Development of Neuronal Cell-Type Inducible GM-CSF Gene Expression System and Combined Cell Therapy in Spinal Cord Injury Model

Youngsang You

Department of Medical Science

The Graduate School, Yonsei University

Development of Neuronal Cell-Type Inducible GM-CSF Gene Expression System and Combined Cell Therapy in Spinal Cord Injury Model

Directed by Professor Yoon Ha

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Youngsang You

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This certifies that the Master's Thesis

Of Youngsang You is approved.

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Thesis Supervisor : Yoon Ha

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Thesis Committee Member#1 : Keung Nyun Kim

_____

Thesis Committee Member#2 : Bae Hwan Lee

The Graduate School

Yonsei University

December 2014

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ABSTRACT

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Youngsang You

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Yoon Ha)

Promising therapy is needed to treat spinal cord injury (SCI) associated with permanent neurological damage. It is difficult to expect a sufficient therapeutic outcome by a single treatment method. Therefore, a strategy combining two or more techniques might be better for improving therapeutic effects.

In this study, it was designed that a merged treatment strategy using neural stem cells (NSCs) introduced with neuronal cell-type inducible transgene expression system controlled by a neuronal specific enolase (NSE) promoter to maximize the therapeutic efficiency. The luciferase gene was chosen to confirm whether this combined system was working properly prior to using a therapeutic gene. The luciferase expression levels of NSCs introduced with a neuronal cell-type inducible luciferase expression system (NSE-Luci) or with a general luciferase expression system (SV-Luci) were measured and compared *in vitro* and *in vivo*.

After verified neuronal cell-specific gene expression by luciferase gene, therapeutic gene, GM-CSF, was substituted with luciferase gene. Neuronal cell type-inducible GM-CSF expression system (NSE-GMCSF) compared with a general GM-CSF

expression system (SV-GMCSF) *in vitro* and *in vivo*.

The results show that neural stem cells introduced with a neuronal cell-type inducible luciferase expression system (NSE-Luci) showed a high level of luciferase expression compared to neural stem cells introduced with a general luciferase expression system (SV-Luci). And also therapeutic gene system, a neuronal cell-type inducible GM-CSF expression system (NSE-GMCSF) was higher expressed GM-CSF than a general GM-CSF expression system (SV-GMCSF).

A neuronal cell-type inducible gene expression system has been demonstrated that it's suitable for NSCs in a merged-treatment strategy. Therefore, it is suggested that this merged-treatment strategy based on NSCs and a neuronal cell-type–inducible gene expression system is a promising tool for treatment of neurodegenerative disorder, including SCI.

Key words: Neuronal specific enolase (NSE), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Neuronal cell-type inducible transgene expression, Neural stem cells (NSCs), Merged treatment strategy, Spinal cord injury (SCI)

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I. **INTRODUCTION**

Spinal cord injury (SCI) is often associated with permanent disability as a result of sensory or motor functional defects caused by neuronal death and demyelination after primary physical damage.[1] A single treatment strategy is unlikely to result in complete recovery. Therefore, a merged-treatment method might be effective for improving the therapeutic efficacy. In the field of gene therapy, therapeutic gene expression controlled by hypoxia-specific or tissue-specific gene expression systems offers a possibility for efficient and safe gene therapy.[2-6]

In the stem cells therapy field, transplantation with neural stem cells (NSCs) showed therapeutic potential in animal models with incurable disorders, including SCI.[7,8] Interestingly, it has been reported that stem cells can be used as a gene delivery system,[9] and stem cell-mediated gene therapy resulted in a better therapeutic outcome and more stable therapeutic gene delivery than single therapy with stem cells or therapeutic genes alone.[10-13]

Granulocyte-macrophage colony-stimulating factor (GM-CSF), synonym is colony stimulating factor (CSF2), which is also known as a hematopoietic factor and is a

14.2kDa globular protein structure secreted by macrophages, T cell, mast cells, NK cells, endothelial cell and fibroblasts.[14] GM-CSF is 125 amino acids and is essential for the *in vitro* proliferation and differentiation of precursor cells into mature granulocyte and macrophage.[15]

GM-CSF recruited monocytes and macrophage and convert to their mature form.[16] After mature macrophages are transfer to a lesion site, they remove the debris that interferes with neuronal regeneration.[17] GM-CSF is possible to pass through the blood-brain barriers or blood-cerebrospinal-fluid barriers so that GM-CSF is potentially pharmacological material for the treatment of CNS injury.[18] The results in many previous studies were shown that GMCSF not only regulates formation of glial scar, also promotes survival of neuronal cells *in vitro* and in SCI and Parkinson's disease model.[19-23] Anti-apoptotic effect of GMCSF was also demonstrated in clinical study.[24,25] Based on the results of previous research, GM-CSF was chosen as a therapeutic gene to combine with the neuronal cell type-inducible gene expression system.

Considering that most stem cell therapies for SCI have used neural stem cells or neural precursor cells, which have high neuronal differentiation potentials, the neuron specific enolase (NSE) promoter was chosen for transgene over-expression in neural stem cells which have it considerably. It was assumed that a combined treatment strategy based on NSCs which were controlled by NSE promoter would increase therapeutic efficiency and neural differentiation. Finally, it was demonstrated that an NSE promoter is suitable with NSCs for the establishment of a gene and neural stem cell therapy platform.

II. MATERIALS AND METHODS

1. Plasmid construction

A neuronal cell type-inducible luciferase expression system (NSE-Luci) and a general luciferase expression system (SV-Luci) were made by pGL3 luciferase reporter vectors to verify that the NSE with mNSCs was suitable for a merged therapy strategy. SV-Luci was purchased from Promega (Madison, WI, USA, Cat.no E1761). Neuron-specific enolase promoter was provided by Professor Lee (Hanyang University, Seoul, Korea). The NSE region was inserted between NheI and BglII sites of a pGL3-basic vector (Promega, Cat.no E1751).

Another experiment 2, SV-GMCSF and NSE-GMCSF vector were made by a backbone of SV-Luci and NSE-Luci vectors. The Luciferase gene was replaced by mouse GM-CSF (Sino biological Inc., Beijing, China). The construction designs of the plasmids are shown in Fig. 1A.

2. Cell culture

Mouse neural stem cells (mNSCs) were used in this study. mNSCs (CRL-2925, ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) / F12 and supplemented with 10 % Fetal Bovine Serum (FBS; Thermo Scientific HyClone, Logan, UT, USA), and 1 % penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). Approximately, 4×10^4 cells/cm^2 were seeded with culture media. the culture dishes were incubated at 37 ℃ in a humidified atmosphere containing 5 % CO_2.

For the other experiment, we used the human embryonic kidney cells (HEK 293FT) which were provided from Professor Kang (Yonsei University, Seoul, Korea). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS, 1 % Penicillin and Streptomycin, 1 X Non-Essential Amino Acid (NEAA, Invitrogen) and 1 X Sodium pyruvate (Invitrogen). Approximately 6×10^5 cells were

plated in 6-well culture dishes (6×10^4 cells/cm^2) and incubated at 37 ℃ in a humidified atmosphere containing 5 % CO_2.

3. Gene transfection

Only transient gene expression method was used throughout the experiment. To generate transfected mNSCs, plasmid vectors (4 μg DNA per well) were used with Lipofectamine 2000 (10 μl per well; Invitrogen, Carlsbad, CA, US) or Viafect™ (10 μl per well : Promega, Madison, WI, USA).

Before experiments were conducted, the transfection efficiency into mNSCs was tested. mNSCs (approximately 2×10^4 cell/cm^2) were seeded with a mixture of DsRed plasmid /Viafect™) in a 6-well plate, then incubated under normal conditions (pO_2, 21 %, 37 ℃) for 48 h. DsRed and DAPI positive cells were counted.

In the first experiment, approximately, 2×10^4 cells/cm^2 mNSCs were seeded with a mixture of plasmids/Lipofectamin with DMEM/F12 media in a 6 well plate and incubated under normal condition (pO_2, 21 %). The day after transfection, culture media was changed and incubated in hypoxia condition (pO_2, 1 %) during 24 h. Over-expression of transfected gene was demonstrated by luciferase assay and IVIS image.

In the second experiment, GM-CSF expression, approximately 2×10^4 cells/cm^2 of mNSCs were seeded with mixture of plasmids/Viafect™ with DMEM/F12 media in a 6 well plate and incubated in 37 ℃ for 48 h. Like a first experiment in hypoxia condition, transfected cells were incubated in hypoxia chamber, with or without DMSO, in 24 h from the day after transfection. After total 48 h, GM-CSF expression was determined by using supernatant of media with the mouse GM-CSF ELISA kit.

Whether the levels of expression of GM-CSF were sustained, mNSCs (approximately 2×10^4 cells/cm^2) were seeded in a 6-well plate and transfected with a mixture of transfection reagent and each group of vectors (Control, SV-GMCSF, and NSE-GMCSF). The supernatant was harvested at 3, 5, and 7 days after transfection. The amount of GM-CSF secreted in culture media was measured using the mouse GM-

CSF ELISA kit.

4. Neural differentiation

For inducing of neural differentiation, mNSCs were cultured in presence of retinoic acid (RA, sigma, USA) for 7 days. RA was dissolved in DMSO (working solution, 10 mM), and diluted with culture media (final concentration, 1 μM). Briefly, mNSCs were seeded on poly-L-lysine (2 $\mu g/cm^2$, Sigma, StLouis, MO, USA) – coated glass cover slips at a density of 2×10^4 cells/cm^2, and then transfected with the plasmid. After 24 h incubation, the medium was replaced with a complete medium with or without RA and then changed every 2 days. Neural differentiation was carried out for 7 days.

5. Cell transplantation

All protocols were approved by the Animal Care and Use Committee of the Medical Research Institute at Yonsei University College of Medicine. All experiments were conducted in accordance with international guidelines on the ethical use of animals, and the number of animals used was minimized. ICR mice (male, 6 weeks, 30 g, OrientBio, Gyeonggi-do, Korea) were intraperitoneally injected with Ketamine (100 mg/kg, Yu-han, Seoul, Korea), and Rompun (10 mg/kg, Bayer Korea, Seoul, Korea) for anesthetization. Laminectomy was performed at thoracic level 11. Spinal cord injury was carried out using a self-closing forcep (compression injury for 10 sec).

7 days after SCI, animals were anesthetized and transfected mNSCs of each group were transplanted into a lesion of a spinal cord. At the experiment 1, mNSCs of each group were prepared by conducting transfection with SV-Luci and NSE-Luci at the day before for transplantation. At the experiment 2, all mice were randomly divided into 4 groups : Sham (only SCI) and Control (Ds-Red), SV-GMCSF and NSE-GMCSF.

mNSCs of all groups were transplanted to spinal cords for 2 min (5×10^5 cells in a 2 μl volume) using a micro-injector pump (KDS310; KD Scientific, US). The exposure site was sealed using suture wound clips and Cyclosporine A (10 mg/kg) and Cefazolin (20 mg/kg, Yu-han) were injected into mice daily until being sacrificed. Mice were sacrificed within 1 day after transplantation to detect a gene expression and sacrificed 4 days to conduct western blotting.

6. In Vivo Imaging System (IVIS)

The IVIS Spectrum (Xenogen, Alameda, CA, USA) was used to detect luciferase expression. For *in vivo* imaging, mice were anesthetized with ketamine (100 mg/kg) and Rompun (10 mg/kg), and then the substrate D-luciferin (150 mg/kg) was injected intraperitoneally. Twenty minutes after injection, bioluminescence images were captured for 10 min. Regions of interest (ROIs) were analyzed and total quantification of bioluminescence was quantified using Living Image® (Xenogen) software. For *in vitro* imaging, the substrate D-luciferin (150 μg/ml) was added to the Control (DsRed), SV-Luci, and NSE-Luci in medium, followed by mounting and bioluminescence detection for 2–3 min.

7. Luciferase Assays

Transfected cells were harvested and lysed with Pro-prep™ (iNtRON biotechnology, Gyeonggi-do, Korea) adding 400 μl for *in vitro* transfection assay. The concentrations of the extracted protein were measured by using BCA protein assay kit (Pierce, Iselin, NJ) and amount of luciferase expression was detected by using Luminometer by relative light units (RLU) for 10 sec. the level of luciferase expression was presented by RLU per mg of total protein. For *ex vivo* assay, anesthetized animals were conducted perfusion with PBS and immediately extracted that spinal cord segments at 4 mm rostral and caudal of the transplanted region. Samples were transferred to the

micro centrifuge and then lysed with lysis buffer 400 μl for 1 h and centrifuged at 13,000 rpm for 10 min. The supernatant was poured into other tubes and measured the quantity of extracted protein and the level of luciferase expression.

8. ELISA Assay

To detect levels of secreted GM-CSF in spinal cords, a kit of mouse GM-CSF ELISA (Young-In Frontier Inc., Seoul, Korea) was used. Before using the ELISA kit, the samples were prepared with collecting sample media *in vitro*. supernatant of media was separated by centrifuging at 2,000 × g for 10 min at 4 ℃ and stored at –20 ℃ until testing took place.

For *in vivo* model, to detect the level of secreted GM-CSF in spinal cords, all spinal cords were removed after saline perfusion to eliminate blood, and temporarily stored at –70 ℃. Before conduct ELISA, spinal cord samples were lysed with 400 μl Pro-prep™ for 1 h to extract protein. Extracted proteins were used to ELISA assay and total concentrations of the extracted protein were measured using BCA protein assay kit. ELISA assay was conducted following the manufacturer's protocol. Standard and samples of serum or supernatant media (100 μl per well) were added to the plate and incubated for 2 h at room temperature. Then secondary antibody solution and streptavidin HRP solution were added to each well and incubated for 1h. Before starting each step, washing the well was conducted 3 times. After substrate were added into each well, stop solution were add in 5 min. ELISA assay was measured by using the ELISA reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA). The values of the sample's optical density (OD) were detected at 450 nm.

9. Immunocytochemistry

Differentiated cells were fixed in 4 % paraformaldehyde, pH 7.4 (Merck, Darmstadt, Germany), for 10-15 min at RT. Samples were washed with phosphate buffered saline

(PBS, Thermo) three times. Blocking was performed with 10 % normal donkey serum in PBS containing 0.3 % Triton X-100 for 60 min at RT. Primary antibodies against beta-III-Tubulin (TUJ1), microtubule-associated protein 2 (MAP2), neural cell adhesion molecule (NCAM), Luciferase and neuro-filament heavy (NF-H) were incubated overnight at 4 °C. Samples were washed three times with PBS, and secondary antibodies were added and incubated for 1 h at RT. Samples were washed three times with PBS, and 4', 6'-diamino-2-phenylinodole (DAPI; Vector Labs, Burlingame, CA, USA) was added. Samples were covered with glass coverslips. A laser confocal microscope (LSM 700; Zeiss, Oberkochen, Germany) was used for analysis.

10. Western blotting

Western blotting was conducted to determine the function of the therapeutic gene GM-CSF. All of the extracted tissue lysates were prepared and loaded onto SDS-PAGE gels (10-12 % gel, Bio-Rad, Richmond, CA, USA). After electrophoresis, transferring and blocking, primary antibodies targeting Bax (dilutions of 1:1,000, Millipore Corp., Milford, MA, USA) and β-actin (1:10,000, Abcam, Cambridge, UK) were applied. After conjugation of secondary antibodies, detection of protein signals was performed with the automatic X-ray film processor (TM-300E; TAEAHN Inc, Incheon, Korea), and densitometry was quantified using ImageJ (NIH, Bethesda, MD, USA).

11. Cell viability assay

The protective effect of GM-CSF was measured in 96-well plates by using the MTT assay. mNSCs were seeded at a density of $0.5\times10^4/cm^2$ and incubated for 3 days with supernatant media from each group (Control, SV-GMCSF, NSE-GMCSF) and treated with a chemically toxic reagent (diluted in 1 % DMSO) for an additional 24 h. MTT

reagent (final concentration of 0.5mg/ml; Sigma) was added to each well. After 3 h, the viability of cells was measured using the ELISA reader (VERSAmax) at 562 nm.

12. Statistical analysis

Statistical analysis was performed using Prism 6 (Graphpad, San Diego, CA, USA) and MedCalc software version 14.12.0 (MedCalc, Mariakerke, Belgium). All data were evaluated using Student's t-test and a one-way ANOVA followed by the Tukey-Kramer Post hoc test. All data are expressed as the mean ± standard error of the mean (S.E.M). A P-value < 0.05 was considered statistically significant.

Table 1. List of Antibodies used in the whole experiments

Antibody	Host	Product Information		Dilution Factor
		Company	Cat.No	
MAP2	Mouse	Abcam	AB11267	1:500
MAP2	Rabbit	Abcam	AB32454	1:200
Luciferase	Goat	Novus	100-1677	1:500
Neurofilament-H	Chicken	Abcam	AB4680	1:1000
TuJ1	Rabbit	Abcam	AB18207	1:1000
NCAM	Rabbit	Abcam	AB75813	1:200
Bax	Rabbit	Upstate biotechnology	06-499	1:1000
β-actin	Mouse	Abcam	AB8226	1:10,000
GMCSF	Mouse	Abcam	AB9741	1:1000

III. RESULTS

1. Establish gene expression system in mouse neural stem cells

Whole experiments were conducted using pGL3 based plasmid. NSE-Luci was constructed using neuron specific enolase (NSE) promoter and pGL3-basic plasmid. SV-GMCSF and NSE-GMCSF was made by substituted luciferase gene of SV-Luci and NSE-Luci to GM-CSF gene (Fig. 1A).

To examine the neuronal differentiation potency of mNSCs used in this study, cells were cultured in differentiation media containing RA (1 μM) for 7 days. Differentiated cells were positive with the neuronal markers TUJ1, MAP2, NCAM, and neuro-filament. This result showed that cells used in this study are neural stem cells which can be differentiated into neurons (Fig. 1B-D).

Before whole experiments were conducted, transfection characteristic was confirmed. Because of using transient gene expression system, transfection efficiency and gene expression in mNSCs was tested.

Transfection efficiency in mNSCs was measured by using DsRed expression vector. 2 days after transfection, positive cells of DsRed or DAPI were counted and compared with them. The average of DsRed positive cell was 40 % percentage compared with DAPI (Fig. 2A).

The target gene expression by transfected mNSCs was confirmed. After mNSCs were transfected with SV-GMCSF or NSE-GMCSF, the immune-fluorescence staining was conducted using anti-GMCSF. Although anti-GM-CSF was positive in mNSCs, but there didn't show a difference between SV-GMCSF and NSE-GMCSF. This means that over-expressed GMC-SF was secreted extra-cellular membrane (Fig. 2B).

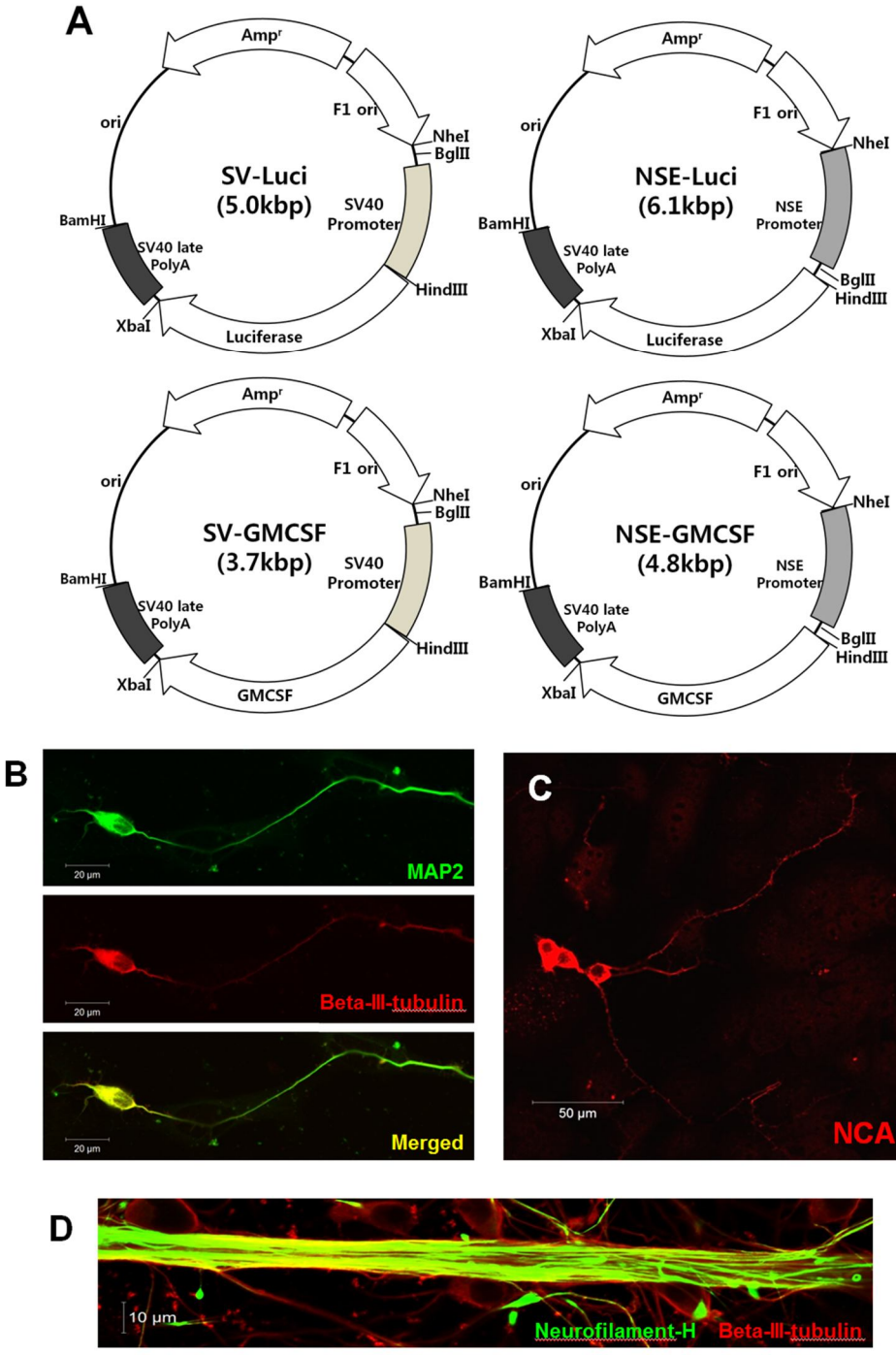
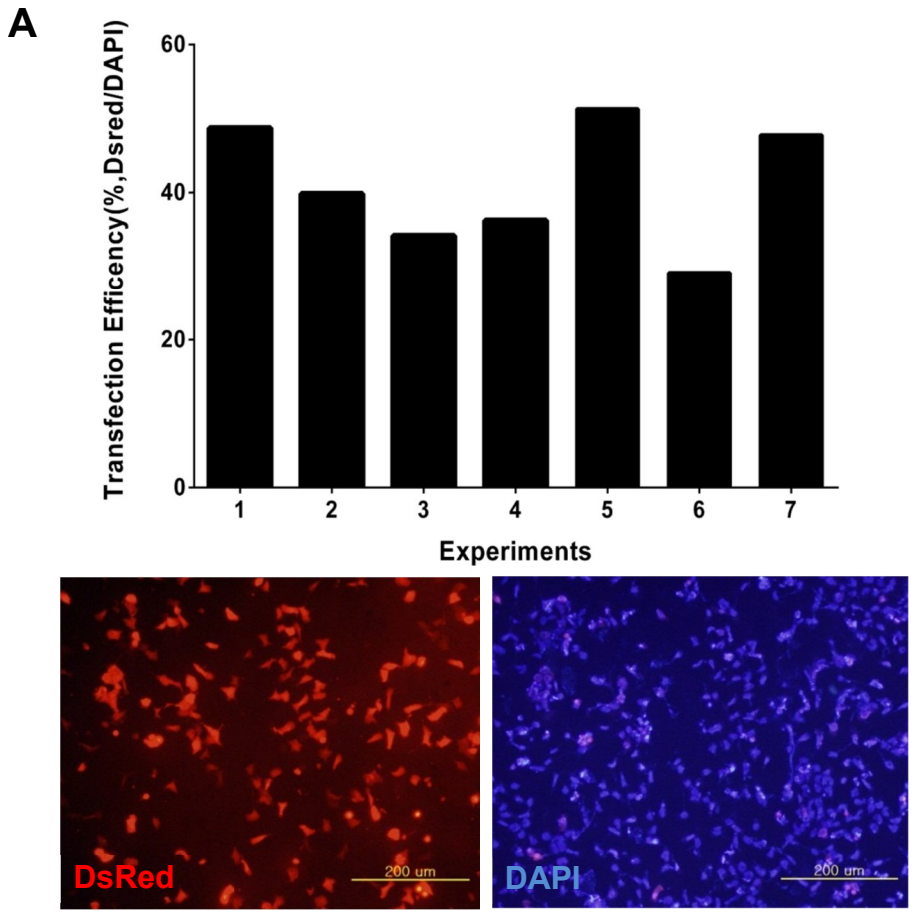

A
Amp^r
ori
F1 ori
NheI
BglII
SV-Luci
(5.0kbp)
SV40
Promoter
BamHI
SV40 late
PolyA
HindIII
XbaI
Luciferase
NSE-Luci
(6.1kbp)
NSE
Promoter
BglII
SV-GMCSF
(3.7kbp)
NSE-GMCSF
(4.8kbp)
GMCSF
B
20 μm
MAP2
Beta-III-tubulin
Merged
C
50 μm
NCAM
D
10 μm
Neurofilament-H
Beta-III-tubulin

Figure 1. Plasmid construction and characterization of mouse neural stem cells

(A) Plasmid construction of neuronal cell type-inducible or general luciferase over-expression vectors. The NSE promoter region was inserted to pGL3-basic vector after digestion (NheI and BglII sites). The mouse GM-CSF was inserted into luciferase site sites of neuronal cell type-inducible or general luciferase expression vector. Neuronal differentiation potency of mNSCs was confirmed by fluorescence staining specific for each neuron. (B and C) Most of the differentiated cells were positive for neuron-specific markers such as beta-III-tubulin, MAP2, and NCAM. (D) The thick axonal bundle was confirmed by staining with neuro-filament and beta-III-tubulin.


A
Transfection Efficency(%,Dsred/DAPI)
60
40
20
0
1
2
3
4
5
6
7
Experiments
DsRed
200 um
DAPI
200 um

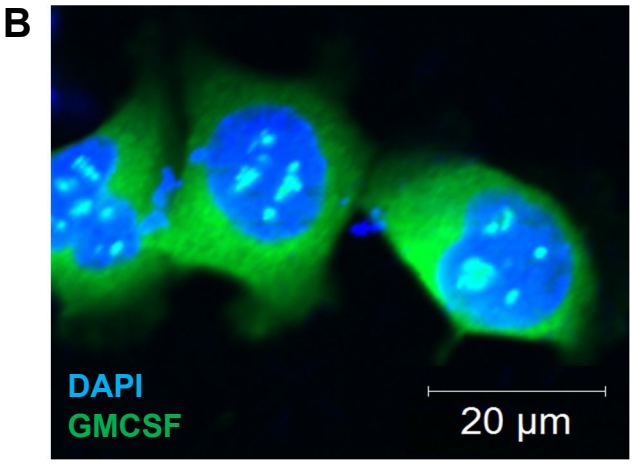

B
DAPI
GMCSF
20 μm

Figure 2. Transfection efficiency and gene expression in mNSCs

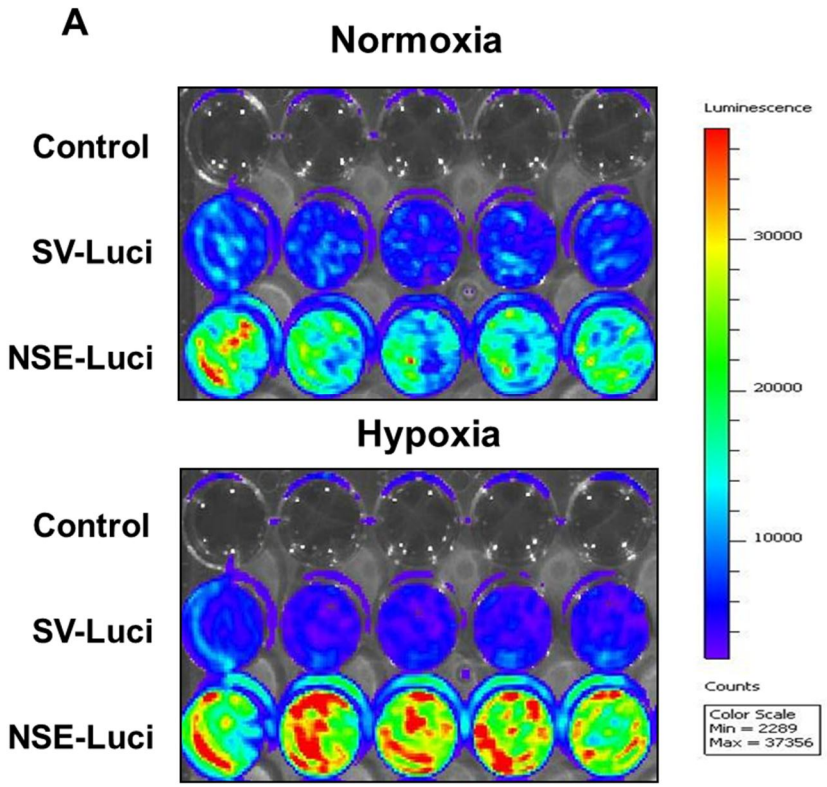
(A) The DsRed expressing plasmid was tranfected into mNSCs to examine the transfection efficiency in mNSCs. 2 days after transfection into mNSCs, DsRed or DAPI positive cells were counted. About 40 % of mNSCs were positive with DsRed compared with DAPI positive cells. Fluorescence scale bar, 200 μm. (B) Immune-fluorescence staining was carried out for detecting of GM-CSF expression in mNSCs which were transfected with SV-GMCSF or NSE-GMCSF. There are no difference between SV-GMCSF and NSE-GMCSF. Fluorescence scale bar, 20 μm.

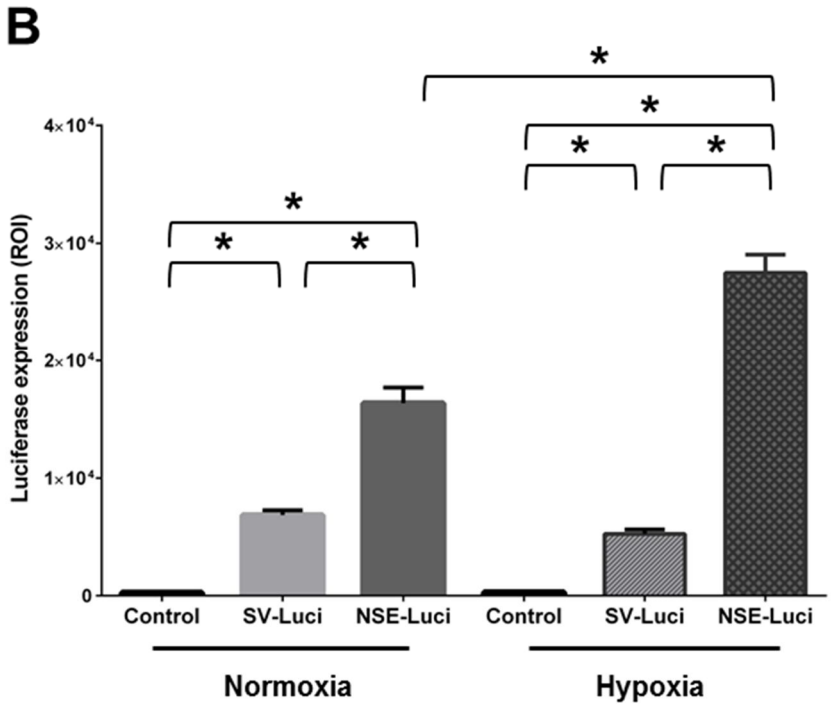
2. Expression pattern of neural stem cells transfected with a neuronal cell type-inducible luciferase over-expression system (NSE-Luci)

The level of luciferase expression in mNSCs was investigated which were transfected with Control (DsRed), SV-Luci, and NSE-Luci plasmid to confirm whether this neuronal cell type-inducible gene over-expression system is suitable for mNSCs. Based on the results of IVIS and the luciferase assay, luciferase expression significantly increased in NSE-Luci compared to SV-Luci (Fig. 3). These results indicate that the neuronal cell type-inducible transgene over-expression system is suitable for mNSCs.

To identify whether the transgene over-expression pattern can be sustained after hypoxic injury (mimicking tissue ischemia after SCI), the luciferase expression levels of Control, SV-Luci, and NSE-Luci were compared after hypoxic injury for 24 h. the luciferase expression level significantly increased in NSE-Luci compared to that in SV-Luci (Fig. 3). These results indicate that the merged therapy strategy with mNSCs and a neuronal cell type-inducible gene over-expression system can be applied to spinal cord injuries that cause tissue ischemia.

In order to assure whether the NSE was selectively working in neuronal lineage cells, the luciferase expression levels were examined in HEK 293FT cells, which are human embryonic kidney cells, transfected with Control (DsRed), SV-Luci, and NSE-Luci plasmid. The luciferase expression level in 293FT transfected with NSE-Luci plasmid was significantly lower than 293FT transfected with SV-Luci plasmid (Fig. 4). This result means that the efficacy of the neuronal cell-type–inducible gene expression system depends on neuronal lineage cell type.


A
Normoxia
Control
SV-Luci
NSE-Luci
Hypoxia
Control
SV-Luci
NSE-Luci
Luminescence
30000
20000
10000
Counts
Color Scale
Min = 2289
Max = 37356


B
Luciferase expression (ROI)
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
Control
SV-Luci
NSE-Luci
Control
SV-Luci
NSE-Luci
Normoxia
Hypoxia

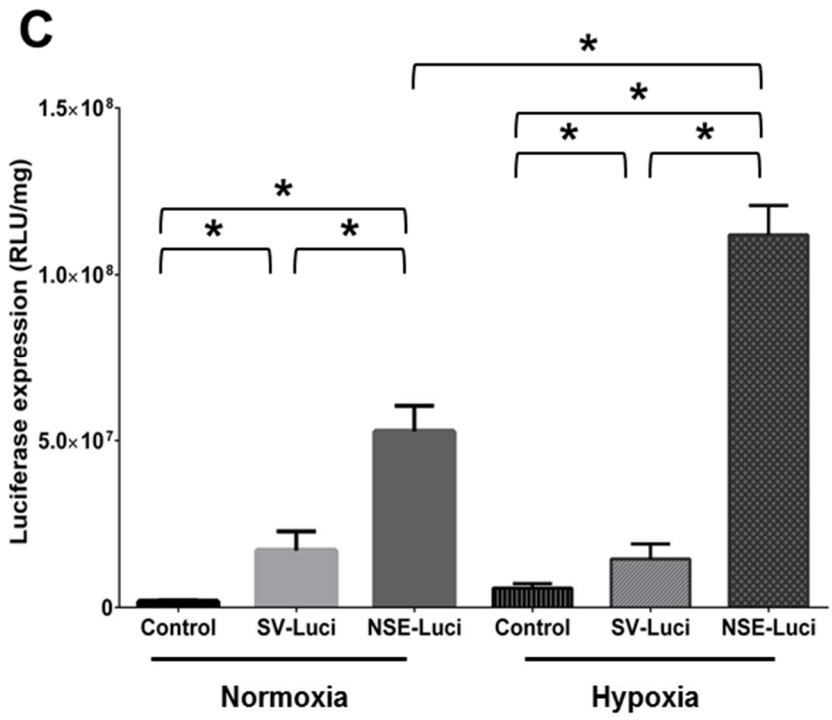

C
Luciferase expression (RLU/mg)
1.5×10⁸
1.0×10⁸
5.0×10⁷
0
Control
SV-Luci
NSE-Luci
Control
SV-Luci
NSE-Luci
Normoxia
Hypoxia

Figure 3. Luciferase expression pattern of neural stem cells introduced with a neuronal cell type-inducible luciferase over-expression system (NSE-Luci) in normoxia and hypoxia

Luciferase expression levels of Control, SV-Luci, and NSE-Luci were examined by luciferase expression imaging, the day after transfection, which were incubated at 24 h more in normoxia or hypoxia. (A) The result of luciferase expression imaging of IVIS indicates that luciferase in NSE-Luci group was highly over-expressed compared to the other groups. The color scale bar indicates the luciferase expression level. (B) Quantitative analysis of luciferase expression imaging in Fig. 3A. (C) Luciferase expression levels of Control, SV-Luci, and NSE-Luci examined by luciferase assay. Luciferase expression of NSE-Luci was much higher than the other groups in normoxia and hypoxia. * $P < 0.05$. Data represent mean ± S.E.M.

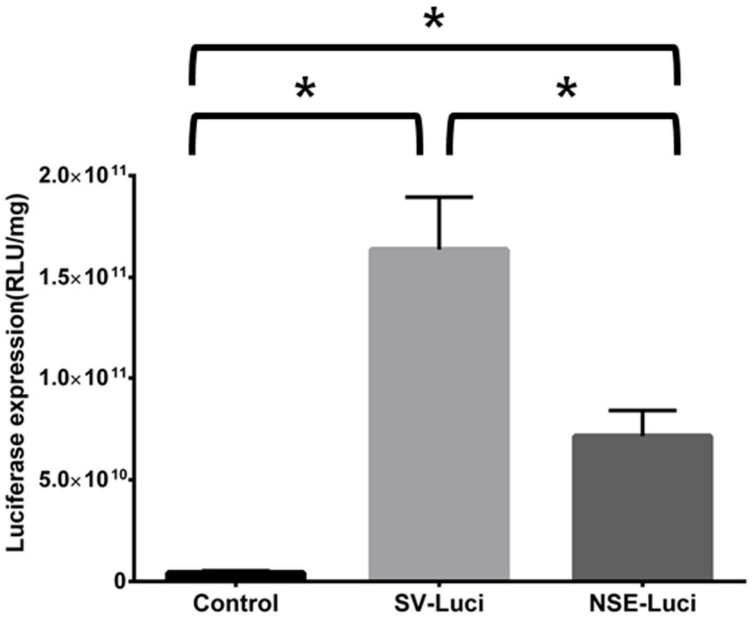


Figure 4. Neuronal cell type-inducible luciferase gene over-expression systems in non-neuronal cells

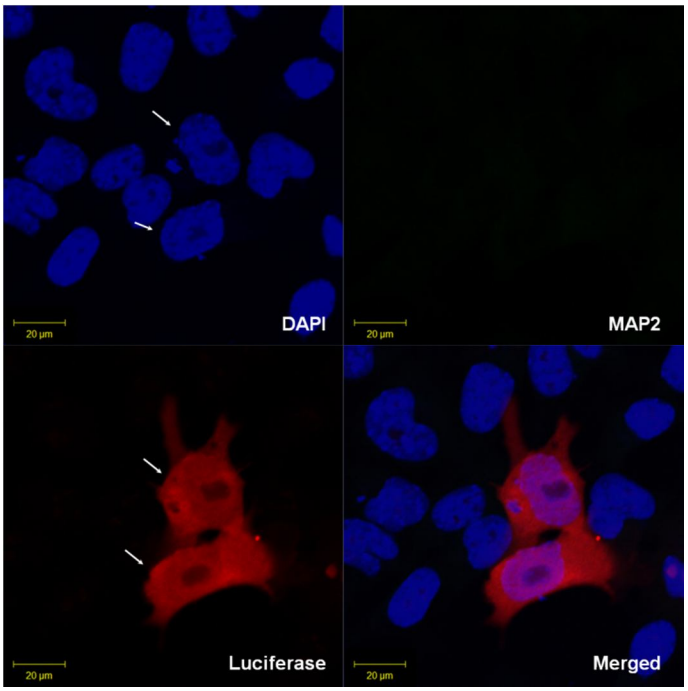
To confirm whether the NSE was selectively working in neuronal lineage cells, Human Embryonic Kidney 293 cells (HEK-293FT), non-neuronal cells, were transfected. Luciferase expression levels of SV-Luci and NSE-Luci were confirmed by luciferase assay at 48 h after transfection. * $P < 0.05$. Data represent mean ± S.E.M.

3. Transgene expression pattern in differentiated neuron

In order to verify whether the luciferase expression pattern of the NSE-Luci can be increased after neuronal differentiation (1 μM RA) compared to neural stem cells stage (0 μM RA), the level of luciferase expression was compared between neural stem cells which were un-differentiated and differentiated neuron and also compared between NSE-Luci and SV-Luci after inducing neuronal differentiation. The luciferase expression level of NSCs transfected with NSE-Luci plasmid was much higher after neuronal differentiation than un-differentiation (Fig. 5). At the neuronal differentiation condition (1 μM RA), the luciferase expression level of NSE-Luci was significantly higher than that of SV-Luci (Fig. 6).

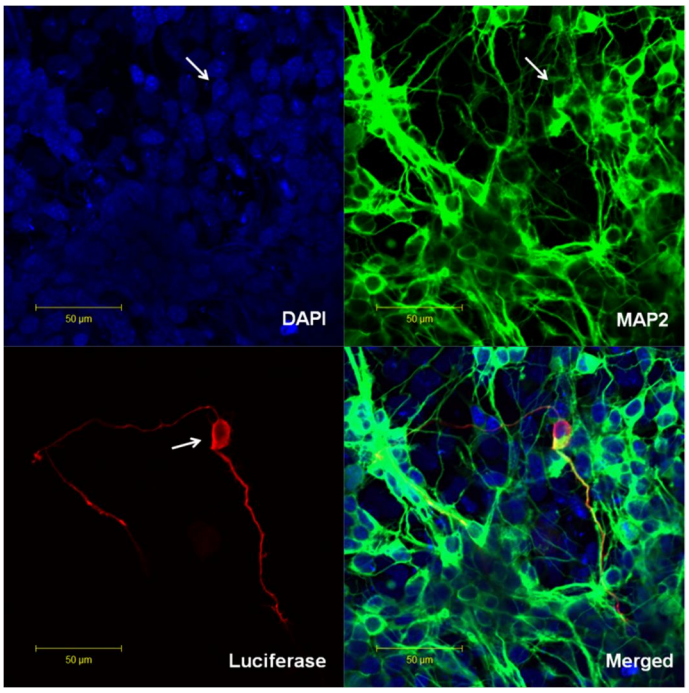
These results mean that the transgene expression by NSE promoter was induced by neuronal differentiation. Thus, these findings indicate that the neuronal cell type-inducible transgene over-expression system appropriates with NSCs which have the neuronal differentiation potency for merged treatment strategy.

A



Undifferentiated mNSCs (0 µM RA)

B



Neuronal Differentiated Cells (1 µM RA)

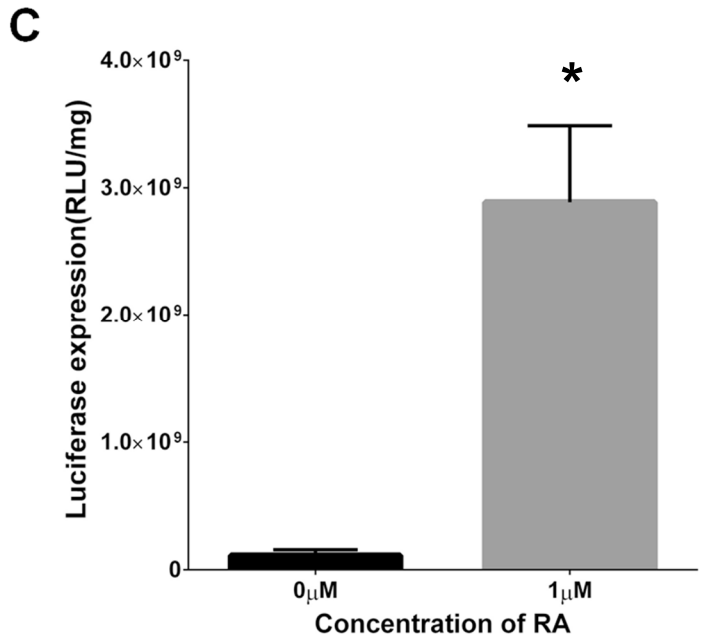


Figure 5. High luciferase expression pattern after differentiation into neurons

Induced cell differentiation with treatment of 1 μM RA for 7 days after transfected with NSE-Luci, Luciferase expression level was compared between undifferentiated (without RA) and differentiated cells (with 1 μM RA). (A and B) Double staining was conducted by using immunocytochemistry with $MAP2^+$ (mature neuronal marker, Green) and $Luciferase^+$ (Luciferase marker, Red). The positive cells by immunostaining were indicated by arrows. (C) The Luciferase expression level of differentiated neurons was compared with undifferentiated neurons by using the luciferase assay. Luciferase expression was significantly greater in differentiated neurons than in neural stem cells. * $P < 0.05$. Data represent mean ± S.E.M.

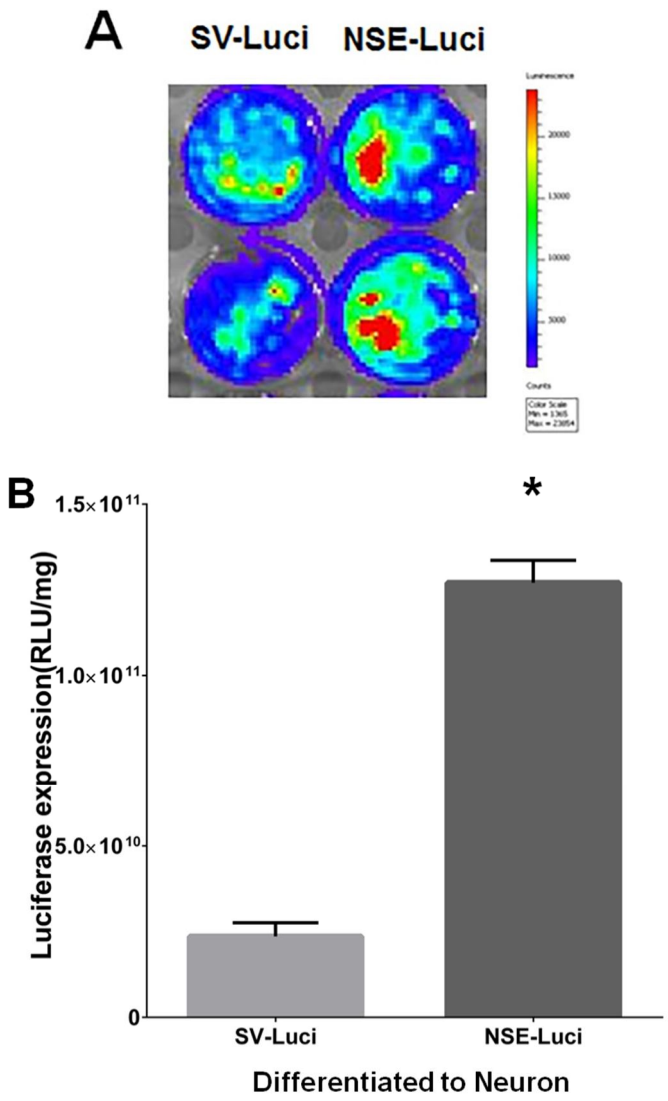


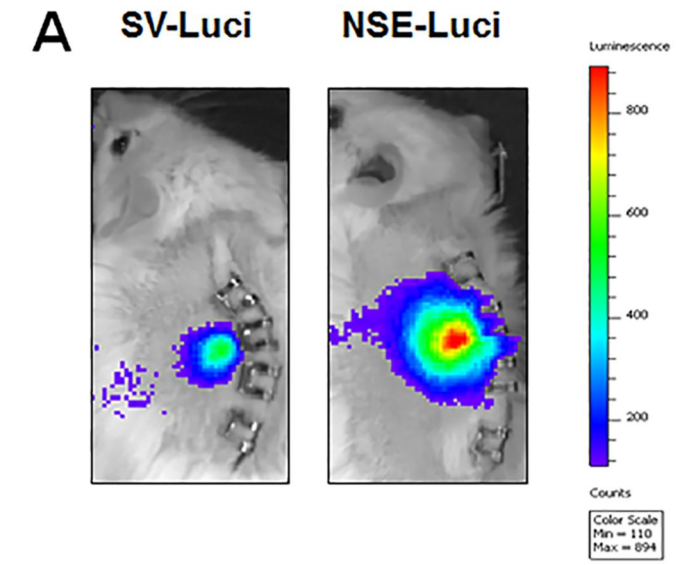
Figure 6. A neuronal cell type luciferase expression pattern after differentiation into neurons

After induced differentiation for 7 days, a neuronal cell-type luciferase expression system was compared with a general luciferase expression system. (A) IVIS imaging

indicated that NSE-Luci was over-expressed luciferase than SV-Luci. (B) Using Luciferase assay, the luciferase expression level of NSE-Luci after neuronal differentiation was significantly greater than that of SV-Luci after differentiation. * $P < 0.05$. Data represent mean ± S.E.M.

4. Transgene expression pattern *in vivo*

To confirm whether NSE-Luci show a consistent high expression pattern *in vivo*, both SV-Luci and NSE-Luci were transplanted into the spinal cord, and carried an IVIS analysis and luciferase assay was carried out 24 h after transplantation. Similar to our results *in vitro*, the luciferase expression level was significantly higher in NSE-Luci than in SV-Luci (Fig. 7A and B). These results indicated that a merged treatment strategy based on neuronal cell type-inducible transgene expression system and NSCs can be applied to spinal cord injury.


A
SV-Luci
NSE-Luci
Luminescence
800
600
400
200
Counts
Color Scale
Min = 110
Max = 894

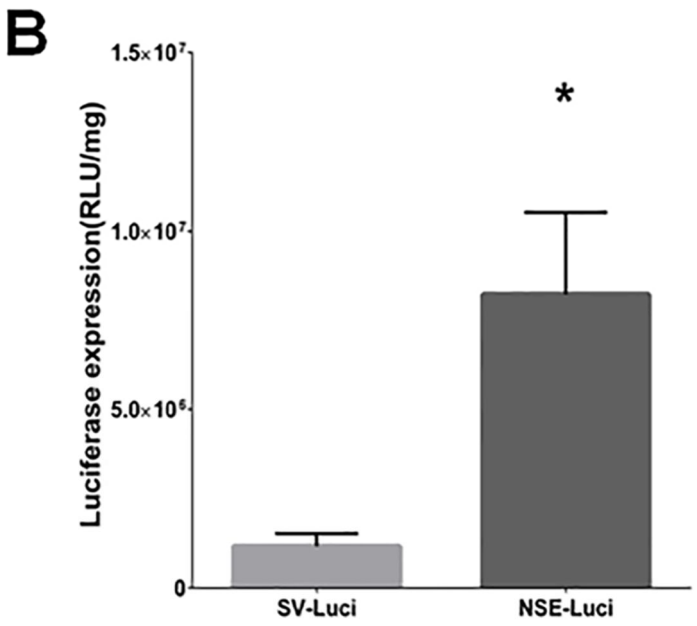

B
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
Luciferase expression(RLU/mg)
*
SV-Luci
NSE-Luci

Figure 7. Luciferase expression pattern of neuronal cell type-inducible luciferase gene over-expressing neural stem cells after transplantation

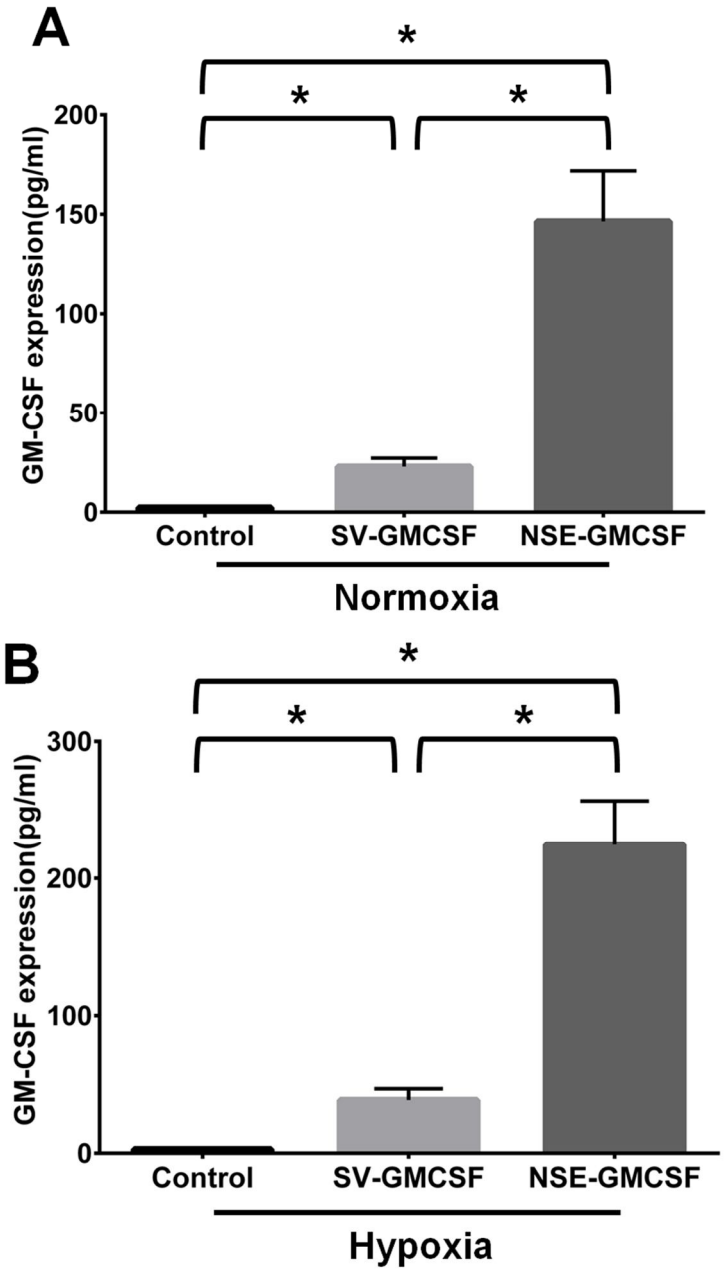
(A) To define over-expression level of luciferase gene by NSE::Luci *in vivo*, transfected mNSCs ($5x10^5$ cells/2μl) were transplanted 7 days after SCI mice model. Luciferase expression pattern of SV-Luci and NSE-Luci confirmed by IVIS 24 h after cell transplantation. NSE-Luci transplanted into spinal cord showed a high luciferase expression compared to SV-Luci after transplantation. The color scale indicates the luciferase expression level, not the cell numbers in this study. (B) Luciferase expression pattern of SV-Luci and NSE-Luci confirmed by luciferase assay 24 h after cell transplantation. NSE-Luci transplanted into the spinal cord consistently showed a high luciferase expression pattern compared to SV-Luci after transplantation. * $P < 0.05$. Data represent mean ± S.E.M.

5. Therapeutic Gene, GM-CSF, expression pattern of a neuronal cell-type inducible gene expression system (NSE-GMCSF)

To confirm expressing therapeutic gene, luciferase gene was substituted to mouse GM-CSF gene which is reported that GM-CSF has the effect survival of neuronal cell (Fig. 1A). GM-CSF is secreted cytokine, thus mature GM-CSF will be secreted out of the extracellular membrane.

The level of secreted GM-CSF was investigated in culture media for 2 days after transfection with the GM-CSF expression vector. The amount of GM-CSF secreted into the culture media was significantly increased in the supernatant of mNSCs transfected with NSE-GMCSF plasmids compared to mNSCs transfected with Control (Ds-Red) or SV-GMCSF plasmid (Fig. 8A).

In order to examine whether the GM-CSF over-expression pattern of mNSCs with the neuronal cell type-inducible gene over-expression system was maintained after transplantation into an ischemic environment, the amount of secreted GM-CSF was measured by conducting ELISA after mNSCs which were transfected with NSE-GMCSF or SV-GMCSF were cultured in hypoxia or cell-toxicity conditions (mimicking an ischemic environment). The GM-CSF over-expression pattern of NSE-GMCSF was consistent in the hypoxia or cell-stress conditions (Fig. 8B and C). Therefore, these results demonstrate that the neuronal cell type-inducible gene over-expression system is stable, even after replacing luciferase with GM-CSF.


A
*
*
*
200
150
100
50
0
GM-CSF expression(pg/ml)
Control
SV-GMCSF
NSE-GMCSF
Normoxia
B
*
*
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300
200
100
0
GM-CSF expression(pg/ml)
Control
SV-GMCSF
NSE-GMCSF
Hypoxia

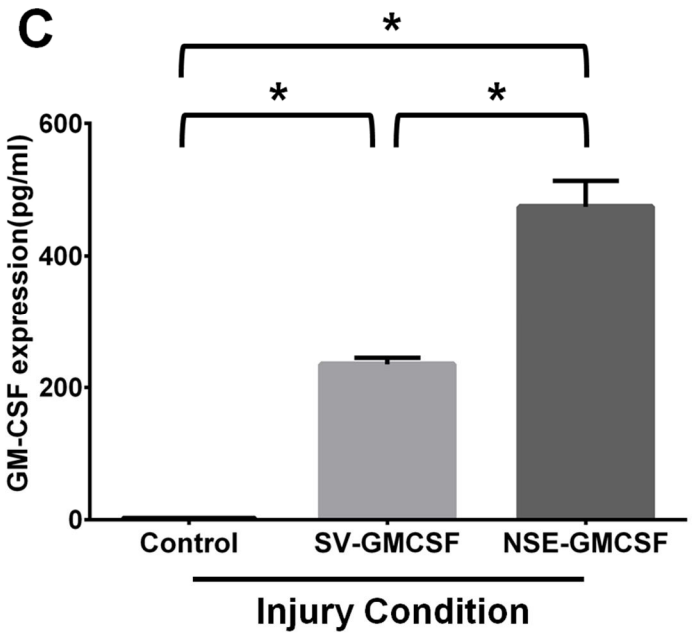

Figure 8. NSE-GMCSF in normoxia, hypoxia and injury condition

A day after transfection, mNSCs were incubated in (A) normoxia, (B) hypoxia and (C) injury condition (hypoxia + 1 % DMSO) for 1 day. The expression level of GM-CSF was measured by conducting the GMCSF ELISA assay with the culture media where GM-CSF was secreted. NSE-GMCSF was higher express GM-CSF than other groups. * $P < 0.05$. Data represent mean ± S.E.M.

6. GM-CSF expression sustains to transplanted cell survival

To neuronal survival, expression of GM-CSF is more sustain than Control (DsRed) group until neuronal differentiation. The secretion period of GM-CSF into the culture media has been checked after transient gene transfection was carried out. The amount of GM-CSF secreted in culture media was measured at 3, 5 and 7 days after conducting transfection with SV-GMCSF and NSE-GMCSF. The result showed that GM-CSF secretion was sustained for about 7 days; also, the GM-CSF over-expression pattern of NSE-GMCSF was sustained after 7 days (Fig. 9).

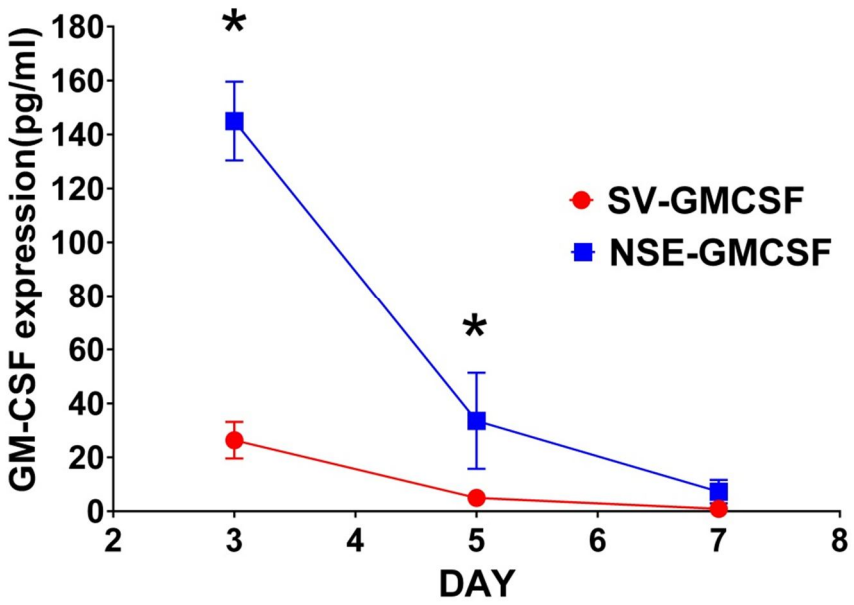

Figure 9. The secretion pattern of GM-CSF following transient expression

To activate the protective effect of GM-CSF to transplanted mNSCs, highly over-expression and continuous secretion of GM-CSF were need. The amount of GM-CSF secreted in culture media at 3, 5 and 7 days after transfection with SV-GMCSF and NSE-GMCSF. Until secretion of GM-CSF became extinct, the expression level of GM-CSF by NSE-GMCSF was higher sustained than SV-GMCSF. * $P < 0.05$. Data represent mean ± S.E.M.

7. Cell survival effect of GM-CSF *in vitro*

The MTT assay was used to test the protective effect of GM-CSF *in vitro*.

To compare with the effect of GM-CSF, mNSCs were incubated for 3 days with supernatant media of each group that had been incubated in normal conditions (Fig. 7A). In order to induce chemical damage into mNSCs, a toxic reagent, 1 % DMSO, was treated to mNSCs for 1 day and then and conducted an MTT assay. The data show that the more the level of GM-CSF increased, more mNSCs survived (Fig. 10). The mNSCs treated with the supernatant media of NSC-GMCSF had the highest survival, compared with other groups of cells. This result indicates that GM-CSF affects to increase the cell viability in injury condition.

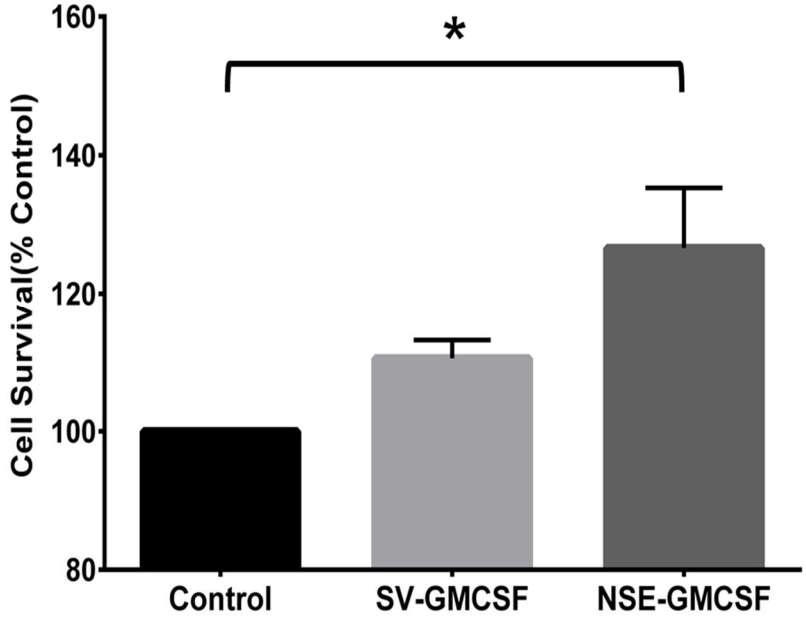

Figure 10. Cell survival as the effect of GM-CSF *in vitro*

To confirm the protective effect of GM-CSF, mNSCs incubated with the culture media of each group for 3 days and then 1 % DMSO was treated 24 h for injury. Cell survival was measured by MTT assay. The higher the level of GM-CSF expressed by mNSCs, the more cell viability increased. * $P < 0.05$. Data represent mean ± S.E.M.

8. GM-CSF expression pattern and anti-apoptotic effect of NSE-GMCSF *in vivo*

To test whether similar pattern of NSE-Luci expression can be found *in vivo*, injured mice model was divided 4 groups : Sham (Only SCI), Control (DsRed), SV-GMCSF and NSE-GMCSF. Each transfected group was transplanted with transfected mNSCs into lesion of spinal cord. Consistently, the amount of secreted GM-CSF significantly increased in the NSE-GMCSF group, compared to other groups (Fig. 11). This result demonstrated that the neuronal cell type-inducible GM-CSF expression on neural stem cells consistently and stably functions *in vivo*. The level of GM-CSF was showed as a unit of pg/mg of total protein.

After confirming expression of GM-CSF in mNSCs *in vivo*, anti-apoptotic effect of GM-CSF was tested *in vivo*. To find out the effect of GM-CSF, the western blotting was performed using the apoptosis marker Bax on tissue samples collected on post-transplantation day 4. The ratio of Bax/β-actin in each transplanted cell group was significantly decreased compared to the Control group. Moreover, the level for NSE-GMCSF was decreased compared with Control and SV-GMCSF (Fig. 12A and B).

These results supported the conclusions of previous studies that GM-CSF has a direct or indirect neuro-protective effect and reduces cell apoptosis.

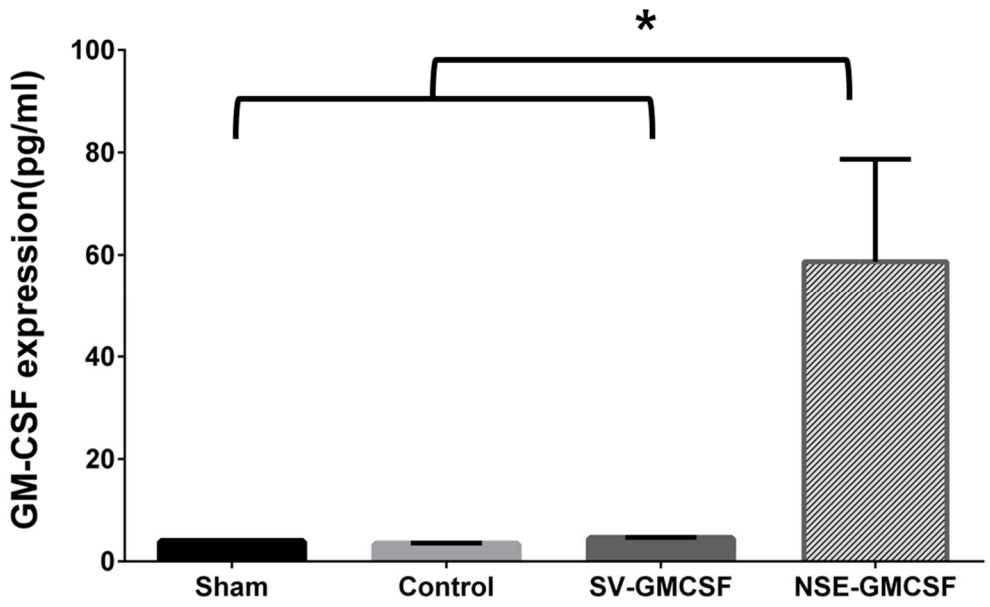

Figure 11. GM-CSF expression pattern of neuronal cell-type inducible GM-CSF expressing neural stem cells after transplantation

mNSCs of each group ($5x10^5$ cell/2μl) was transplanted to spinal cord injury model. A day after transplantation, spinal cord samples were used to ELISA assay. The amount of GM-CSF secreted from transplanted mNSCs, which were transfected by NSE-GMCSF, over-expressed compared to other groups. * $P < 0.05$. Data represent mean ± S.E.M.

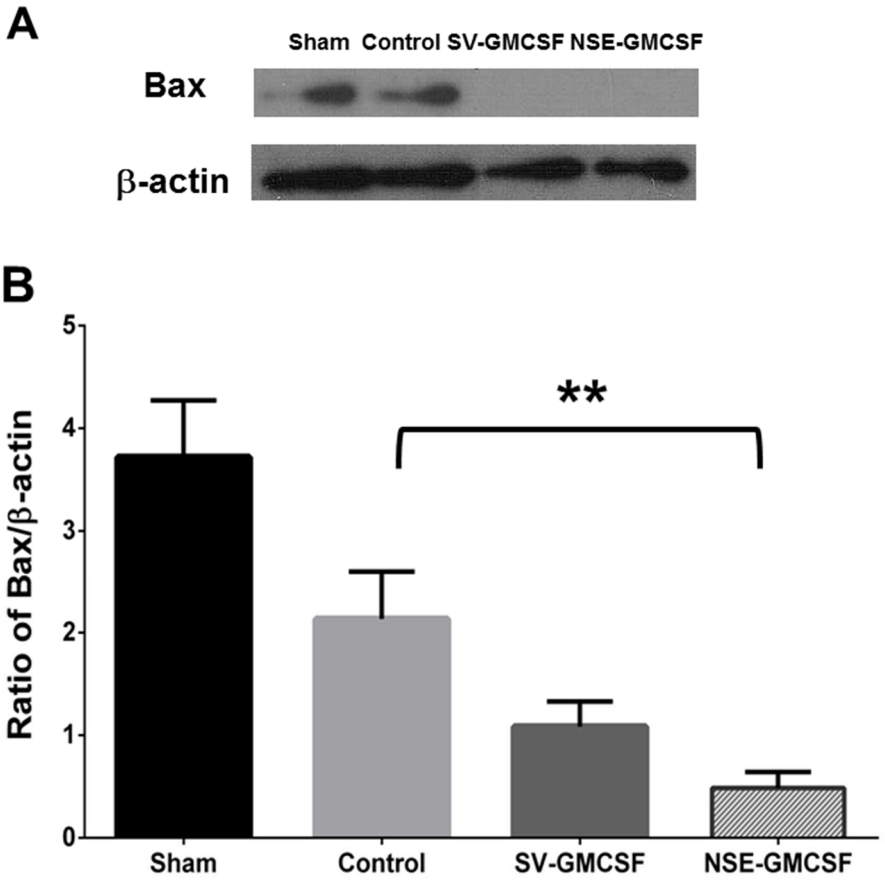

Figure 12. The anti-apoptotic effect of GM-CSF *in vivo*

4 days after transplanted mNSCs into SCI model, extracted spinal cord lysates were examined by western blotting with apoptotic marker, Bax. (A and B) Western blotting data showed that transplanted mNSCs expressed decreased levels of Bax and NSE-GMCSF expressed highly decreased levels of Bax, compared with other groups. ** $P < 0.01$. Data represent mean ± S.E.M.

IV. DISCCUSION

As one of the pathologies that appear after spinal cord injury, an ischemic environment in the spinal cord is caused by the destruction of blood vessels; this ischemic condition lasts from several days to several weeks. The survival rate of the stem cells transplanted into the ischemic environment would normally rapidly decline. Therefore, a gene and stem cell therapy platform based on neural stem cells and a neuronal cell-type–inducible gene expression system were designed to protect transplanted stem cells and the injured spinal cord.

The gene over-expression by neuron specific enolase (NSE) promoter is already known as working on mature neurons. It was confirmed that the luciferase expression level of NSE-Luci was significantly higher than the luciferase expression level of SV-Luci; however, the luciferase expression level of 293FT cells (used as a mean non-neuronal cell type) transfected with NSE-Luci was significantly lower than that of 293FT cells transfected with SV-Luci plasmid. This result means that transgene over-expression by NSE promoter is specifically working at the neural stem cell stage. The previous study also investigated the difference of gene over-expression based on NSE promoter in between neurons and non-neuronal cells such as HEK293 and HeLa cells.[4] In addition to, it has been known that NSE is expressed not only in mature neuron, but also in other immature cell types such as a neuroblastoma, small cell lung cancer, medullary thyroid cancer, carcinoid tumors, and melanoma.[26-32] Thus, it suggested that NSE promoter may function extensively in neuronal lineage cells.

NSE-Luci showed a higher luciferase expression level than that of SV-Luci under normoxia and hypoxia. Interestingly, the luciferase expression level of NSE-Luci in hypoxia was higher than that of NSE-Luci in normoxia. This result consider that the response of mNSCs stimulated by hypoxic condition.[33-36] In addition to, the luciferase expression level of NSE-Luci was much higher after differentiation into neurons. All of these features can be used advantageously, if the luciferase used in this study were replaced by other therapeutic genes.

In previous study, it was confirmed that NSCs transfected with a hypoxia-inducible

gene expression system should be maintained for at least 1 day under hypoxic conditions for transgene over-expression.[3] However, the gene expression level of NSCs transfected with a neuronal cell type-inducible gene over-expression system was sufficiently greater in normoxia, namely before transplantation (saving time, as there is no need to wait 24 h). Thus, the high amount of secreted therapeutic protein may be useful for improving the survival of transplanted stem cells, as well as protecting the injured spinal cord from exposure to an ischemic environment. Subsequently, the high amount of secreted therapeutic protein from transplanted NSCs would be sustained under the ischemic environment and, would also increase further neuronal differentiation.

As known as one of the therapeutic gene, GM-CSF is a secreted cytokine, and mature GM-CSF is secreted out of extracellular membranes. As examining the GM-CSF expression pattern in cell lysates of SV-GMCSF or NSE-GMCSF by Western blot analysis, GM-CSF expression level of NSE-GMCSF was similar to SV-GM-CSF (the data was not shown). Finally, the amount of GM-CSF secreted in culture media was significantly increased in NSE-GMCSF. These results indicate that mature therapeutic protein was properly made by neural stem cells transfected with the neuronal cell type-inducible therapeutic gene over-expression system. The blood brain barrier or blood spinal cord barrier are present in the central nervous system so that it is very difficult to deliver a relatively large sized therapeutic protein to a lesion site by oral ingestion or injection. Based on this problem, direct transplantation of therapeutic gene expressing stem cells at the injured region of the central nervous system may provide the maximal therapeutic benefit by allowing a relatively large sized therapeutic protein to be provided directly and sustainably to the neural tissues or other cells.

In this study, it has a limited part that only focused on the reproducibility regarding the stability or consistency of the neuronal cell type-inducible therapeutic gene over-expression system in neural stem cells. Thus, the biological effect of the therapeutic gene did not further investigated. Because the transient gene transfection method was

used in all experiments, the secretion of GM-CSF couldn't be expected beyond 7 days (Fig. 9). In future studies, it will be necessary to establish a stably therapeutic gene-expressing neural stem cell line for long-term secretion of therapeutic protein after transplantation. If that happens, it could be expected that the high amount of the therapeutic gene secreted by therapeutic gene-expressing NSCs will be performed effectively their innate biological function.

Thus, these findings suggest that a neuronal cell type-inducible gene expression system is suitable for use combined with NSCs for expressing a high amount of therapeutic genes, and such gene and neural stem cell therapy methods based on present systems would be very promising tools as potential treatment for spinal cord injury.

V. CONCULSION

In this study, a combined treatment strategy using a neuronal cell type-specific transgene expression system with NSCs was established for spinal cord injury. It was confirmed that the luciferase gene expression level of NSE-Luci was significantly higher than that of SV-Luci. This pattern was sustained after hypoxic injury and *in vivo*. After verified by using Luciferase gene, GM-CSF gene expression level of NSE-GM-CSF was compared with SV-GMCSF. The amount of secreted GM-CSF is significantly increased in the supernatant of NSCs transfected with NSE-GMCSF. The GM-CSF over-expression of NSE-GMCSF was consistently stable in hypoxic or cell stress conditions as well as *in vivo*. It is suggest that a neuronal cell type inducible transgene expression system is suitable for neural stem cells; furthermore, a combined treatment strategy based on a neuronal cell type-inducible therapeutic gene over-expression system and NSCs may provide an effective therapeutic outcome for neurological disease treatment.

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ABSTRACT (IN KOREAN)

척추신경손상모델에서 신경계열 세포 특이적 유전자 발현시스템과 신경줄기세포를 이용한 병합 치료 기술 개발

<지도교수 하 윤>

연세대학교 대학원 의과학과

유 영 상

영구적인 신경손상과 관련된 척수 손상 치료에 있어서 유망한 치료기술이 필요하다. 단일 치료 기술로 충분한 치료효과를 기대하기 힘들기에 두 가지 이상 병합된 치료 기술이 치료 효과를 보다 향상시킬 수 있을 것이다.

본 연구에서, 치료 효율을 극대화하고자 신경세포 계열 특이적인 프로모터로 조절되는 신경세포 계열 특이적 발현시스템과 신경줄기세포 병합 기술을 개발하였다. 치료유전자를 사용하기 전에 병합 시스템이 적절히 작동하는지 확인하고자 루시퍼레이즈 유전자를 선택하여 신경 세포 계열 특이적 유전자 발현시스템 (NSE-Luci)과 신경세포 계열 비특이적 유전자 발현시스템 (SV-Luci)을 생체 내와 생체 외 조건에서 비교 분석하였다. 루시퍼레이즈 유전자로 신경세포계열 특이적 유전자 발현시스템을 확인 한 뒤, 치료유전자인 과립대식세

포 자극인자 발현 시스템을 제작하였으며 생체 내와 생체 외 조건에서 신경세포 계열 특이적 발현시스템을 비교하였다.

실험 결과로 신경줄기세포에 도입된 신경세포계열 특이적 유전자 발현 시스템이 비특이적 유전자 발현시스템보다 루시퍼레이즈 유전자가 과발현되는 것을 확인하였고 또한 치료유전자 발현시스템에서 과립대식세포 자극인자가 과발현되는 것을 확인하였다.

신경세포 계열 비특이적 유전자 발현시스템보다 신경 세포 계열 특이적 유전자 발현시스템이 신경 줄기 세포와 병합 치료 기술에 있어서 적합하다는 것을 증명해보았다. 따라서, 척수손상과 같은 신경퇴행성 질환 치료에 있어서 이와 같은 병합치료기술이 유용한 기법으로 사용될 수 있음을 제안을 해본다.

핵심되는 말: 신경계열 특이적 프로모터, 과립대식세포 자극인자, 신경계열 특이적 유전자 발현, 신경줄기세포, 세포병합치료기술, 척수손상