSCs have trophic effects on motor neurons but trophic factor delivery to the right target was low when it was centrally administered. To increase correct targeting to the damaged motor neuron, especially the neuromuscular junction, improvement of axonal transport might be helpful for delivery of trophic factors. Inventions of novel transplantation methods and type of cells also are needed. Improvement of survival rate of donor cells is another concern. The sexual discrepancy of SOD1 mice could be a crucial matter when choosing a therapeutic approach. Despite trophic factor delivery is mainly conducted presently, cell of tissue replacement is the main part of the stem cell research. Because present pathologies imply the non-neuronal neighbors is also important, replacement of damaged astrocytes and microglias with normal counterparts could be effective for the disease. But, after all, accompany with establishments of the pathophysiology of the disease and developmental process of the motor neurons, substitution of the damaged motor neuron with trophic support will be the ultimate goal of stem cell research.

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ABSTRACT (IN KOREAN)

근위축성 측삭경화증 생쥐모델에 lentiviral vector 이용 GDNF 발현 인간 신경줄기세포의 이식

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박 성 주

근위축성 측삭 경화증(ALS)은 점진적인 운동신경원세포의 소실로 인하여 사망에 이르는 대표적인 신경퇴행성 질환이다. 2~3% 정도의 ALS가 SOD1 유전자의 돌연변이에 의해서 발생하는데, SOD1 형질전환 쥐가 대표적인 ALS 동물모델이다. 이 동물모델을 이용해서 많은 병리학 연구 및 치료법 연구가 진행되고 있으나 아직까지 특별한 치료방법은 없는 상황이다. 신경줄기세포는 간편하게 신경계 이식이 가능하고, 이주 및 생착 능력을 가지며, 손상된 조직에서 염증반응을 감소시킬 뿐만 아니라 각종 신경영양인자를 분비하도록 할 수 있다. 그리고 GDNF는 대표적인 운동신경원세포의 신경영양인자이다. 본 연구에서는 GDNF를 발현하는 lentivirus를 만들어 인간 신경줄기세포에 이식한 후, 이식한 세포의 분화 방식을 관찰하고, 행동 검사와 생존 분석을 실시하여 질병에 미치는 영향을 분석하였다. GDNF를 발현하는 인간 신경줄기세포는 뇌 및 척수에 걸쳐 광범위한 이주 능력을 보여 주었으며, 상당부분 척수에서 excitatory amino acid transporter 2 (EAAT2)를 발현하는 성상세포로 분화하였다. 이는 GDNF와 함께 공동작용을 하여 생체내의 운동신경원세포의 크기를 증가시켰다. 하지만, 전체적으로 운동능력의 증가는 없었으며, 생존일수는 오히려 감소하였다. 생존일수의 감소는 현저히 악화된 암컷 쥐의 생존일수 때문이었다. 신경영양인자의 중추신경계로의 장기발현이 좋지 않은 역할을 한 것으로

보이며, 특히 GDNF는 암컷 쥐에서 과도한 발현이 일어나 부작용을 일으킨 것으로 보인다. 이번 실험을 통해서 적적한 수준의 신경영양인자의 발현과 올바른 위치로의 신경영양인자 전달이 중요한 요소임을 알 수 있었다. 이와 더불어 ALS 동물 모델의 성적이형이 질병 연구에 큰 영향을 미칠 수 있음을 알 수 있었다.

핵심 되는 말 : 근위축성 측삭 경화증, 인간 신경줄기세포, SOD1, 이식, GDNF, 유전자 치료