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신경 전구세포에서 뉴로제닌 1 에 의해 유도된 신경세포 분화활성에 대한 생체 내 생물발광 리포터 유전자 영상

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오 현 정

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지도교수 정 재 민

이 논문을 의학석사 학위 논문으로 제출함

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In vivo bioluminescence reporter gene imaging for the activation of neuronal differentiation induced by neuronal activator neurogenin 1 (Ngn1) in neuronal precursor cells

By Hyun Jeong Oh.

(Directed by Professor Jae Min Jeong Ph.D)

A Thesis submitted in Partial Fulfillment of the Requirement for the Degree of Master of Philosophy in Medicine (Nuclear Medicine) to Seoul National University College of Medicine

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Approved by Thesis Committee:

Professor _____Chairman

Professor _____Vice chairman

Professor _____

ABSTRACT

In vivo bioluminescence reporter gene imaging for the activation of neuronal differentiation induced by neuronal activator neurogenin 1 (Ngn1) in neuronal precursor cells

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Purpose: The facilitation capability of neuronal lineages derived from transplanted stem cells is essential to improve the low efficacy of neuronal differentiation in stem cell therapy in vivo. Neurogenin 1 (Ngn1), a basic helix-loop-helix factor, has been used as an activator of neuronal differentiation. In this study, we monitored the in vivo activation of neuronal differentiation by Ngn1 in neuronal precursor cells using neuron-specific promoter-based optical reporters.

Methods: The NeuroD promoter coupled with the firefly luciferase reporter system (pNeuroD-Fluc) was used to monitor differentiation in F11 neuronal precursor cells. In vitro luciferase activity was measured and normalized by protein content. The in vivo-jetPEITM system was used for in vivo transgene delivery. The IVIS 100 imaging system was used to monitor in vivo luciferase activity.

Results: The Ngn1-induced neuronal differentiation of F11 cells generated neurite outgrowth within 2 days of Ngn1 induction. Immunofluorescence staining demonstrated that early and late neuronal marker expression (βIII-tubulin, NeuroD, MAP2, NF-M, and NeuN) was significantly increased at 3 days after treatment with Ngn1. When Ngn1 and the pNeuroD-Fluc vector were co-transfected into F11 cells, we observed an approximately 11-fold increase in the luciferase signal. An in vivo study showed that bioluminescence signals were gradually increased in Ngn1-treated F11 cells until 3 days.

Conclusions: In this study, we examined the in vivo tracking of neuronal differentiation induced by Ngn1 using an optical reporter system. This reporter system could be used effectively to monitor the activation efficiency of neuronal differentiation in grafted stem cells treated with Ngn1 for stem cell therapy.

Key Words: Neurogenin 1 (Ngn1), Activation of neuronal differentiation, Reporter gene, Neuronal precursor cells, *In vivo* optical imaging

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LIST OF FIGURES

LIST OF ABBREVIATIONS

Ngn1: Neurogenin 1

bHLH: basic helix-loop helix

GFAP: glial fibrillary acidic protein

GFP: green fluorescence protein

qRT-PCR: quantitative reverse transcription-PCR

Fluc: Firefly luciferase

Gluc: Gaussia luciferase

CMV: Cytomegalovirus

CONTENTS

I. INTRODUCTION

The use of multipotent stem cells to restore damaged tissue in terms of stem cell therapy has been well documented. With their effective differentiation potential, neural stem cells (NSCs) or neural precursor cells can provide the ideal cell source for the treatment of neurodegenerative diseases of the brain. Transplantation of NSCs or neuronal precursor cells has the potential to restore the functional deficits associated with injury to the central nervous system [1]. Cell replacement and gene transfer to the injured brain have provided an ideal source of new therapeutic strategies for human neurodegenerative diseases including Parkinson's disease, Huntington's disease, and Alzheimer's disease [1–6]. Indeed, intravenously transplanted human NSCs effectively migrated to the damaged region, differentiated into neurons and astrocytes, and ultimately induced a functional improvement in rats with focal cerebral ischemia [7–8]. Although stem cell-based research has been studied intensively, the low efficiency of differentiation into functioning neuronal cells has hampered functional improvements. Therefore, the development of critical factors to promote neuronal differentiation by modulating endogenous neuron-specific genes is necessary for effective stem cell-based therapy.

Neurogenin 1 (Ngn1) is a basic helix-loop-helix (bHLH) transcription factor that is expressed in early neuronal progenitor cells during the development of the nervous system [9]. The overexpression of Ngn1 increases the number of neuronal cells in Xenopus embryos and in uncommitted pluripotent stem cells, e.g., embryonic carcinoma P19 cells, by the activation of downstream proneural bHLH transcription

factors, including NeuroD [10]. Ngn1 suppresses gliogenesis by sequestering CREB binding protein/p300 and Smad1 away from the signal complexes that are required for the expression of glial fibrillary acidic protein [9, 11]. Kim et al. reported that the overproduction of Ngn1 alone is sufficient to induce neurite outgrowth in F11 neuronal precursor or mouse embryonal carcinoma P19 cells [10, 12]. F11 cells are a hybrid cell line between neuroblastoma N18TG2 and dorsal root ganglionic neurons. These cells have unique characteristics in that they can be differentiated into neuron-like cells in the presence of cAMP and express various neuron-specific markers, including specific receptors for prostaglandin and N- and L-type voltage-dependent Ca2+ channels, suggesting that F11 cells are similar to neuronal precursor cells [13].

An in vivo molecular imaging approach has emerged as a powerful technique to identify the in vivo characteristics of implanted cells. To assess the location and proliferation or differentiation of the grafted stem cells in vivo, molecular markers, e.g., β-galactosidase, alkaline phosphatase, or green fluorescent protein, are used for immunochemical detection or in situ hybridization, particularly to prove the localization or neuronal commitment of grafted cells [14–20]. However, this method is too invasive and requires a tissue biopsy, which is difficult to repeat in the same animal. The application of in vivo noninvasive and quantifiable methods to estimate the differentiation potential of stem cells in vivo could confer important information at the level of small animals. Previously, we demonstrated a new noninvasive method to evaluate neuronal differentiation in vivo and in vitro by examining the expression of a reporter gene under the transcriptional control of a neuron-specific promoter in F11 cells [14]. In the present study, we monitored the activation process of neuronal differentiation induced by the neuronal activator Ngn1 using optical imaging reporters

coupled to the NeuroD promoter in the mouse brain and subcutaneous regions.

II. MATERIALS AND METHODS

Plasmid DNA vectors

The plasmid vector containing full-length mouse Ngn1 cDNA was provided by Professor Haeyoung Suh-Kim, Ajou University. Full-length Ngn1 cDNA (GeneBank U63841) was obtained by polymerase chain reaction (PCR) using the oligonucleotides TGC AAG ATG CCT GCC CCT TT (forward) and GCC ATA GGT GAA GTC TTC TGA AGC CGA GGG ACT ACT G (reverse) and subcloned into the pCR2.1-TOPO plasmid. The cDNA was inserted into the *Eco*R I site of pcDNA3.1/His (B) to yield pcDNA/His-Ngn1 [10]. pNeuroD(-2.2)-Fluc contained a firefly luciferase reporter gene under the control of 2.2 kb of the mouse NeuroD promoter. This reporter gene contains cis-elements for the tissue-specific expression of NeuroD in the nervous tissue and pancreatic islets [21].

Cell culture and transfection protocol

F11 cells, rat dorsal root ganglion and mouse neuroblastoma hybrid cells, and HeLa cells (human cervical cancer cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 10 U/ml penicillin, and 10 μg/ml streptomycin. The cells were maintained at 37℃ in a humidified 5% CO_2 atmosphere. We plated 1.5×10^5 F11

cells in 6-well plates. The pNeuroD(-2.2)-Fluc reporter gene, pcDNA/His-Ngn1, and pcDNA3.1/His(B) were transfected into F11 cells using lipofectamine (Invitrogen, Carlsbad, CA) and diluted in OPTI-MEM medium (Gibco, Grand Island, NY). The transfected cells were incubated in DMEM supplemented with 0.5% FBS for 2 or 3 days.

Quantitative analysis of the expression of neuronal markers using quantitative reverse transcription-PCR

Using Trizol (Invitrogen, Grand Island, NY), total RNA was isolated during the neuronal differentiation of F11 cells. Total RNA (1 μg/ml) was reverse transcribed using reverse transcriptase (Invitrogen, Carlsbad, CA) for quantitative reverse transcription-PCR (qRT-PCR) analysis. qRT-PCR was performed using neuron-specific primers. The PCRs were performed in triplicate using an ABI® 7500 (Applied Biosystems™, Carlsbad, CA) with EvaGreen qPCR Mastermix (abm™, Richmond, BC). The reactions were incubated at 50℃ for 2 min, 95℃ for 10 min, and 40 cycles of 95℃ for 15 s and 60℃ for 1 min. To normalize the experimental samples, β-actin was used as a control.

Immunofluorescence staining

Mock-treated and Ngn1-treated F11 cells were seeded on cover slips in 6-well plate. After discarding the medium and rinsing with phosphate-buffered saline (PBS),

the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature. Afterward, the cells were washed 3 times with PBS and incubated in 0.5% H_2O_2 in MeOH for 5 min at 20℃. After washing with PBS 3 times, the cells were treated with 0.5% Triton X-100 in PBS for 5 min at 4℃. The samples were rinsed 3 times with PBS and non-specific binding sites were blocked with 1% normal horse serum in PBS for 1 h at room temperature. Anti-Tuj-1 (1:1300 dilution; Cell Signaling, Danvers, MA), anti-NeuroD (1:500 dilution; Abcam, Cambridge, FL), anti-MAP2 (1:1000 dilution; Sigma, Saint Louis, MO), anti-NF-M (1:2000 dilution; Sigma, Saint Louis, MO), and anti-NeuN (1:100 dilution; MILLIPORE, Temecula, CA) antibodies were diluted in PBS and incubated with the samples at 4℃ overnight. Alexa Fluor 488-conjugated anti-mouse and anti-rabbit secondary antibodies (Invitrogen, Grand Island, NY) were used to visualize the antibody reactions. Nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA) and observed at 461 nm. Fluorescent images were acquired using a confocal laser scanning microscope (Carl Zeiss LSM 510; Carl Zeiss, Jena, Germany).

Quantitative analysis of the expression of neural markers

Confocal data were used for quantitative analysis using TissueFAXS2.0. Firstly, the field of view for the areas of interest was set after pre-scanning to identify tissue sections. The fluorescent signals from the areas of interest were detected using TissueFAXS2.0. TissueFAXS yields rapid high resolution images of tissue sections

for subsequent analysis. Quantitative analysis was performed using TissueQuest, the cell analysis software for fluorescent images.

In vitro luciferase assay

F11 and HeLa cells were washed with PBS and lysed using a lysis buffer (Promega, Madison, WI) at 48 h after transfection. The cell lysates were collected with a cell scraper and redistributed into a 96-well plate. The luciferase assay was carried out using a luciferase assay kit (Promega, Madison, WI). The bioluminescence intensity of each cell lysate was measured using a microplate luminometer (TR717; Applied Biosystems^{TM}, Carlsbad, CA). Luciferase activity was normalized by protein content.

In vivo bioluminescence imaging

First, at 24 h after F11 cells were transfected with the pNeuroD-Fluc and pCMV-Gluc vectors, they were detached by trypsinization and harvested in PBS. An injection volume of 100 μl containing 1.5×10^6 cells mixed with *in vivo*-jetPEI^{TM}-Mock or Ngn1 were injected subcutaneously (s.c.) into the right thigh of male BALB/c nude mice with *in vivo*-jetPEI^{TM}-Ngn1 complexes and into the left thigh with *in vivo*-jetPEI^{TM}-Mock complexes. The mice were anesthetized with 10 μl zoletil-rumpun (2:1) solution, and 3 mg D-luciferin suspended in 100 μl PBS was injected intraperitoneally at 10 min before the acquisition of the bioluminescence images.

Whole-body images for Fluc activity were acquired after 10 min. The Gluc signal from the gaussia luciferase gene, which is under the control of the cytomegalovirus (CMV) promoter, was used for Fluc signal normalization. To acquire Gluc images, 12 h after the Fluc images were taken, 5 μg coelenterazine substrate (Biotium, Hayward, CA) were injected directly into the implanted F11 cells of the subcutaneous region. The Gluc images were acquired at an acquisition time of 1 min. Then, the mice were placed in an IVIS-100 imager (Xenogen, Alameda, CA,). Bioluminescence images for Fluc activity were acquired for 1 and 3 min. The Fluc and Gluc signals were analyzed quantitatively by region of interest (ROI).

In vivo bioluminescence imaging for orthotopic implantation

For the *in vivo* imaging study of the brain, F11 cells transfected with the pNeuroD-Fluc vector in a T75 flask were harvested by trypsinization. The cells were spun, rinsed, and resuspended in 500 μl PBS. A CM-DiI cell-labeling solution was mixed directly with PBS (2 μl CM-DiI labeling solution per mL of solution). Then, the F11 cells were incubated with CM-DiI/PBS for 20 min at 37℃. We injected BALB/c nude mice (n = 6) stereotactically after CM-DiI cell-labeling with 4.5×10^5 cells in a total fluid volume of 5 μl PBS into the right striatum (coordinates: anteroposterior, 0.5; mediolateral, 1.5; dorsoventral, 2.0) with *in vivo*-jetPEI^{TM}-Ngn1 complexes or *in vivo*-jetPEI^{TM}-Mock complexes. Quantitative ROI data from the acquired bioluminescence images are expressed as photon/s/cm^2/sr.

Histology and immunohistochemistry

The mice were sacrificed using CO_2 at 3 days after cell transplantation. The F11 cells that were injected s.c. into the thighs were removed and then post-fixed with 4% PFA. Post-fixation, the isolated F11 cells were embedded in paraffin and 4 μm-thick sections were prepared. The brains were removed surgically, embedded in OCT, and frozen on a bed of liquid nitrogen. Serial (10 μm thick) cryosections were prepared and some of the cryosections were stained with hematoxylin and eosin. For immunohistochemistry, the sections were blocked with 1% normal goat serum in PBS for 1 h at room temperature. The sections were then incubated with antibodies against Tuj-1 (1:100 dilution; Cell Signaling, Danvers, MA), NeuroD (1:500 dilution; Abcam, Cambridge, FL), MAP2 (1:100 dilution; Sigma, Saint Louis, MO), NF-M (1:2000 dilution; Sigma, Saint Louis, MO), and NeuN (1:100 dilution; MILLIPORE, Temecula, CA) at 4℃ overnight. The primary antibody reactions were visualized with Alexa Fluor 488-conjugated anti-mouse and anti-rabbit secondary antibodies (Invitrogen, Grand Island, NY). Fluorescent signals were detected using a confocal laser scanning microscope (Carl Zeiss LSM 510; Carl Zeiss, Jena, Germany).

Statistical analysis

Data are displayed as means ± standard deviation (SD) and assessed using Student's *t*-test. Statistical significance was accepted at *P*-values < 0.05.

III. RESULTS

Induction of neurite outgrowth by overproduction of Ngn1 in F11 cells

To confirm the potential ability of neurogenic Ngn1 to induce neuronal differentiation, F11 cells were treated with a Mock or Ngn1-containing plasmid vector for 1 or 3 days to promote neuronal differentiation. The Mock vector was the pcDNA3.1/His expression vector of Ngn1 that does not contain Ngn1 cDNA and was used as a negative control. F11 cells were transfected with the Ngn1 transgene under two different serum concentrations to determine the optimal condition to induce the activation of neuronal differentiation by Ngn1.

Because the optimal condition for neural induction was reported to be 0.5% FBS [12], 0.5% and 10% FBS conditions were used. Mock- and Ngn1-transfected F11 cells (F11-Mock or F11-Ngn1 cells, respectively) were incubated for either 1 or 3 days in 0.5% or 10% FBS. The round cell shape morphology was unchanged in F11-Mock cells, indicating their undifferentiated state. In addition, there was no significant change of F11 cell morphology in the groups treated with Ngn1 or Mock in 10% FBS (data not shown) and Mock in 0.5% FBS (Fig. 1a, c). In contrast, a significant neurite outgrowth pattern was observed in F11-Ngn1 cells incubated in DMEM containing 0.5% FBS (Fig. 1b, d). The length of neurite outgrowth was also estimated quantitatively from differential interference contrast images of confocal analysis of F11-Mock and F11-Ngn1 cells in 0.5% FBS. The individual length of neurite outgrowth was measured using LSM Image Examiner and showed a distinct

pattern between F11-Mock and F11-Ngn1 cells (n = 20). Neurite outgrowth in F11-Ngn1 cells was, on average, 16-fold longer than in F11-Mock cells (Fig. 1e).

Mesurement of neuron-specific gene expression after neuronal activation by treatment of Ngn1

To investigate the ability of Ngn1 to activate neuronal differentiation, we firstly examined changes in the mRNA expression levels of a variety of neuron-specific genes at each neuronal developmental stage, including early and late neuronal markers. The expression levels of the early neuronal marker Tuj-1 and the late neuronal genes NeuroD, MAP2, and NF-M were evaluated using real-time RT-PCR analysis. Because dramatic neurite outgrowth was induced by Ngn1 in 0.5% FBS, RNA was isolated from F11 cells that were maintained in DMEM containing 0.5% FBS after transfection with the Mock or Ngn1 expression plasmid vector. Quantitative analysis by real-time RT-PCR revealed increased expression levels of Tuj-1, NeuroD, MAP2, and NF-M, except for the neuronal nuclear antigen (NeuN), in F11-Ngn1 cells compared to F11-Mock cells (Fig. 2). An immunostaining assay was also conducted to confirm whether Ngn1 overproduction induces neuronal differentiation at the protein level. The highly specific expression of early and late neuronal markers, i.e., Tuj-1, NeuroD, MAP2, and NF-M, was found in the cytoplasm of F11-Ngn1 cells, whereas they were expressed at minimal levels in F11-Mock cells. In addition, the nuclear expression of Ngn1 was detected in F11-Ngn1 cells (Fig. 3). However, NeuN, a mature neuronal marker that is expressed in the final stage of

neuronal differentiation, was not expressed in F11-Ngn1 cells, indicating that these cells do not reflect the last stage of neuronal differentiation, at least in an *in vitro* environment.

To examine the changes of neuron markers induced by Ngn1 more accurately, a TissueFAX imaging analyzer, which is a quantitatively analyzable imaging microscope, was used to acquire total fluorescence signals from whole cells on a slideglass. Consistent with the immunostaining results, the immature neuronal marker Tuj-1, mature neuronal marker NeuroD, and neuron-specific cytoskeletal protein NF-M exhibited approximately 2.5-, 1.8-, and 5.6-fold increased expression levels, respectively (data not shown). In particular, the MAP2 marker showed the greatest increase in expression when the Ngn1 gene was overexpressed. These data were significantly correlated with the higher level of MAP2 expression in the cytoplasm of F11-Ngn1 cells, as measured by immunocytochemistry. These results indicate that Ngn1 overexpression alone in F11 cells is sufficient to induce neuronal cell fate with changes of neuronal markers as well as of neuron-specific phenotypes.

Evaluation of the effect of Ngn1 using a neuronal promoter-driven reporter gene

A recombinant plasmid DNA vector carrying the firefly luciferase gene regulated by the NeuroD promoter (pNeuroD-Fluc) was designed to examine the activation of neuronal differentiation by Ngn1. HeLa cells, which have non-neuronal characteristics, were chosen as a negative control to confirm whether the NeuroD

promoter is only influenced by the overproduction of Ngn1. F11 and HeLa cells were transiently transfected with Mock or Ngn1 and pNeuroD-Fluc as a reporter gene. F11 cells co-transfected with both Ngn1 and pNeuroD-Fluc genes exhibited approximately 11-fold higher luciferase activity than F11-Mock cells (Fig. 4a). In contrast, only 1.6-fold higher luciferase signals were observed in HeLa cells overexpressing Ngn1 compared to Mock-transfected HeLa cells. These results indicate that although the activation of the NeuroD promoter was, to a certain degree, affected by Ngn1 itself, the level of activation was very low. Thus, Ngn1 is critical for the activation of neuronal differentiation by its interaction with a variety of other neurogenic factors in F11 cells.

In vivo bioluminescence imaging to analyze the effect of Ngn1 in a subcutaneously injected F11 cell implantation group

To monitor the activation pattern of neuronal differentiation *in vivo* by the induction of Ngn1, 1.5×10^6 F11 cells were collected in 100 μl PBS after transfection with the pNeuroD-Fluc vector. The gaussia luciferase gene driven by the CMV promoter (pCMV-Gluc) was also used as an internal control for *in vivo* normalization. As for the *in vivo* delivery of the transgene, we used the *in vivo*-jetPEI™ system, which is an *in vivo* delivery reagent for efficient nucleic acid delivery in many species, including the mouse and rabbit.

Firstly, to evaluate the efficiency of transgene delivery into F11 cells by *in vivo*-jetPEI™, a retroviral vector carrying the enhanced luciferase (effluc) gene was tested

in F11 cells. After the effluc vector was mixed with 20 μl *in vivo*-jetPEI^{TM} for 15 min, F11 cells were injected s.c. into the right thigh of male BALB/c nude mice with *in vivo*-jetPEI^{TM}-effluc vector complexes. Bioluminescence signals in the injected region were detected at 24 and 48 h after injection (Fig. 4b). After pNeuroD-Fluc and pCMV-Gluc-transfected F11 cells mixed with *in vivo*-jetPEI^{TM}-Ngn1 complexes or -Mock complexes were injected into the thighs of nude mice, serial images were acquired using an *in vivo* optical imaging device. The F11-Ngn1 injected group (right thigh) showed higher luciferase intensity than the F11-Mock injected group at 2 and 3 days after injection. In contrast, Gluc activity in the right thigh of the F11-Ngn1 group gradually decreased (Fig. 4c), possibly because the proliferation rates of the F11-Ngn1 group might be reduced continuously due to the induction of neuronal differentiation by Ngn1. The ROI value was calculated by dividing the firefly luciferase activity of pNeuroD-Fluc by the gaussia luciferase signal of the pCMV-Gluc vector in the F11-Ngn1 and F11-Mock groups. The ROI value for the Fluc/Gluc activity ratio indicated that the luciferase signal in the F11-Ngn1 group increased continuously compared to the F11-Mock group (Fig. 4d). These results demonstrated that the increased luciferase activity in the F11-Ngn1 group was due to the activation of neuronal differentiation by Ngn1 in the transplanted F11 cells.

To verify the activation of *in vivo* neuronal differentiation of the grafted F11 cells by Ngn1, we performed immunohistochemistry analysis for Ngn1 and neuronal-specific markers, i.e., Tuj-1, NeuroD, MAP2, NF-M, and NeuN. At 3 days after the *in vivo* bioluminescence images were acquired, the injected F11 cells were isolated from the subcutaneous region for the immunohistochemistry assay. The expression of most of the neuron-specific markers, including NeuroD, was increased dramatically in the

F11-Ngn1 implanted region. In contrast, under the same conditions, these neuron-specific proteins were expressed at a minimal level in the F11-Mock implanted region. We also performed quantitative analysis of the immunohistochemistry assay using TissueFAXS2.0. TissueFAXS analysis showed the increased expression of the neuron-specific markers, including NeuroD, except for NeuN, in the F11-Ngn1 group (data not shown). Interestingly, consistent with the *in vitro* immunofluorescence staining results, no fluorescent signal for NeuN expression was found, even in the F11-Ngn1 group (Fig. 5). These results indicated that the s.c. implanted F11-Ngn1 cells were successfully induced into a neuronal cell lineage, but not the final stage of neuronal differentiation.

In vivo orthotopic implantation of F11 cells to examine the activation of neuronal differentiation in the brain

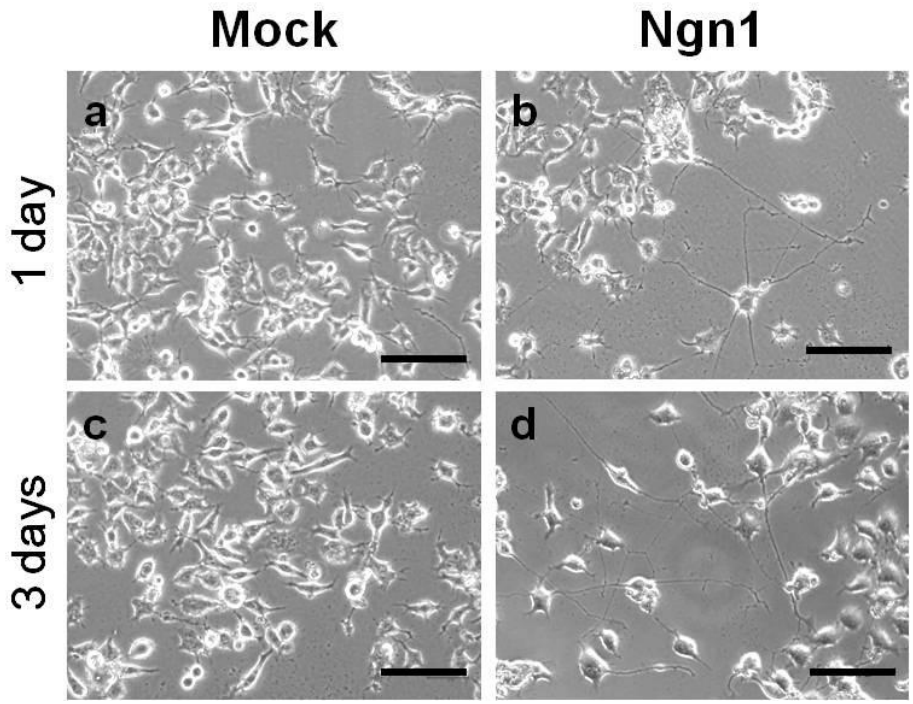
To evaluate the possibility of inducing neuronal activation of the implanted cells in the brain, we performed the orthotopic injection of F11 cells transfected with pNeuroD-Fluc into the right striatum region of nude mice after mixing them with *in vivo*-jetPEI^{TM}-Ngn1 or -Mock complexes. In addition, to track the injected F11 cells in the brain, they were stained with the fluorescent tracker DiI before they were transplanted into the brain. At 3 days after the cells were injected intracranially, we detected a significant difference in brain luciferase intensity between the F11-Ngn1 and F11-Mock groups (Fig. 6a). Quantitative analysis of the bioluminescence signal ratio (each day/0 day ratio) revealed similar luciferase intensity between the F11-

Ngn1 and F11-Mock implantation groups until 2 days after cell implantation. However, the luciferase intensity ratio was significantly increased at 3 days in the F11-Ngn1 group (Fig. 6b). In these results, a somewhat different pattern of luciferase signal was found between the groups implanted in the subcutaneous environment and those implanted in brain tissue.

For the immunohistochemistry assay of brain tissue, brain tissue was also isolated at 3 days after the bioluminescence imaging was performed. Ngn1 was expressed only in the F11-Ngn1 group. The increased expression of the neuron-specific markers Tuj-1, NeuroD, MAP2, and NF-M markers was found in the implanted F11-Ngn1 cells. The fluorescence signal for the neuronal markers was matched with the DiI fluorescence signal. Interestingly, compared with the results acquired from *in vitro* immunostaining and the *in vivo* subcutaneous injection study, the high expression of the late neuronal marker NeuN was detected in brain sections from the F11-Ngn1 group (Fig. 7). This result indicated that the brain environment surrounding the implanted cells can induce a different neuronal differentiation capability compared to the subcutaneous regions of mice.

Quantitative analysis by TissueFAXS2.0 for the expression of immature and mature neuron-specific markers also supported the activation of neuronal differentiation with more accurate analysis than the immunostaining data. Consistent with the immunostaining findings, the immature neuronal marker Tuj-1 and mature neuronal marker NeuroD showed an approximately 1.6- and 38-fold increase in their expression levels, respectively (data not shown). The expression of the neuron-specific cytoskeletal protein NF-M was 3.2-fold higher than in the F11-Mock group. In particular, MAP2 was expressed at the highest levels out of all of the examined

neural markers. The TissueFAXS analysis data were consistent with the immunohistochemistry findings, indicating the increased expression of neuronal markers in F11-Ngn1 cells isolated from the brain.


Mock
Ngn1
1 day
3 days
a
b
c
d

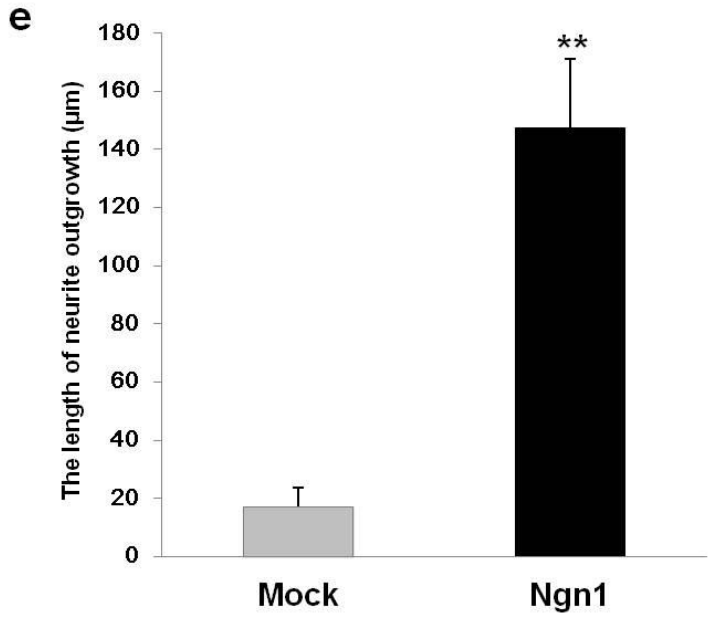
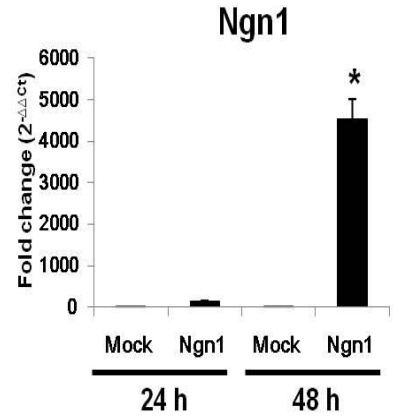

e
The length of neurite outgrowth (μm)
180
160
140
120
100
80
60
40
20
0
**
Mock
Ngn1

FIGURE 1 Induction of neuronal differentiation by Ngn1 alone in F11 cells. To examine the ability of Ngn1 to activate neuronal differentiation, F11 cells were transfected with 1 μg Mock or Ngn1 plasmid vector. These transfected F11 cells were incubated in DMEM containing 0.5% FBS for 3 days. (a, c) Mock (0.5% FBS) and (b, d) Ngn1 (0.5% FBS). Phase contrast photomicrograph showing Ngn1-induced F11 cells had neuron-like morphological neurite outgrowth features (b, d). (e) Quantitative analysis for the length of neurite outgrowth was performed in F11-Mock and F11-Ngn1 cells. The average length of neurites extending from the cell body was measured from immunofluorescence confocal data. $^{**}P < 0.001$.


Ngn1
Fold change (2-ΔΔct)
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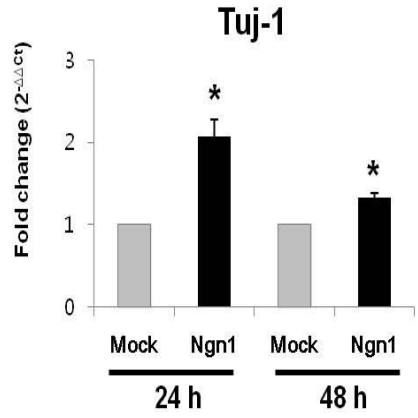
5000
4000
3000
2000
1000
0
*
Mock
Ngn1
Mock
Ngn1
24 h
48 h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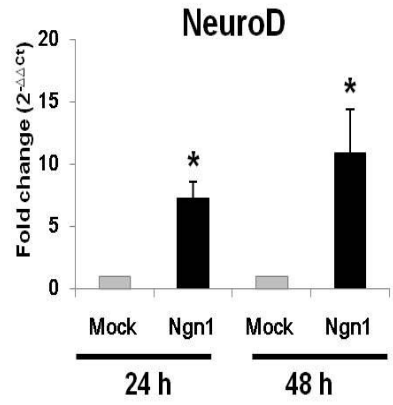
NeuroD
Fold change (2-ΔΔct)
20
15
10
5
0
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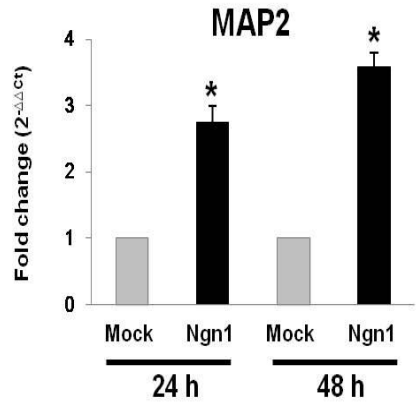
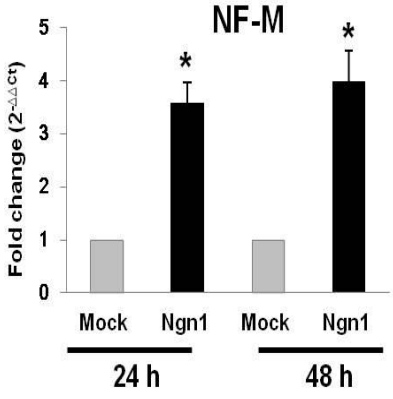
Mock
Ngn1
Mock
Ngn1
24 h
48 h


MAP2
Fold change (2-ΔΔct)
4
3
2
1
0
*
*
Mock
Ngn1
Mock
Ngn1
24 h
48 h


NF-M
Fold change (2-ΔΔct)
5
4
3
2
1
0
*
*
Mock
Ngn1
Mock
Ngn1
24 h
48 h

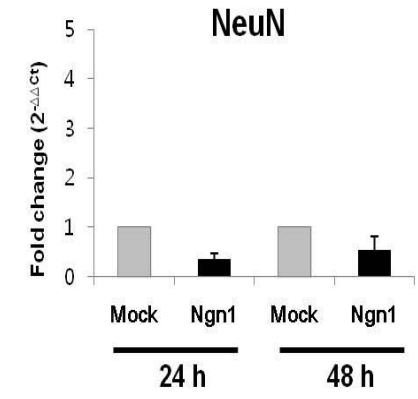

NeuN
Fold change (2-ΔΔct)
5
4
3
2
1
0
Mock
Ngn1
Mock
Ngn1
24 h
48 h

FIGURE 2 Neuronal marker expression of Mock or Ngn1 treated F11 cells. Real-time RT-PCR was performed on total RNA extracted from F11 cells treated with Mock or Ngn1. In F11-Ngn1 cells, the expression of several neuron-specific markers including NeuroD was higher than in the F11-Mock cells at 48 h. β-actin was used as an internal control. *$P < 0.05$.

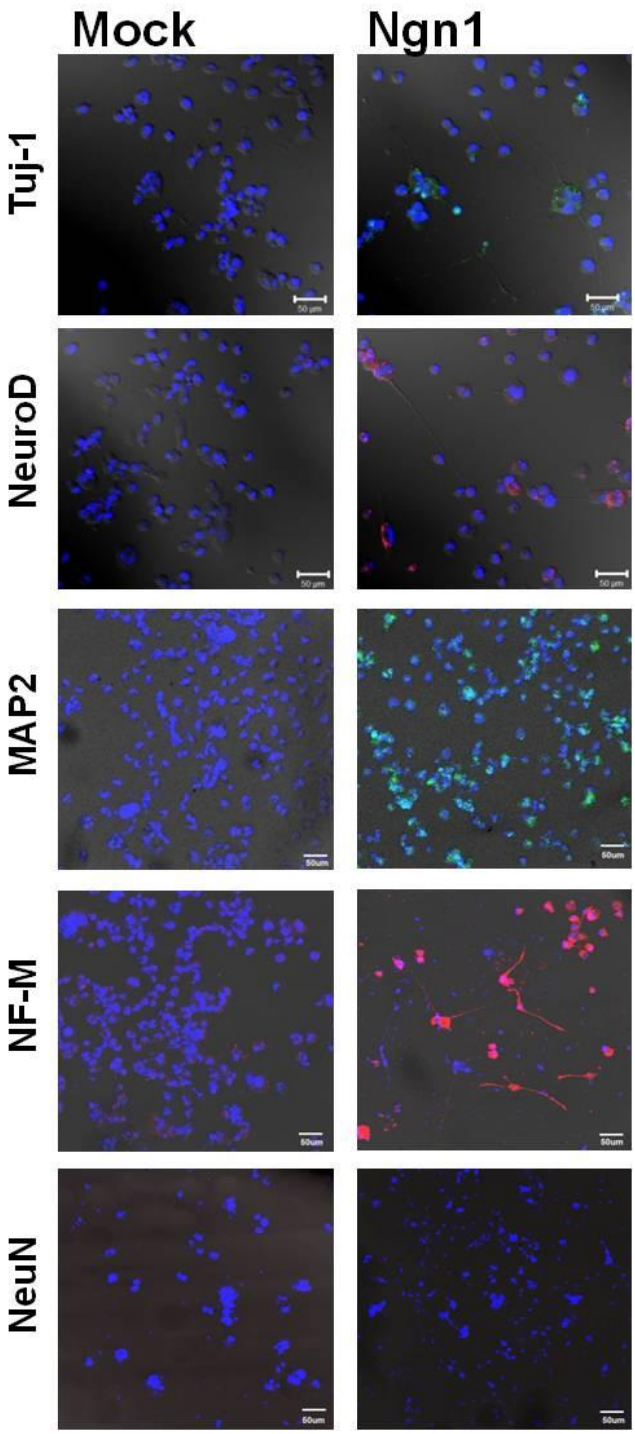
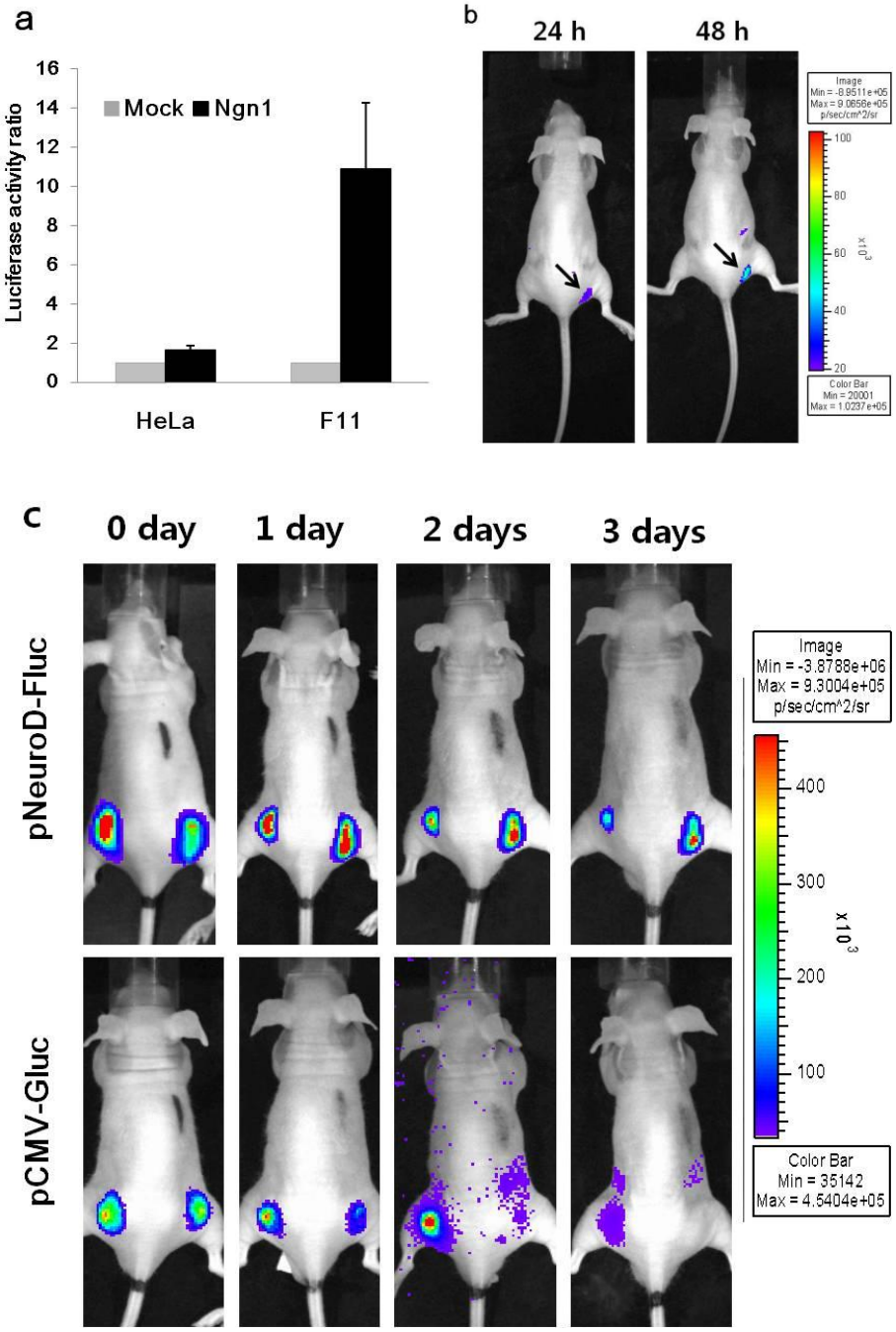

Mock
Ngn1
Tuj-1
NeuroD
MAP2
NF-M
NeuN

FIGURE 3 Expression level in neuron-related proteins by Ngn1. Immunofluorescence studies to examine neuron-related proteins were conducted in F11-Mock and F11-Ngn1 cells. Immunofluorescence staining revealed the increased expression levels of Ngn1 (red), Tuj-1 (green), NeuroD (red), MAP2 (green), and NF-M (red) in F11-Ngn1 cells compared to F11-Mock cells. Nuclei were counterstained with DAPI (blue). Scale bar = 50 μm.


a
Luciferase activity ratio
16
14
12
10
8
6
4
2
0
Mock
Ngn1
HeLa
F11
b
24 h
48 h
Image
Min = -8.9511e+05
Max = 9.0656e+05
p/sec/cm^2/sr
100
80
60
40
20
x10^3
Color Bar
Min = 20001
Max = 1.0237e+05
c
0 day
1 day
2 days
3 days
pNeuroD-Fluc
pCMV-Gluc
Image
Min = -3.8788e+06
Max = 9.3004e+05
p/sec/cm^2/sr
400
300
200
100
x10^3
Color Bar
Min = 35142
Max = 4.5404e+05

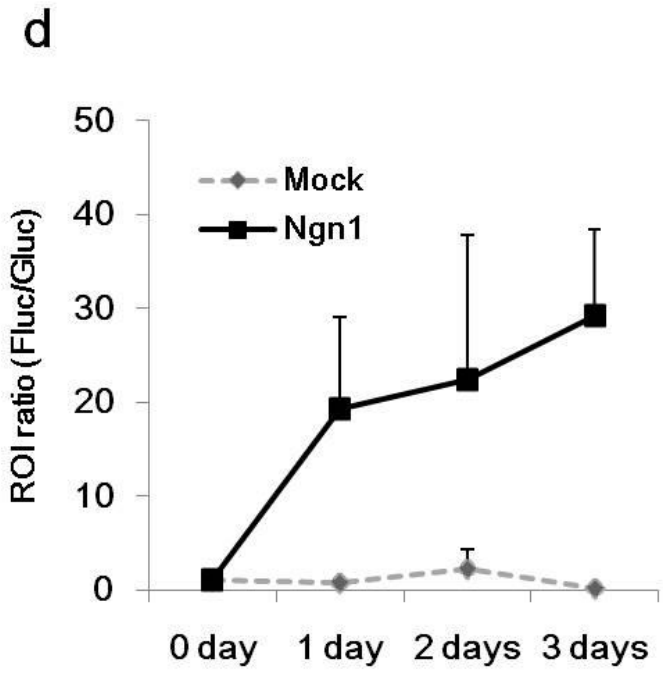


FIGURE 4 Enhanced luciferase reporter activity in F11 cells treated with Ngn1. (a) HeLa and F11 cells were transiently transfected with Mock or Ngn1 and pNeuroD-Fluc as a reporter gene. Ngn1 overproduction increased the luciferase reporter signals in pNeuroD-Fluc-transfected F11 cells. Slightly increased luciferase activity was observed in the F11-Ngn1 cells. The acquired values are represented as the average luciferase activity ratio ± SD that was normalized using the bicinchoninic acid assay. (b) After the plasmid vector containing enhanced luciferase (effluc) gene was mixed with in vivo-jetPEITM, F11 cells (5×10^5) mixed with effluc plasmid-polymer complex were subcutaneously (s.c.) injected into the right thigh of nude mouse. After 24 h, markedly higher bioluminescence intensity was observed in injection site of F11 cells mixed with in vivo-jetPEITM -lucferase gene complexes. No bioluminescence

signal was detected in F11 cells only mixed with luciferase vector. Arrow indicates cell injection site. (c) At 24 h after F11 cells were transfected with the pNeuroD-Fluc and pCMV-Gluc vectors, 1.5×10^6 cells were injected s.c. into the right thigh of nude mice with *in vivo*-jetPEI^{TM}-Ngn1 complexes and into the left thigh with *in vivo*-jetPEI^{TM}-Mock complexes. A gaussia luciferase reporter vector was used for *in vivo* normalization. Bioluminescence images were acquired using an IVIS-100 optical imaging device from 0 to 3 days. (d) ROI analysis from the bioluminescence images was carried out for each cell-implanted region. The Fluc/Gluc activity ratio from the right thigh increased over time. The measured ROI values are shown as photon/s/cm^2/sr.

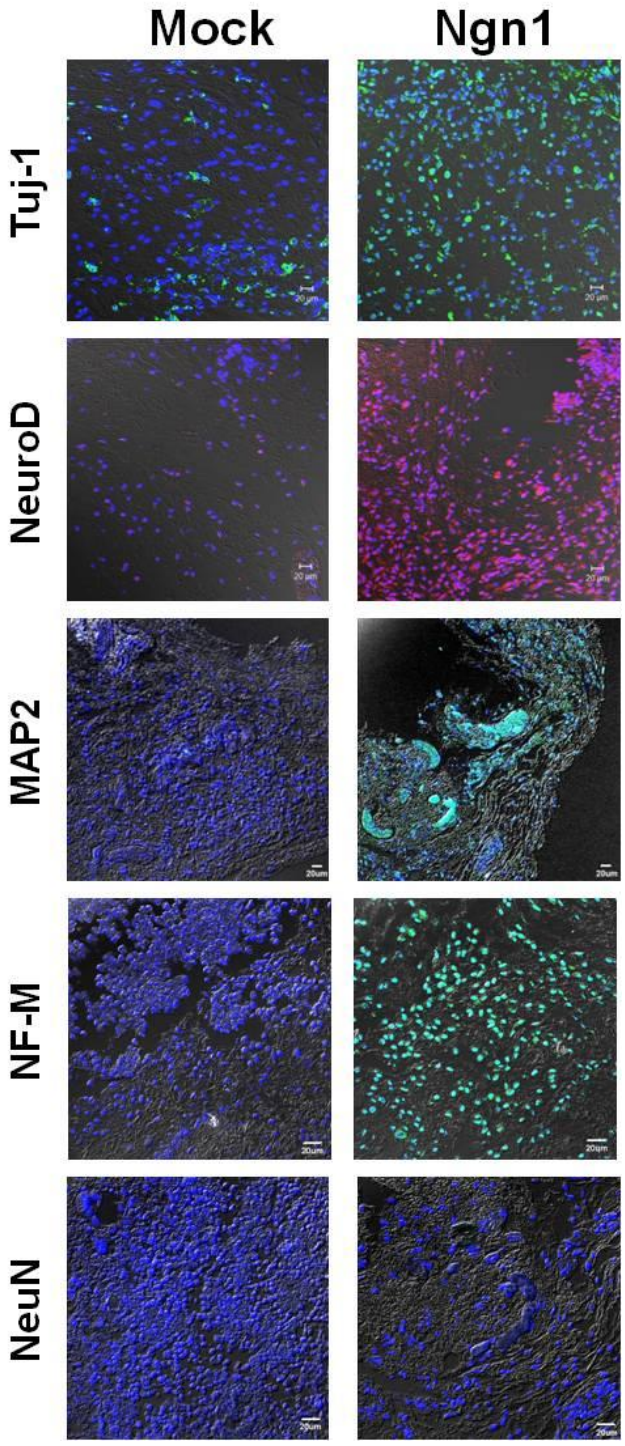

Mock
Ngn1
Tuj-1
NeuroD
MAP2
NF-M
NeuN

FIGURE 5 *In vivo* neuronal differentiation of F11 cells injected s.c. into thighs at 3 days after cell implantation. The implanted F11 cells were isolated from each thigh of the nude mice, and each sample was fixed with 4% PFA for paraffin slide sections. Immunohistochemistry analysis demonstrated the increased expression of the transcription factor Ngn1 (red), immature neuronal marker Tuj-1 (green), and mature neuronal markers NeuroD (red), MAP2 (green), NF-M (green), and NeuN (green) in the F11-Ngn1 group. Scale bars = 20 μm.

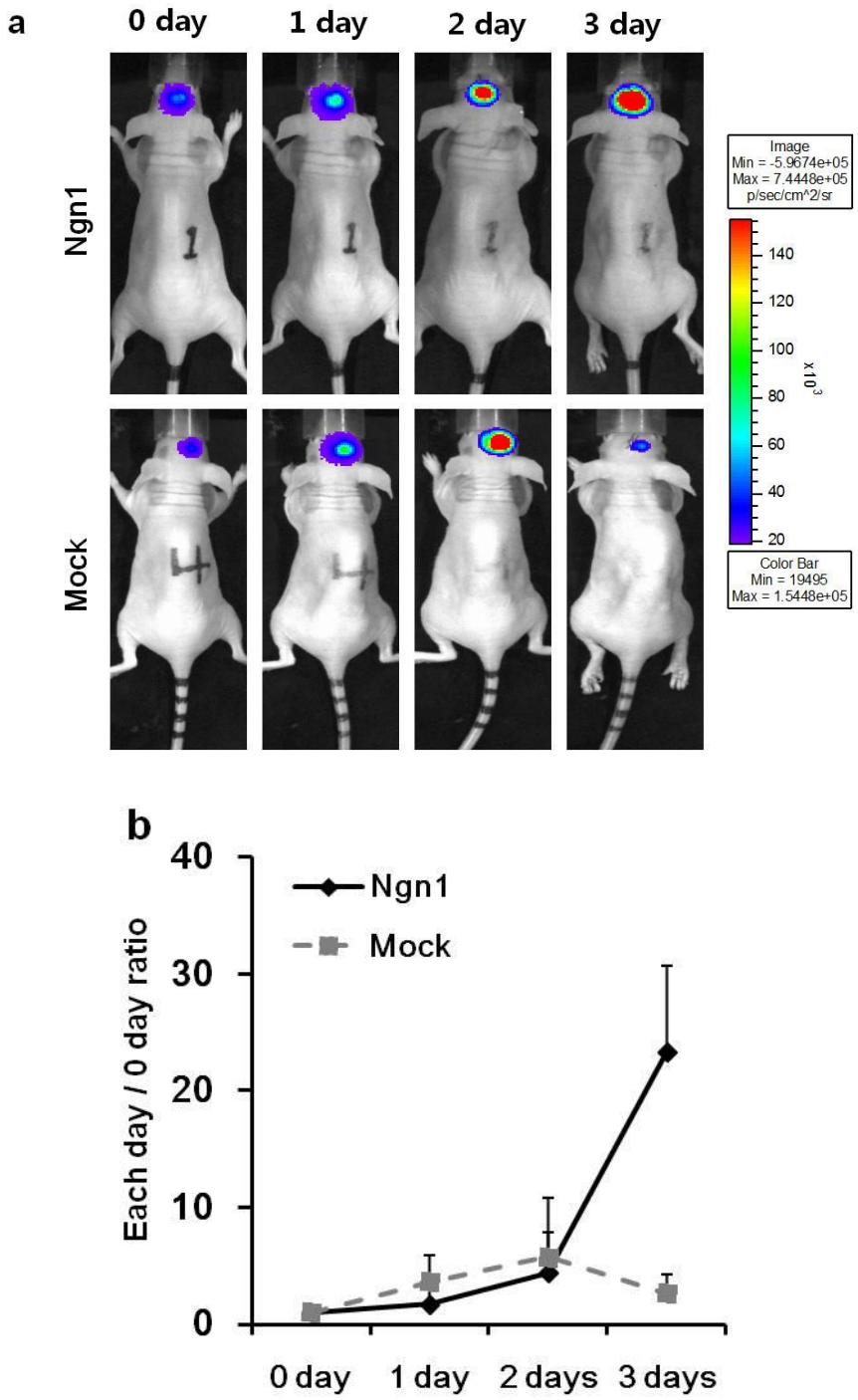

a
0 day
1 day
2 day
3 day
Ngn1
Mock
Image
Min = -5.9674e+05
Max = 7.4448e+05
p/sec/cm^2/sr
140
120
100
80
60
40
20
x10^3
Color Bar
Min = 19495
Max = 1.5448e+05
b
Each day / 0 day ratio
40
30
20
10
0
Ngn1
Mock
0 day
1 day
2 days
3 days

FIGURE 6 *In vivo* bioluminescence imaging of Ngn1-induced F11 cells injected orthotopically into the brain. (a) At 24 h after F11 cells were transfected with pNeuroD-Fluc, 4.5×10^5 cells were injected stereotactically into the right striatum of nude mice with *in vivo*-jetPEI^{TM}-Ngn1 complexes or *in vivo*-jetPEI^{TM}-Mock complexes. Bioluminescence images were acquired from 0 to 3 days. The enhanced bioluminescence signals in the F11-Ngn1 cell implantation group were detected at 3 days after implanting the cells into the brain. (b) ROI values are expressed as the ratio each day/0 day ratio. Significantly increased luciferase intensity was found in the F11-Ngn1 group mixed with *in vivo*-jetPEI^{TM}-Ngn1 complexes at 3 days after cell injection. Data are expressed as means ± SD.

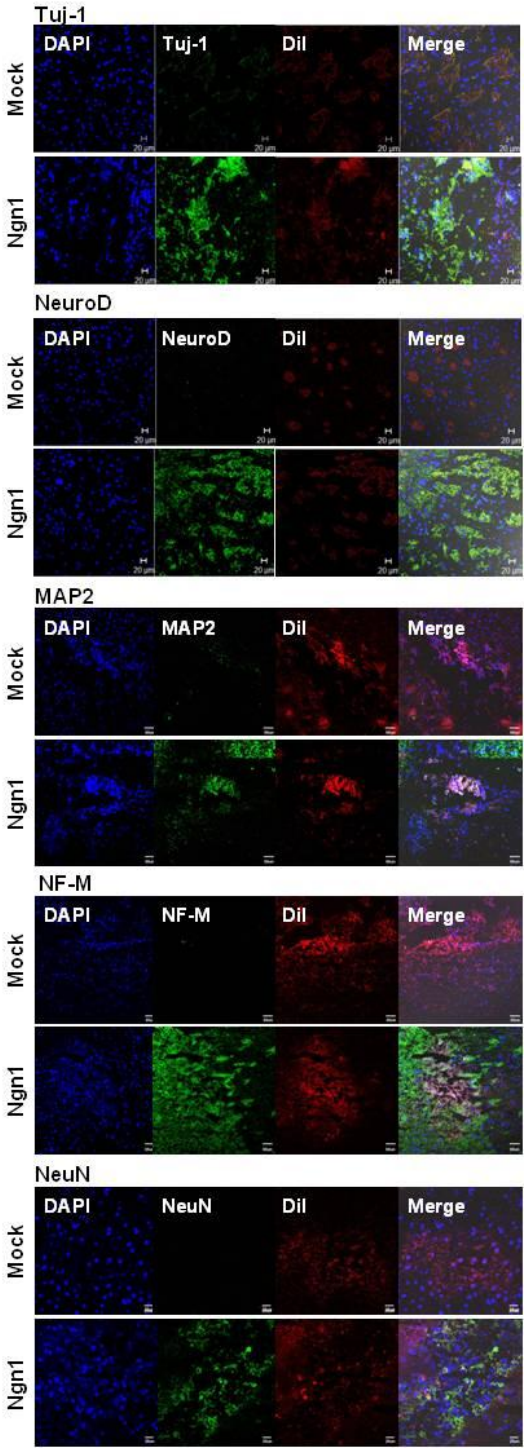

Tuj-1
DAPI
Tuj-1
DiI
Merge
Mock
Ngn1
NeuroD
DAPI
NeuroD
DiI
Merge
Mock
Ngn1
MAP2
DAPI
MAP2
DiI
Merge
Mock
Ngn1
NF-M
DAPI
NF-M
DiI
Merge
Mock
Ngn1
NeuN
DAPI
NeuN
DiI
Merge
Mock
Ngn1

FIGURE 7 *Ex vivo* validation study for the activation pattern of neuronal differentiation in brains implanted with F11-Ngn1 cells. As soon as the brain tissue was isolated from nude mice at 3 days after cell transplantation, it was fixed with 4% formaldehyde. Neuron-specific antibodies to the transcription factor Ngn1, the marker for early differentiating neurons Tuj-1, and the mature neuronal markers NeuroD, MAP2, NF-M, and NeuN were used to analyze the degree of neuronal differentiation induced by Ngn1 in the brain. The expression of all of the markers is shown in green. Scale bars = 20 μm.

IV. DISCUSSION

In vivo monitoring of the activation process of grafted stem cells into specific cell lineages by neuronal-inducing factors enables us to track the activation efficacy of neuronal differentiation in living subjects. Indeed, an *in vivo* differentiation imaging approach can provide useful information not only for the evaluation of differentiation efficacy for the development of novel differentiation-inducing factors but also a better understanding of the therapeutic mechanism in terms of stem cell-based therapy. Currently, stem cell replacement therapy has been recognized as a unique therapeutic approach to repair neurodegenerative disorders such as Parkinson's disease [22, 23]. However, since the grafted stem cells show low efficiency for their differentiation into functioning neurons *in vivo*, the development of effective neuron-inducing factors is required to enhance its therapeutic efficacy. In this study, we used the neurogenic transcription factor Ngn1 that represses gliogenesis directly and activates neurogenesis simultaneously by promoting the expression of NeuroD, which may become an ideal candidate for facilitating the induction of neuronal differentiation [9]. In this study, we showed that Ngn1 activated neuronal differentiation using a validation study with a variety of neuron-specific markers in neuronal precursor F11 cells. We also established an *in vivo* imaging system to track the activation process of the grafted cells into a neuronal lineage using optical luciferase regulated by a neuron-specific promoter. Using this system, we observed the significant change of F11 cells to a neuronal phenotype from the overexpression of the Ngn1 gene alone and culture in a low percentage of FBS. The low concentration of FBS, which reduces cell growth,

is one of the critical factors that accelerate the neuronal differentiation of neuronal precursor cells. Neuronal gene expression profiling using real-time RT-PCR was investigated to confirm whether the morphological changes induced by Ngn1 were caused by the extreme stress of exogenous gene transfection. In this study, we found the enhanced expression of several neuron-specific genes including NeuroD in F11-Ngn1 cells (Fig. 2).

It is of interest that Ngn1 activated neuronal differentiation in the neuronal precursor cells within a short period of time. This result indicates that it is not necessary to use a virus-based construct for long-term transgene expression; indeed, virus-mediated transduction may cause insertional mutagenesis or unexpected side effects [24, 25]. Transient transfection techniques using plasmid DNA vectors are also problematic in that they do not show the long-term effect of the transgene due to their "dilution" when the cells start to undergo division. However, in this case, since the activation of neuronal differentiation by Ngn1 occurred in a short period of time, the use of a plasmid vector encoding Ngn1 in this study is suitable in terms of safety.

Immunofluorescence staining results also supported the activation of neuronal differentiation by Ngn1, showing the enhanced expressions of late neuronal proteins as well as early neuronal markers. Especially, the expression levels of MAP2, which forms the neuronal cytoskeleton, were the most distinctive between Mock and Ngn1 induction. We analyzed various neuron-specific markers related to different neuronal stages between the F11-Mock and F11-Ngn1 groups. In addition, more accurate neuronal differentiation analysis using quantitative measurements based on individual fluorescence was included in this study. From those results, we acquired concrete information for the extent of differentiation induced by Ngn1 according to neuronal

stage. Especially, the acceleration process into the neuronal lineage *in vivo* was monitored successfully in the mouse model via the orthotopic as well as subcutaneous injection of neuronal precursor cells.

To deliver the transgene of interest into cells *in vivo*, an efficient *in vivo* gene delivery system is required. In this study, we utilized the cationic polyethyleneimine (PEI) polymer that has been widely used for gene delivery *in vivo* [26, 27]. In a previous study, there was no induction of major pro-inflammatory cytokines such as TNF-α, IL-6, and IL-12/IL-23 using *in vivo*-jetPEI^{TM} systemic delivery of DNA and siRNA *in vivo* compared to other lipophilic reagents. Therefore, we used the *in vivo*-jetPEI^{TM} system for our *in vivo* systemic delivery experiments instead of lipofectamine. After the effective delivery of the transgene via *in vivo*-jetPEI^{TM} was verified using a general luciferase viral vector system, F11 cells carrying pNeuroD-Fluc and pCMV-Gluc vectors were mixed with Ngn1 or Mock complexes within *in vivo*-jetPEI^{TM} and then injected into each thigh of nude mice. Acquired serial images indicated a higher luciferase signal over time in the right thigh injected with F11-Ngn1 cells compared to the left thigh that was injected with F11-Mock cells. In contrast, the Gluc signal from F11 cells transfected with pCMV-Gluc was decreased gradually in the right thigh injected with F11-Ngn1 until 3 days. (Fig. 4c). These results indicated that the *in vivo* induction of Ngn1 effectively represents its functional action by differentiating the grafted cells into the neuronal lineage and, at the same time, proliferation of the grafted cells was reduced by Ngn1-mediated neuronal differentiation. In addition, these findings were supported by the results from the microscopically observed immunohistochemistry data.

The *in vitro* study revealed that the expression of the early neuronal marker Tuj-1 and mature neuronal markers MAP2 and NeuroD were significantly enhanced in F11-Ngn1 cells, but not in F11-Mock cells (Fig. 3). In addition, neuronal differentiation was activated in F11 cells isolated from the subcutaneous and brain injection sites and they expressed Tuj-1, NeuroD, MAP2, and NF-M (Figs. 5,7). In contrast, NeuN, which is a late neuronal marker, was not expressed even after Ngn1 induction in the subcutaneous area or *in vitro*. However, compared to the above results, F11-Ngn1 cells in the brain showed significantly increased NeuN expression. This result demonstrated that the injected F11-Ngn1 cells in the brain environment, which contains various neuronal or glial cell populations, may be affected by various factors secreted from host neuronal or glial cells, and allow for the progression of neuronal differentiation.

In the optical imaging analysis of the brain, the *in vivo* normalization of the implanted F11 cells using gaussia luciferase expression is inaccessible because coelenterazine, which is the substrate for gaussia luciferase, cannot penetrate the blood-brain barrier. Thus, the development of a novel luciferase substrate that is capable of crossing the blood-brain barrier, which is similar to D-luciferin, is necessary for *in vivo* optical imaging in the brain.

The neuron-specific reporter imaging technique offers powerful information to track the time course of neuronal differentiation using a noninvasive approach. Our approach regarding *in vivo* imaging for assessing the activation of neuronal differentiation by a neuron-inducing gene can represent a variety of applicable aspects for the therapeutic purpose. The *in vivo* efficiency of the extent of neuronal differentiation of grafted cells can be detected easily. In addition, this monitoring

system can provide useful knowledge to determine the capability of candidate Ngn1-like neuronal-inducing factors to promote neuronal differentiation *in vivo*. Moreover, in terms of the enhancement of therapeutic efficacy in gene-based therapy, an *in vivo* imaging strategy to track the activation of neuronal differentiation induced by a neuronal activator can help to understand the therapeutic mechanisms underlying findings from behavior analysis for functional improvement. We expect that the ability to monitor the activation of neuronal differentiation by Ngn1 can be helpful to determine an enhanced therapeutic effect in a variety of neurological diseases.

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국문초록

목적: 생체 내에 이식된 줄기 세포는 신경세포로의 분화 효율이 매우 낮기 때문에 이를 높이기 위해서는 신경세포로의 분화촉진을 유도 하는 유전자가 필요 하다. 뉴로제닌 1은 신경 줄기 세포 및 신경 전구 세포에서 효과적으로 신경세포 분화 활성을 나타내게 하는 전사 인자이다.

본 연구에서는 신경세포 특이적인 프로모터를 가진 광학 리포터 유전자를 이용하여 신경 전구 세포에서 뉴로제닌 1에 의한 신경세포로의 분화 활성을 생체 내에서 모니터링 하고자 하였다.

연구방법: 신경세포 특이적인 NeuroD 프로모터의 조절을 받아 firefly luciferase 리포터 유전자의 발현이 일어나도록 재조합 된 플라스미드 벡터 (pNeuroD-Fluc) 를 이용하여 신경전구 세포인 F11 세포에서 일어나는 신경세포 분화 활성을 모니터링 하였다. 뉴로제닌 1에 의해 유도된 신경세포의 신경세포 특이적인 성질을 확인하기 위해 면역 염색을 실시하여 신경세포 특이적인 마커들을 확인하였으며 마커 발현의 정량적인 분석을 위해 TissueFAXs 분석을 추가적으로 진행하였다. 생체 내 pNeuroD-Fluc 의 광학 시그널의 보정을 위해서 cytomegalovirus (CMV) 프로모터 조절을 받는 gaussia luciferase 리포터 유전자 (CMV-Gluc) 를 사용하였다, 생체 내 유전자 전달 시스템으로는 생체 내 jetPEI^{TM} 을 사용하였다.

결과: 뉴로제닌 1이 처리 된 후 2 일 안에 신경전구 세포인 F11 세포에서는 신경돌기가 뻣어 나오는 것을 관찰 할 수 가있다. 면역염색을 통해 뉴

로 제닌 1이 처리된 그룹에서 신경세포 분화 초기 마커인 *β*III-tubulin (Tuj-1)과 성숙한 신경세포에서 발현하는 NeuroD, MAP2 그리고 NF-M 의 발현이 유의하게 증가 하는 것을 확인 할 수 있었다. 신경 전구 세포인 F11 세포에 pNeuroD-Fluc 리포터 유전자와 뉴로제닌 1 혹은 Mock 벡터를 처리 하였을 때 뉴로제닌 1이 처리 된 그룹에서는 Mock 이 처리 된 그룹에 비해 광학 시그널이 11배 정도 증가 하는 것을 확인 할 수가 있었다. 또한 생체 적합성 *in vivo* PEI를 이용하여 생체 내에 뉴로제닌 1 유전자를 전달하여 신경세포 분화 활성을 관찰 하였을 때 pNeuroD-Fluc 와 pCMV-Gluc 가 동시에 처리된 F11 세포에 뉴로제닌 1을 처리한 그룹이 Mock 벡터가 처리된 그룹에 비해 3일째 까지 발광 시그널이 점차 증가 하는 것을 확인 할 수 있었다. 그리고, 마우스 뇌에 F11 세포를 이식한 후 뉴로제닌 1을 유도 시켰을 때 이식 3일 후 Mock 벡터가 처리된 그룹에 비해 뉴로제닌 1이 처리된 그룹에서 광학 시그널이 유의하게 증가하는 것을 생체 내에서 영상화 할 수 있었다.

결론: 신경 전구세포에서 신경세포 특이적 광학 리포터 유전자를 이용하여 뉴로제닌 1에 의한 신경세포 분화 활성을 생체 내에서 실시간 영상으로 추적할 수 있음을 확인하였다. 이런 신경세포 분화 활성을 모니터링 하는 리포터 유전자 이미징 시스템은 줄기세포 치료 시 신경세포 분화 유도 활성제인 뉴로제닌 1에 의해 이식된 줄기 세포가 신경세포 분화 효율을 모니터링 하는데 효과적으로 사용될 수 있을 것이라 기대한다.

주요어: 뉴로제닌 1, 신경세포 분화활성, 리포터 유전자, 신경전구 세포, 생체 내 광학 영상

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