Combination of GSK3 and HDAC Inhibitors Increases Gene Expression Efficiency to the Hypoxia/Neuron-Specific System: An Optimal Experimental Scheme in Treating Spinal Cord Injury

Zhimin Pan

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Yoon Ha

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Zhimin Pan

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This certifies that the Doctoral Dissertation of Zhimin Pan is approved.

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Thesis Supervisor : Yoon Ha

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Thesis Committee Member#1: Bae Hwan Lee

_____

Thesis Committee Member#2: Inbo Han

_____

Thesis Committee Member#3: Sung-Rae Cho

_____

Thesis Committee Member#4: Jinsoo Oh

The Graduate School

Yonsei University

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ABSTRACT

Combination of GSK3 and HDAC inhibitors increases gene expression efficiency to the hypoxia/neuron-specific system: an optimal experimental scheme in treating spinal cord injury

Zhimin Pan

Department of Medicine
The Graduate School, Yonsei University

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Spinal cord injury (SCI) tends to damage neural tissue and generate a hypoxic environment. Studies have confirmed that single therapy with gene or stem cells was inefficient, but combining stem cells and gene therapy in treating tissue damage has been studied to overcome some limitations, such as low gene delivery efficiency and therapeutic outcome. Thus, a combination of stem cells, gene therapy and hypoxia-specific system may be useful for the reconstruction of injured spinal cord.

To synergistically treat SCI, I designed a combined platform (HNIS-hiNSCs) using a hypoxia/neuron-inducible gene expression system (HNIS) and human induced-neural stem cells (hiNSCs) produced by direct reprogramming. Sox2- or Nestin-positive hiNSCs were differentiated to Tuj1-, MAP2-, or NeuN-positive neurons. HNIS showed consistent hypoxia/neuron-specific gene expression in hiNSCs cultured under hypoxia. In particular, HNIS-hiNSCs revealed a complex

pattern with higher gene expression compared with a single platform, such as a neuron-specific gene expression system (NSE). In addition, we found that an optimal combination of small molecules, such as CHIR99021, valproic acid, glycogen synthase kinase-3β (GSK3β) and histone deacetylase (HDAC) inhibitors, which could significantly enhance gene expression with HNIS-hiNSCs in the hypoxic environment. This experiment demonstrated that HNIS-hiNSCs combined with GSK3 and HDAC inhibitors may present another promising strategy in the treatment of SCI.

Key words: Glycogen synthase kinase-3, Histone deacetylase, Hypoxia/neuron-specific system, Spinal cord injury

Combination of GSK3 and HDAC inhibitors increases gene expression efficiency to the hypoxia/neuron-specific system: an optimal experimental scheme in treating spinal cord injury

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I. INTRODUCTION

Spinal cord injury (SCI) is a common and severe disease with a complex underlying mechanism. Given that it is possible for spinal cord damage to occur without fracture [1], and the predominant damage in traumatic SCI derives from cell death in the second stage [2], surgery may not be an applicable or the only approach for treating SCI. Fortunately, researchers found that transplantation-based therapy provided numerous key benefits because grafts could be precisely delivered to relevant lesions to achieve neuroprotection [3] and support long-term cellular integration and therapeutic replacement. More specifically, researchers have demonstrated that transplantation of neural stem cells (NSCs) or neural progenitor cells was a promising treatment for SCI and neurodegenerative diseases [4]. In recent years, induced pluripotent stem cells (iPSCs) have been used to overcome the ethical issues of embryonic stem cells

(ESCs) and to achieve autologous stem cell reprogramming from many mature cell types [5]. As autologous stem cells from patients or animals, iPSCs have been intensively investigated for treating intractable diseases [5,6].

Although directly reprogrammed stem cells have been used for the treatment of neurodegenerative diseases, including SCI in basic research [7], and stem cell therapy has shown effective outcomes in some preclinical studies [8,9], the therapeutic effectiveness of stem cells against SCI still needs to be improved because SCI has a complicated pathophysiology and possibly demands a combined strategy for adequate treatment [10-12]. The therapeutic challenges include how to address the damaged nerve existing in an ischemic environment following SCI [13]. Therefore, our research team developed a tissue-specific gene expression platform based on hypoxia- and neuron-specific promoters to improve ischemia in the injured spinal cord [14,15]. However, the previous study confirmed that a single gene delivery strategy is inefficient for SCI [14]. Although cell transplantation is feasible for SCI but the efficacy remain unproven [16], and only stem cells are not significantly effective in spinal lesion treatment [17].

Both hypoxia- and neuron-specific gene expression systems have shown that the overexpression systems functioned well in the hypoxic-ischemic environment. In combination with stem cells, this special gene expression system could overcome critical hypoxic limitations, such as low gene delivery efficiency. Both Azmitia et al. and our research team could obtain gene overexpression and specific autologous stem cells by direct reprogramming technology with tissue-specific transcription factors or small molecule-based methods [18,19]. In the present study, I investigated the application of a hypoxia/neuron-inducible gene expression system (HNIS) combined with human induced NSCs (hiNSCs) in a hypoxic environment. Unexpectedly, I observed that the optimal combination of

HNIS-hiNSCs and small molecules could increase the gene expression efficiency of HNIS.

II. MATERIALS AND METHODS

1. Cell culture and neural differentiation

Fibroblast-derived hiNSCs were donated by the Korea Research Institute of Bioscience and Biotechnology, and hiNSCs were identified by a research article (Cell Research, Janghwan Kim, KRIBB). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in a culture medium consisting of neurobasal medium and advanced Dulbecco's modified Eagle's medium/nutrient mixture F-12 at a 1:1 ratio, with 1% N-2 supplement (100×), 2% B-27 supplement (50×), 0.05% bovine serum albumin, 1% penicillin/streptomycin, 1% Glutamax-I (100×), and 0.1% 2-mercaptoethanol (1000×; all purchased from Life Technologies, Carlsbad, CA, USA), as well as 0.1 μM A83-01 (Tocris Bioscience, Bristol, UK), 3 μM CHIR99021 (Tocris Bioscience), and leukemia inhibitory factor (LIF, 1 unit; Millipore, Temecula, CA, USA), which were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA). To induce differentiation of hiNSCs, the cells were seeded on plates coated with 1% basement membrane matrix (BD Biosciences, San Jose, CA, USA) at a density of 1×10^4 cells/cm^2 and incubated at 37°C for 24 h. The next day, I replaced the media containing A83-01, CHIR99021, and LIF with one containing brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, and neurotrophin-3 (all 10 ng/mL, all purchased from Life Technologies). The medium was replaced every 2-3 days.

Mouse NSCs (mNSCs; 30~40 passages; CRL-2925, ATCC, Manassas, VA,

USA) were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT, USA) and 1% penicillin streptomycin (Gibco). Cells were cultured under normoxic conditions at 37℃ in 5% CO_2. For neural differentiation of mNSCs, cells were seeded in a Matrigel (BD Biosciences, Bedford, MA, USA) coated a 12-mm glass coverslip. The following day, it was replaced with basal media containing retinoic acid (1 μM) and then maintained for 7 days. The medium was replaced every 2-3 days.

2. Plasmid establishment and transfection

The pSV-Luc, pNSE-Luc, and pEpo-SV-Luc plasmids were kindly provided by Hanyang University (Prof. Minhyung Lee). The HNIS plasmid was established in our previous study [14]. Briefly, Epo cDNA was amplified using polymerase chain reaction (PCR) (Hotstart Pfu PCR PreMix, Bioneer, Daejeon, Korea) using pEpo-SV-AP (laboratory DNA stock). Two Epo fragments were used to construct the pEpo-NSE-Luc plasmid. First, the primers for PCR were 5′-MluI-Epo and 3′-NheI-AscI-Epo. The amplified Epo enhancer was inserted into the MluI and NheI sites upstream of the NSE promoter. The second PCR of the Epo enhancer was conducted using the primers 5′-KpnI-SpeI-EcoRV-Epo and 3′-SacI-PmeI-Epo. The PCR products of Epo were inserted into KpnI and SacI.

For transfection, hiNSCs (30~40 passages) were seeded at a density of 1×10^5 cells/cm^2 in a multiwell plate and cultured under normoxic conditions (21% O_2). Plasmid (250 ng plasmid/cm^2) and Viafect transfection reagent (3 μl Viafect/1 μg plasmid, Promega, Madison, WI, USA) were added to 50 μl serum-free media. The Viafect/plasmid complexes were briefly mixed and incubated at room temperature for 10 min. The Viafect/plasmid mixture was added to cells and then

maintained under normoxic conditions for 24 h. The next day, I changed the culture media to one containing small molecules (A83-01, 0.1 μM; CHIR99021, 3 μM; LIF, 1 unit; valproic acid, 1 mM) and then transferred the cells to hypoxic conditions (1% O_2). After 24 h, the luciferase expression level was analyzed.

3. Luciferase expression detector

A luciferase expression detector (LED; Xenogen, Alameda, CA, USA) was used to measure the expression level of luciferase in terms of promoter activity in HNIS. For the in vitro study, D-luciferin (150 μg/ml) was added into each well and then kept in the dark for 10 min. The cell culture plates were transferred to the LED imaging chamber, and then, the bioluminescence was measured for 10-30 s.

For the in vivo study, D-luciferin (30 mg/kg) was intraperitoneally injected before anesthesia. After 10 min, both ketamine (100 mg/kg, Yuhan, Seoul, Korea) and Rompun (10 mg/kg, Bayer Korea, Seoul, Korea) were injected to induce anesthesia (I had used the above anesthetic drugs by the suggestion of our institution). The spinal cord tissues containing transplanted human induced NSCs (hiNSCs) were harvested from the animals. After washing with saline, the tissues were transferred to culture media containing D-luciferin (300 μg/ml) and then kept in the dark for 10 min. The samples were transferred to the LED chamber, and then, the bioluminescence was measured for 10-30 s. The luciferase level was quantified using the LED software (Xenogen, Alameda, CA, USA). For blinding analysis, different researchers performed the analysis for the LED imaging study.

4. Immunofluorescence staining

Samples were washed with phosphate-buffered saline (PBS, HyClone), and

then, the cells were fixed using 4% paraformaldehyde (pH 7.2, Millipore, Bedford, MA, USA) for 10 min at 20°C. The samples were washed three times for 3 min with 0.3% Tween 20 (Life Technologies) in PBST and incubated in blocking buffer for 30 min at 20°C. Primary antibodies against Nestin (1:1000, Millipore), Sox-2 (1:250; Abcam, Cambridge, MA, USA), rabbit anti-Tuj1 (1:1000, Abcam), NeuN (1:1000, Millipore), MAP2 (1:1000, Millipore), Synapsin-1 (1:205, Abcam), and neurofilament (1:1000, Abcam) were diluted in blocking buffer and allowed to react with the samples for 60 min at 20°C.

The samples were washed with PBST three times (3 min each). Cy3- or FITC-conjugated secondary antibodies (1:500) were also diluted in blocking buffer and incubated in the dark for 30 min at 20°C. After three washes with PBST, the samples were stained using 4′,6′-diamino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA).

5. Flow cytometry

After removing the medium and washing with PBS, the cells were detached from the culture plates using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (Life Technologies) and harvested in 1.5-mL Eppendorf tubes (Eppendorf, Hamburg, Germany). Fixation was performed with 4% paraformaldehyde for 10 min, and the samples were then washed twice with PBST for 3 min. Then, the cells were incubated with primary antibodies against Nestin (1:1000) and Sox-2 (1:250) for 60 min at 20°C. Following three washes with PBST, the cells were incubated with secondary antibodies in the dark for 30 min at 20°C. Finally, the pellet was washed in 200 μL PBST three times. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Rutherford, NJ, USA) and Cell Quest software (Becton Dickinson).

6. Spinal cord injury and cell transplantation

For transplantation of iNSCs transfected with the HNIS-Luc plasmid, the cells (at a density of 1×10^5 cells/cm^2) were seeded in Matrigel-coated 60-mm dishes and then cultured under normoxic conditions (21% O_2). The HNIS plasmids (250 ng plasmid/cm^2) were transfected into hiNSCs using the previously mentioned transfection protocol and then maintained under normoxic conditions for 24 h. On the next day, I changed the culture media to one containing small molecules (A83-01, 0.1 μM; CHIR99021, 3 μM; LIF, 1 unit; VPA, 1 mM) and then transferred the cells to hypoxic conditions (1% O_2). After 24 h, cells were harvested using trypsin and then resuspended in PBS at a density of 2.5×10^5 cells/μL.

For inducing anesthesia, both ketamine (100 mg/kg, Yuhan) and Rompun (10 mg/kg, Bayer Korea) were intraperitoneally injected into C57BL/6 mice (male, 6-week-old, 30 g; OrientBio, Seongnam, Gyeonggi-do, Korea). I performed a laminectomy at the thoracic level 11; SCI was induced using a self-closing forcep (a compression injury for 1 s).

I divided into two groups using a simple randomization method: Group 1: HNIS-NSCs (n=10); Group 2: HNIS-iNSCs with small molecules (n=10). Subsequently, the transfected cells (5×10^5 cells, 2 μL) were immediately injected into the SCI site at a 1 μL/min injection rate using a syringe pump (KDS 310 Plus, KD Scientific, Inc., Holliston, MA, USA). Cyclosporin A (10 mg/kg, Chong Kun Dang, Seoul, Korea) and cefazolin (20 mg/kg, Yuhan) were also intraperitoneally injected. Kerorolac (0.1 mg/kg) was intraperitoneally injected to minimize animal suffering.

Our protocols were approved by the Animal Care and Use Committee of the

Medical Research Institute at Yonsei University Health System. The experimenter was unaware of the animal's group during experimentation and statistical analysis. This randomized animal experiment was conducted in accordance with international guidelines on the ethics of animals, and I made efforts to minimize the number of animals used.

7. Statistical analysis

Statistical analysis was performed with SAS version 9.4 and SPSS version 22 (IBM; Chicago, IL, USA). Student's t-test was performed to assess the differences between two groups. One-way analysis of variance and Tukey's post hoc test were performed to assess differences among three groups. Normally distributed data are presented as the mean (standard deviation), and p-value less than 0.05 was considered statistically significant.

III. RESULTS

1. hiNSC characterization

I first performed characterization, such as assessment of proliferation and marker expression, of the hiNSCs produced by direct reprogramming. The hiNSCs showed a rosette morphology and rapid growth, and most of the cells were positive for Ki67 in complete growth medium (Fig. 1A, B and C). At this stage, >90% of the hiNSCs were positive for Nestin and Sox-2 (Fig. 1D and E).

Next, I investigated the neural differentiation potency of the hiNSCs in vitro and in vivo. In the neural differentiation medium, many of the hiNSCs differentiated into neurons, which were positive for Tuj1, MAP2, and NeuN 2 weeks after induction of neural differentiation (Fig. 1F). Furthermore, green fluorescent protein (GFP)-expressing hiNSCs were transplanted into the injured

spinal cord and maintained from 1 week to 8 weeks. At 1 week, I confirmed that many of the hiNSCs had survived in the injured spinal cord tissue, and these were positive for human-specific nuclei and Nestin (Fig. 1G). At 8 weeks, some of the hiNSCs remained Nestin-positive (Fig. 1H), and many of the hiNSCs differentiated into MAP2-, Synapsin-1- and NeuN-positive cells (Fig. 1I). These results indicate that the hiNSCs used in these experiments are indeed NSCs.

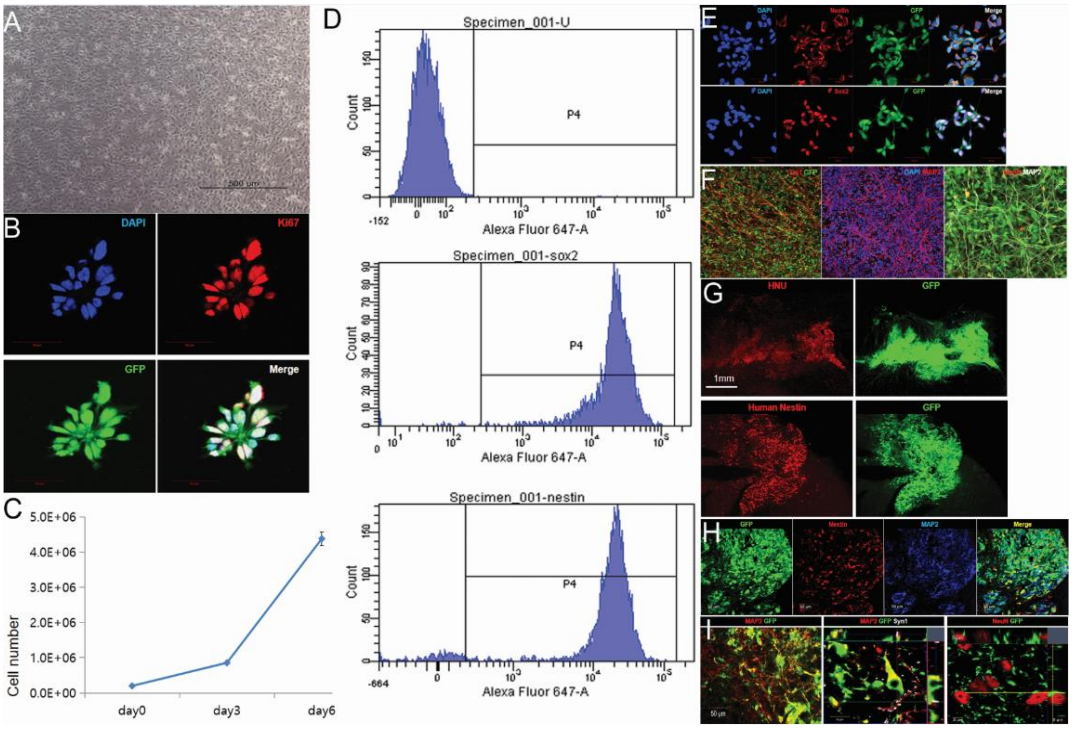

Fig. 1 Characterization of induced neural stem cells derived from human fibroblasts

A. Representative phase contrast images of human induced neural stem cells in proliferation media; B. Representative fluorescence images of human induced neural stem cells stained for Ki-67 and green fluorescent protein (GFP); C. Quantification of the cell proliferation rate of human induced neural stem cells maintained under normal conditions for 6 days; D and E. Representative flow cytometry scatter plots for Nestin- and

Sox-2-positive human-induced neural stem cells (D). Representative fluorescence images for Nestin and Sox-2 in undifferentiated neural stem cells (E); F. Representative fluorescence images of differentiated neural stem cells that were maintained for 14 days in differentiation condition and stained for the neural markers Tuj1, MAP2, and NeuN; G. Representative images of human induced neural stem cells transplanted into the injured spinal cord. Tissues were stained for human-specific nuclei, Nestin, and green fluorescent protein (GFP) 1 week after transplantation; H and I. Triple-stained image of human induced neural stem cells transplanted into the injured spinal cord. Tissues were stained for green fluorescent protein (GFP), human-specific Nestin, and MAP2 8 weeks after transplantation (H). Fluorescence image of differentiated neural stem cells transplanted into the injured spinal cord. The GFP-positive neural stem cells were colocalized with MAP2 and synapsin-1. Furthermore, GFP-positive fibers were attached to NeuN-positive neurons (I). Scale bars represent 20 μm (white) and 100 μm (yellow).

2. Gene expression pattern of HNIS in hiNSCs

Next, I examined the function of HNIS in hiNSCs maintained under normoxia or hypoxia. To investigate neuron-specific expression, I assessed luciferase expression in hiNSCs transfected with SV- or NSE-systems. In LED imaging, the hiNSCs transfected with NSE showed high gene expression compared with the SV system (Fig. 2A). This implies that the NSE system is more specific to NSCs than the SV system. Furthermore, I designed the HNIS by combining the Epo enhancer and NSE promoter. To confirm the hypoxia/neuron-specific expression, I compared the luciferase expression level in the hiNSCs transfected with the NSE or HNISs under normoxia or hypoxia. In LED imaging, the hiNSCs transfected with HNIS showed a higher gene expression level than that of the NSE system under hypoxia (Fig. 2B). Next, I verified the gene expression level by switching the oxygen concentration (i.e., from normoxia to hypoxia as well as from hypoxia

to normoxia). I again confirmed that gene expression controls depended on oxygen concentration (Fig. 2C-E).

I applied the HNIS to mNSCs to verify hypoxia/neuron-specific gene expression in different types of NSCs. The mNSCs were also positive for Sox-2 (Fig. 2F) and could be differentiated into Tuj1-, MAP2-, and neurofilament-positive cells in neural differentiation medium containing retinoic acid (Fig. 2G). Therefore, I demonstrated that the HNIS functions optimally in different types of NSCs (Figs. 2H and I).

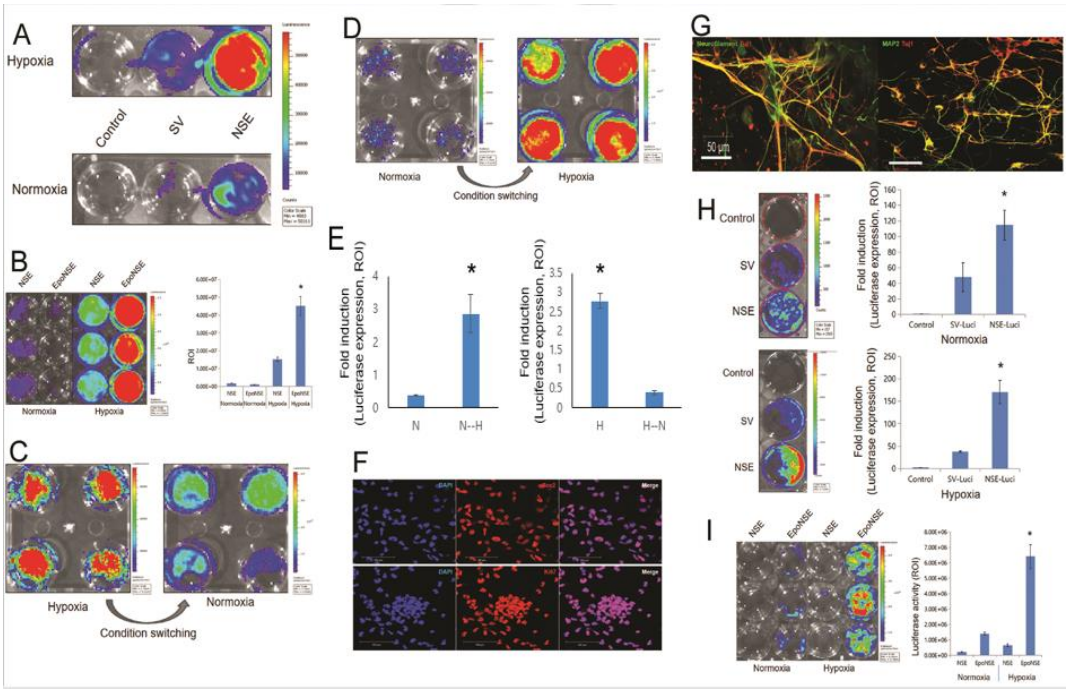

Fig. 2 Hypoxia/neuron-specific gene expression system in human induced neural stem cells (hiNSCs) and mouse neural stem cells

A. Luciferase expression detector (LED) image acquired 24 h after transfection of hiNSCs with SV- or NSE-Luc plasmids. The NSE promoter system is more inducible than the SV promoter in a neuronal cell type (hiNSCs); B. LED image and quantification

of luciferase expression in hiNSCs. Transfected cells were maintained under normoxia and hypoxia for 24 h after transfection with NSE- or HNIS-Luc plasmids; C-E. LED images (E and F) and quantification (G) of luciferase expression in hiNSCs transfected with HNIS. The hiNSCs were maintained under normoxia for 24 h following exposure to hypoxia (E). In contrast, hiNSCs were maintained under hypoxia for 24 h following exposure to normoxia (F); F. Representative fluorescence images of undifferentiated mouse neural stem cells (mNSCs) stained for Sox-2 and Ki-67; G. Representative fluorescence images of differentiated mNSCs stained for Tuj1, MAP2, and neurofilament following differentiation in vitro; H. LED imaging after transfection of mNSCs with SV- or NSE-Luc plasmid. Transfected mNSCs were maintained under normoxia or hypoxia for 24 h; I. LED images and quantification of luciferase expression 24 h after transfection of mNSCs with NSE- or HNIS-Luc plasmids. The HNIS is more inducible than NSE in hypoxic conditions. *p<0.05. Data are presented as the mean±standard error of the mean. Scale bars represent 500 μm.

3. Treatment with a HDAC inhibitor improves the efficiency of HNIS

In Fig. 2, I show the results confirming the specificity of HNIS to both hypoxic environments and neuronal cells. Next, I performed an experiment to identify small molecules that may improve the efficiency of HNIS. First, I assessed whether valproic acid (VPA) treatment increases the gene expression level in hiNSCs transfected with the NSE system (NSE-iNSCs). To this end, VPA, a HDAC inhibitor, was added to the complete iNSC culture media containing A83-01, leukemia inhibitory factor (LIF), and CHIR99021. The luciferase expression level was significantly increased in the VPA-treated group (Fig. 3A), and this phenomenon was also observed with the HNIS (Figs. 3B and C). I also compared the gene expression level between HNIS and NSE under hypoxia; the HNIS showed an increased gene expression pattern compared with the NSE

system (Fig. 3D). Finally, I verified the combination effect of HNIS and small molecules in a SCI model. The HNIS-hiNSCs was transplanted into the injured spinal cord after treatment with VPA for 24 h under hypoxia. Luciferase expression was increased in the VPA-treated group (Fig. 3E). Thus, these results imply that the combination of VPA with A83, LIF, and CHIR99021 is critical to increase the gene expression in HNIS.

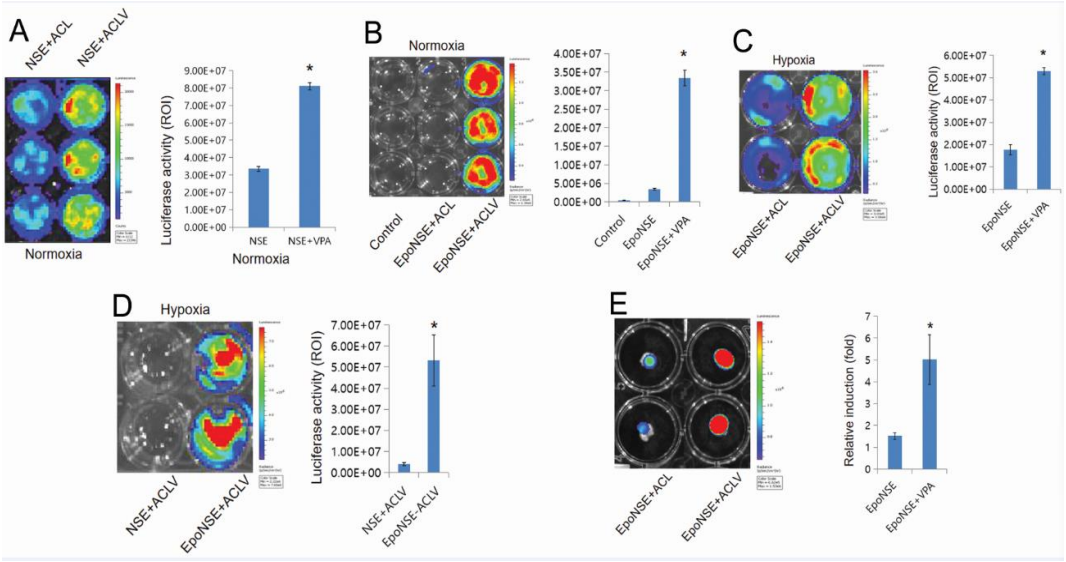

Fig. 3 Treatment with valproic acid as a histone deacetylase inhibitor improves the efficiency of the hypoxia/neuron-inducible gene expression system (HNIS)

A and B. Representative luciferase expression detector (LED) images and quantification of luciferase expression in induced neural stem cells (hiNSCs) transfected with NSE (A) or HNIS plasmids (B). Transfected hiNSCs were maintained under normoxia for 24 h after treatment with an HDAC inhibitor; C. Representative LED image and quantification of luciferase expression in hiNSCs transfected with HNIS plasmid. Cells were maintained under hypoxia for 24 h after treatment with a HDAC inhibitor; D. Comparison of luciferase expression in hiNSCs transfected with NSE or HNIS plasmids. NSCs were

maintained under hypoxia for 24 h after treatment with a HDAC inhibitor; E. Representative LED image and quantification of luciferase expression in HNIS-hiNSCs transplanted into the injured spinal cord 24 h after transplantation. *p<0.05.
A, A83-01; C, CHIR99021; L, leukemia inhibitory factor; V, valproic acid.

4. The combination of GSK3 and HDAC inhibitors increases luciferase expression in HNIS

In Fig. 3, I show results confirming that VPA treatment with complete media containing A83-01, LIF, and CHIR99021 increases the gene expression level in HNIS-hiNSCs. However, it is unclear which molecule combination plays a role in increasing gene expression in HNIS. Therefore, I further screened small molecule combinations that may be pivotal in increasing the gene expression in HNIS. First, I excluded a single small molecule, one by one, in mixed combinations. The luciferase expression level was remarkably decreased in the absence of CHIR99021 or VPA (Fig. 4A-D). This phenomenon was observed in other hiNSCs derived from different donors and in the combination of VPA and LiCl. These results indicate that CHIR99021 or VPA plays a pivotal role in controlling gene expression and that the combination of CHIR99021 and VPA is synergistic.

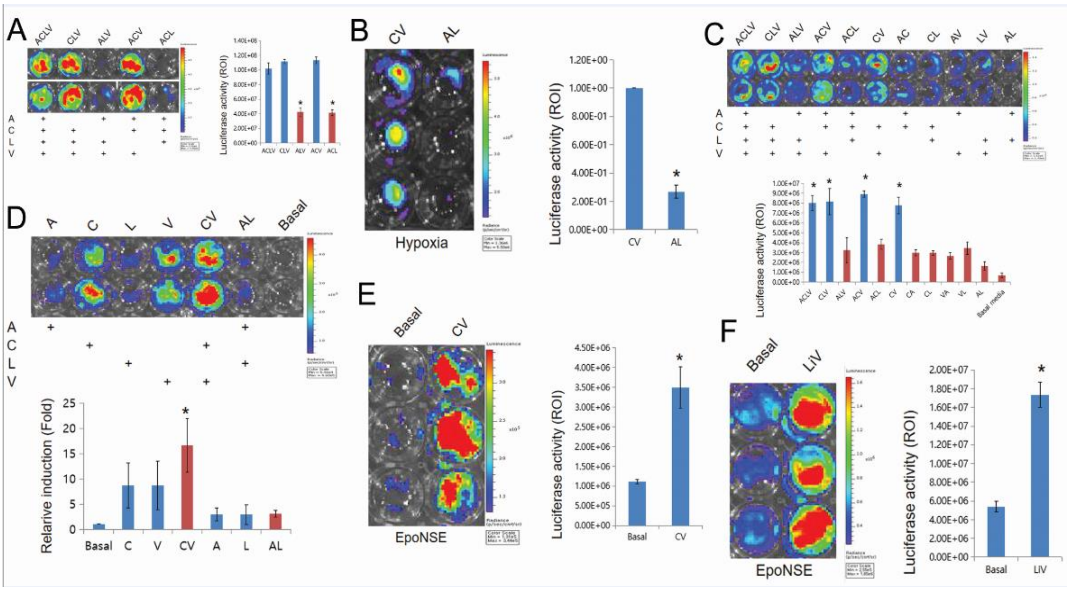

Fig. 4 The combination of GSK3 and HDAC inhibitors increases the gene expression efficiency of the hypoxia/neuron-inducible gene expression system (HNIS)

A–D. Representative luciferase expression detector (LED) images and quantification of luciferase expression in combination with different types of small molecules. Human induced neural stem cells (hiNSCs) transfected with the HNIS plasmid were maintained for 24 h under hypoxia in the absence of one small molecule per condition. The results indicate that CHIR99021 or valproic acid plays a pivotal role in increasing the gene expression efficiency of HNIS, and the combination has a synergistic effect in this context; E. Representative LED image and quantification of luciferase expression in hiNSCs derived from a different donor. The hiNSCs were transfected with HNIS plasmid and cultured under hypoxia for 24 h after CHIR99021 and valproic acid treatment; F. Representative LED image and quantification of luciferase expression in HNIS-hiNSCs. Transfected hiNSCs were treated with valproic acid and LiCl, used as another GSK3 inhibitor, and maintained under hypoxia for 24 h. *p<0.05.

A, A83-01; C, CHIR99021; L, leukemia inhibitory factor; V, valproic acid.

Forskolin is widely used for the neural differentiation of NSCs. Thus, I investigated whether forskolin treatment increases the gene expression level in hiNSCs transfected with HNIS (HNIS-iNSCs). I added forskolin or VPA to complete iNSC culture media containing A83-01, LIF, and CHIR99021 and then compared the gene expression level. In LED imaging, the luciferase expression level was significantly increased in the VPA-treated group. In contrast, this increasing phenomenon was not observed for the combination of forskolin with A83-01, LIF, and CHIR99021 (Fig. 5). These results indicate that the combination of forskolin, a common neural differentiation inducer, has no synergistic effect on increasing the gene expression efficiency.

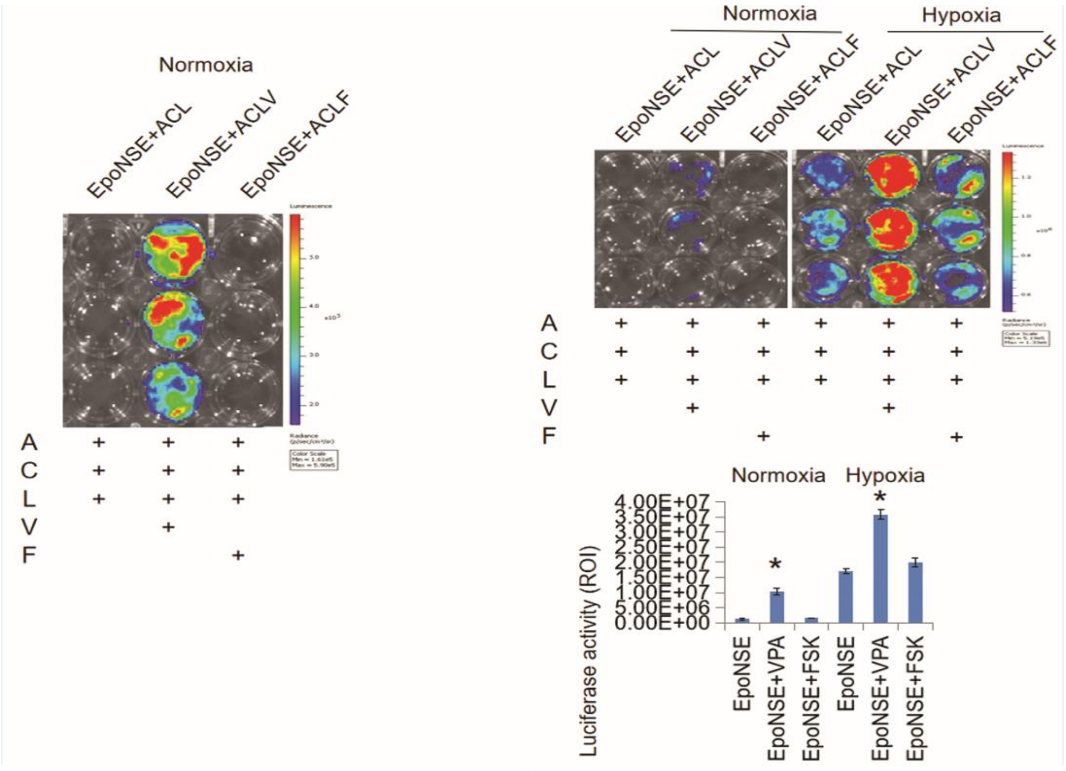

Fig. 5 A HDAC inhibitor rapidly increases the activity of HNIS in combination with a GSK3 inhibitor rather than forskolin as a common neural differentiation inducer.
Representative LED image of luciferase expression in HNIS-hiNSCs treated with different types of small molecules in combination with valproic acid and forskolin. This result indicates that combination with forskolin, a neural differentiation inducer, does not show a synergistic effect within a short time, unlike valproic acid. *p<0.05.
A, A83-01; C, CHIR99021; L, leukemia inhibitory factor; V, valproic acid; F, forskolin.

To investigate whether a combination of CHIR99021 and VPA shows specific activity in the NSE system only, I compared the gene expression level in the SV or NSE systems after treatment with CHIR99021 and VPA. Treatment with VPA increased luciferase expression in both the NSE and SV systems (Fig. 6A), and this phenomenon was also observed in hiNSCs derived from different donors (Fig. 6B). Thus, these results indicate that the combination effect of CHIR99021 and VPA is not specific to the NSE promoter.

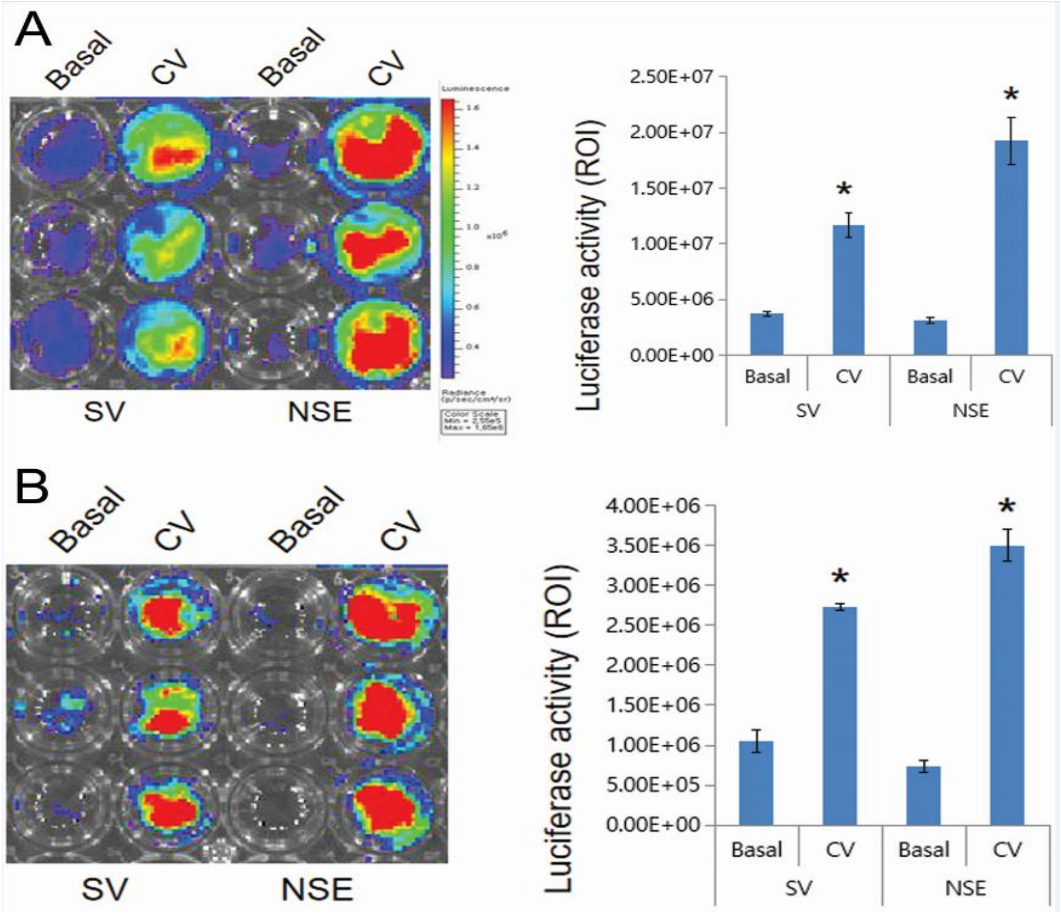

Fig. 6 The combination of GSK3 and an HDAC inhibitor also increases the activity of common promoter as well as a NSE promoter

A and B. Representative LED images and quantification of luciferase expression in induced neural stem cells (hiNSCs) transfected with SV or NSE plasmids. This experiment was performed in two different types of iNSCs; hiNSCs were maintained under hypoxia for 24 h after CHIR99021- and valproic acid-treatment. This result indicates that the combination of two small molecules is suitable for a common promoter and is not specific to the NSE promoter. *p<0.05. C, CHIR99021; V, valproic acid.

IV. DISCUSSION

A clinical study analyzed 63,109 patients with acute traumatic SCI from 1993 to 2012 and revealed that the SCI population is increasing in the USA [20]. SCI treatment is difficult because the condition is closely associated with an extended period because of post-traumatic cell death and neurological deficits [21], and secondary degeneration after SCI requires an even longer time for adequate management [4]. To maximally restrict the extent of secondary injury following SCI, transplantation of stem cells and pharmacological treatment are considered valuable approaches for preventing continuous cell injury and neural functional loss [22,23].

Currently, direct reprogramming technology is being actively studied in the field of regenerative medicinal research [24,25]; in particular, the direct conversion of skin fibroblasts to neurons through a combination of small molecules is appealing [26]. In our previous study, we observed that the efficiency of the NSE system was increased in mouse neural stem cells (mNSCs) [27]. However, the hypoxic-ischemic environment within the spinal cord after SCI is detrimental to therapeutic effectiveness, while the amount of preserved spinal cord tissue is closely related to post-traumatic neural functional recovery, and even small improvements in neural protection against secondary destruction could significantly enhance functional recovery [28]. In the present study, therefore, I employed a hypoxia/neuron-specific gene expression system (HNIS) that was specific for the SCI environment with a hypoxic status. Additionally, the gene expression efficiency of HNIS was significantly improved in combination with small molecules, including GSK3β and HDAC inhibitors.

1. **Gene and stem cells synergistically increase gene delivery efficiency**

Given that SCI leads to an ischemic environment with nerve damage in the spinal cord, a hypoxia-inducible system has the potential to be applied in SCI treatment. However, our previous study confirmed that the single gene delivery strategy is inefficient but efficient after employing stem cells as the gene delivery system in vivo [14]. Therefore, a combination strategy is the superior choice to overcome the limitations of gene delivery alone. In our previous study, a neuron-specific gene expression system (NSE) was used to enhance the gene expression efficiency in the neural lineage, such as mouse adult NSCs [15], since the first intron of NSE gene as a strong enhancer can confer a high level of neuron-specific expression in neuronal cells [29]. In another previous study, we also identified that exogenous NSE promoter actively increased gene expression not only in terminally differentiated neurons but also in NSCs [14]. However, hiNSCs have more advantages compared to adult neural stem cells, such as their autologous nature. In addition, hiNSCs show a high reproducibility in combination with HNIS, the experiment proved that pEpo-NSE consistently shows hypoxia/neuron-specific gene expression in NSCs [14]. As an enhancer, erythropoietin (Epo) had been used to increase gene expression within hypoxia [30].

2. **Small molecules, including HDAC and GSK3 inhibitors used in stem cell research**

Recently, small molecules have been widely used in stem cells or reprogramming research fields. Small molecules have been verified to facilitate cell reprogramming and regulate cell fate, and they have cell-permeable and non-immunogenic attributes [31]. Abematsu et al. employed the restorative benefits of exogenous NSCs and VPA-induced neuronal differentiation to treat

SCI [32]. Their study demonstrated that grafted NSCs predominantly differentiated into neurons after daily VPA injection for 7 days. Other research teams have found that the combination of small molecules with inhibitors of transforming growth factor beta (TGF), HDAC, and GSK3 signaling could efficiently convert skin fibroblasts to NSCs [33,34]. In addition, a combination of A83-01 and CHIR99021 has been used to generate neural progenitor cells from iPSCs [35]. In particular, the combination of CHIR99021 and VPA could affect neural reprogramming and the regulation of promoter activity [36].

Among the small molecules used in the present study, A83-01 is a TGF-β kinase/activin receptor-like kinase inhibitor, and LIF is a factor used for maintaining ESC pluripotency by acting downstream of the phospho-ERK pathway to block ESC differentiation. This molecule could restore cell viability in combination with a GSK3 inhibitor, which could improve ESC propagation and maintain pluripotency by activating Wnt/β-catenin signaling [34,37]. Yu et al. revealed that a combination of A83-01 and LIF is effective in maintaining and generating iPSCs [38]. As a multifunctional inhibitor of both HDAC and GSK3, VPA induces neuronal differentiation and promotes neuroprotection as well as neurogenesis by activating the ERK pathway and inducing the neurotrophic effects mediated by this pathway [21,39]. This beneficial effect on neurogenesis has encouraged researchers to explore the combination effect of VPA and stem cell therapy in an effort to promote neuronal differentiation of stem cells grafted in injured spinal cords [32,40]. Furthermore, VPA could suppress ischemia-induced neuronal caspase-3 activation [41]. In addition to preventing the permeability of the blood-spinal cord barrier, VPA can attenuate the extensive inflammation, neurotoxicity, and autophagy observed during secondary injury in SCI [42]. By preventing pathological autophagy from exacerbating secondary cell

death, VPA exerts potent neuroprotective benefits following SCI [43]. Additionally, Li et al. demonstrated that another GSK3 inhibitor (CHIR99021) can convert human ESCs to neuroepithelium in vitro [44].

In the current study, there are some similarities between the inhibition of HDAC and GSK3: both HDAC and GSK3β inhibition can upregulate the expression of neuroprotective and neurotrophic factors in the context of SCI [45,46]. The underlying mechanism may be associated with multiple interconnected signaling pathways mediated by the potent effects of VPA on HDAC and GSK3 inhibition. Jin et al. demonstrated that combined treatment with VPA and hypothermia led to enhancement of neuroprotective effects since hypoxia-inducible factor-1α and phospho-GSK3β expression are synergistically affected (the number of viable cells increased by 17.6%) [47]. In addition, Huang et al. revealed that inhibition of GSK-3β and HDAC by urocortin resulted in decreased caspase-3 activation, and treatment with a caspase inhibitor deterred dopaminergic neuronal degeneration [48]. Leng et al. further found that the synergistic neuroprotective effects of HDAC and GSK3 inhibition might be warranted for clinical trials against glutamate-related neurodegenerative diseases such as stroke, Alzheimer's disease, Parkinson's disease, and SCI [49]. Apart from enhancing the expression level of target genes, combinations with TGF-β, GSK3, and HDAC inhibitors have been successfully used in studies to investigate cell reprogramming [50,51].

In fact, I applied forskolin in this study because it is widely used in studies on neural differentiation or direct reprogramming. In a previous study, we confirmed that glioma cells can be converted to neurons by treatment with CHIR99021 and forskolin for 5 weeks [18]. In our preliminary study, hiNSCs were frequently differentiated into neurons within 7 days of being treated with forskolin. Thus, I

hypothesized that a combination of CHIR99021 and forskolin can improve the gene expression efficacy of the HNIS in hiNSCs by inducing neuronal differentiation. However, the combination with forskolin did not enhance the gene expression level of HNIS within 24 hours. This result indicates that the combination of VPA and CHIR99021 rapidly enhances promoter activity and has a synergistic effect on increasing the gene expression efficiency in HNIS.

V. CONCLUSION

This experiment demonstrated that HNIS has consistent hypoxia/neuron-specific gene expression in hiNSCs cultured under hypoxia. A complex pattern of HNIS-hiNSCs possesses higher gene expression compared with a single platform, such as the neuron-specific gene expression system (NSE). In addition, an optimal combination of small molecules, including GSK3 and HDAC inhibitors, combined with HNIS-hiNSCs may present a promising strategy in the treatment of SCI.

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Abstract (in korean)

GSK3β 와 HDAC 억제제를 활용한 허혈 및 신경조직 특이적 유전자 발현 시스템의 효율 개선 방법

<지도교수 하 윤>

연세대학교 대학원 의과학과

판 지 민

이전 연구를 통해 유전자 혹은 줄기세포 단일 치료 전략은 각각의 전략이 갖는 한계점 (낮은 유전자 전달 효율 및 줄기세포 치료 효능)으로 인해 난치성 척수손상을 효과적으로 치료하는데 충분하지 못하다는 것을 확인했다. 따라서, 줄기세포와 유전자 치료를 결합한 치료 전략은 각각의 치료 전략이 갖는 단점을 보완할 수 있는 방법으로 많이 연구되어왔다.

척수손상(SCI)은 신경 조직의 손상과 혀헐성 환경을 유발한다. 따라서 줄기세포와 유전자 치료 그리고 허혈 특이적 시스템의 조합은 손상 받은 척수손상 치료를 향상시키기 위해 좋은 전략이 될 수 있다.

이를위해 허혈/신경조직 특이적 유전자 발현시스템 (HNIS)과 리프로그래밍 기술에 의해 제작된 인간 유래 유도신경줄기세포를 이용하여 연구를 디자인했다. Sox2- 혹은 Nestin-양성 hiNSCs 는 Tuj1-과 MAP2-, 혹은 NeuN-양성의 신경세포로 분화되었다. 허혈/신경조직 특이적 유전자 발현 시스템이 도입된 인간 유도신경줄기세포는 허혈성 조건에서 허혈/신경세포 특이적인 유전자 발현 양상을 일관적으로 보여주었다. 특히, 허혈/신경조직 특이적 유전자발현 시스템이 도입된 인간 유도신경줄기세포는 각각의 단독 시스템 (예를들면 신경조직 특이적 유전자발현시스템)과 비교하여 월등한 유전자 발현 효율을 보였다. 게다가, glycogen synthase kinase-3β (GSK3β) 억제제인 CHIR99021 과 histone deacetylase (HDAC)

inhibitors 인 valproic acid 저분자화합물 조합이 허혈/신경조직 특이적 유전자발현 시스템의 발현 효율을 유의하게 향상시키는 것을 찾았다. 본 실험은 GSK3β 및 HDAC 억제제와 허혈/신경조직 유전자 발현 시스템 그리고 유도신경줄기세포가 척수손상 치료에 가능성을 보여준다.

핵심되는 말: Glycogen synthase kinase-3, Histone deacetylase, 허혈/신경조직 특이적 시스템, 척수손상

PUBLICATION LIST

I. First author/Co-first author

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