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Comparison of supportive effect among hydrogel, matrigel, poly-L lactic acid (PLLA) scaffold incorporating neural stem cells in the motor cortex-ablated corticectomy rat model via *in vivo* bioluminescence imaging

뇌 운동영역 피질절제 **rat** 에서 **hydrogel, matrigel, poly-L lactic acid (PLLA)** 지지체내 이입된 신경줄기세포의 생존률 비교를 위한 생체 광학 영상화

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Comparison of supportive effect among hydrogel, matrigel, poly-L lactic acid (PLLA) scaffold incorporating neural stem cells in the motor cortex-ablated corticectomy rat model via *in vivo* bioluminescence imaging

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ABSTRACT

Purpose: Recent studies have tried to enhance efficiency of cell therapy for neurodegenerative disease using biocompatible scaffolds that can provide machenical support. Among a wide variety types of scaffolds, evaluating which scaffolds play an effective roles in promoting the implanted cell growth and proliferation *in vivo* is important for determining the properly available scaffolds capable of enhancing thearpeutic effect. In this study, we compared three different scaffolds, hydrogel, matrigel, poly-L lactic acid (PLLA) scaffold to evaluate the supportive effect of individual scaffold using *in vivo* optical imaging system in corticectomized rat model.

Material and Method: Enhanced firefly luciferase (effluc) retroviral vector system was introduced to evaluate the survival of human neural stem cells within each scaffold *in vivo*. The gelatin-polyethylene glycol-tyramine (GPT) hydrogel was prepared from a Tet-SA-TA solution in the presence of horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) under physiologic condition. PLLA scaffold was also prepared in 24 well plate by wetting in 70% ethanol over 24 hr before cell incorporation step. The corticectomized rat model that excises an area of cerebral motor cortex was established. The immune suppressor was daily administered via intraperitoneal injection into

corticectomized rat model after cell implantation. The behavioral test was performed to evaluate functional recovery in brain injury model. *In vivo* optical images were acquired using IVIS-100 optical imaging device.

Results: The increasing F3-effluc cell number was linearly correlated with luciferase signal intensity. The luciferase signals were gradually increased in F3-effluc cells incorporated within three different scaffolds, showing highest luciferase intensity in F3-effluc cells within matrigel or PLLA, compared to F3-effluc/hydrogel complex group. While F3-effluc cells implanted into ablated cortical region were only maintained for 6 days in their survival, implanted F3-effluc cells encapsulated within three different scaffolds in corticectomized rat exhibited prolonged survival until 10 days. In quantitative analysis, F3-effluc cells within PLLA scaffold showed the best supportive effect in brain damaged rat model. Immunohistochemistry results revealed that nestin (neural stem cell marker)-positive cell population in cell/scaffold group was higher than cell only-injected group. The expression of Tuj-1, early neuronal marker was co-localized with DiI-labeled implanted F3-effluc cells wihin matrigel or PLLA, compared to cell-only and cell/hydrogel group.

Conclusion: In this study, we compared the supportive effect among different biocompatible scaffolds to evaluate the survival and

proliferation pattern of F3-effluc incorporated within hydrogel, matrigel, PLLA scaffolds using optical imaging reporter system *in vivo*. We expect that *in vivo* optical monitoring system could be helpful to determine the optimal selection for appropriate biocompatible scaffold favorable to the treatment of specific brain injury disorder.

KeyWords: Corticectomized rat model, Matrigel, Hydrogel, PLLA scaffold, Neuronal stem cells, *In vivo* bioluminescence imaging

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LIST OF FIGURES

LIST OF ABBREVIATIONS

GPT: Glycol-tyramine

PLLA: Poly-L lactic acid

EGF: Epidermal growth factor

FGF: fibroblast growth factor

IGF: Insulin-like growth factor

HRP: Horse radish peroxidase

GFP: green fluorescence protein

effluc: enhanced firefly luciferase

CMV: Cytomegalovirus

TBI : Traumatic brain injury

SEM: Sacnning electric microscope

CONTENTS

Ⅰ. INTRODUCTION

Stem cell transplantation has been recognized as a unique therapeutic way for treatment of various central nervous system (CNS) diseases because the damaged neuronal cells cannot be regenerated. Neural stem cells capable of generating functional neurons have been utilized to treat neurodegenerative disease such as Parkinson's disease and traumatic brain injury [1-5]. In spite of recognizing the importance of cell therapy-based research in the regenerative medicine area, critical issues such as poor survival rate and low therapeutic efficacy have hampered the rapid advances for cell therapy in clinical field. In recent years, considerable interest has been given to biologically active scaffolds which are based on similar structure of the extracellular matrix that is necessary to provide mechanical support for either endogenous or the implanted cells. In clinical aspect, scaffold-based cell therapy has been proven to be effective therapeutic strategy by directly enhancing the survival of implanted cells with high therapeutic efficacy in various disease models [6-9]. The gel-types or microfibrous-types of various scaffolds have been introduced with emphasizing their unique characteristics in terms of promoting cell adhesion and proliferation [10-14]. It can be critical to determine proper scaffolds as

a cell-type or environmental condition. In particular, use of appropriate biocompatible scaffold to find optimal therapeutic condition is more important for better therapeutic efficiency in terms of treatment of degenerative disease. Hydrogel has widely been studied as excellent biomaterials for a wide range of biopharmaceutical and biomedical application such as drug or cell delivery and bio-tissue engineering for regenerative medicine, because of their tissue-like properties, high water contents, and high permeability for nutrients and metabolites [15,16]. In particular, in situ gel formation based on injectable hydrogel has received much attention because of the simple application using minimally invasive techniques for tissue regenerative medicine and drug delivery systems. Hydrogel scaffolds can be manipulated with soft biological tissue such as the brain (<1 kilopascal, kPa), and thus have been widely used in regenerative medicine community to provide biomechanical support for neural stem cells by filling up lost tissue space [17]. Matrigel is known as a soluble and sterile extract of basement membrane proteins derived from the EHS (Engelbreth-Holm-Swarm) sarcoma tumor that can form a 3D gel at 37 ˚C. It provides cells with supportive function of extracellular matrix contributing to the structural organization to be utilized to improve cell survival and to increase tumor growth [18,19]. Poly L-lactic acid (PLLA) is FDA-approved fibrous polymer that is most commonly used for

biocompatible scaffold in tissue engineering field [20,21].

Many different forms of imaging modalities such as optical, radionuclide, and magnetic resonance (MR) imaging have been available to understand the *in vivo* characteristics of implanted stem cells such as their survival and proliferation. In particular, optical imaging technique has been widely used to track the implanted stem cells due to the potential merit such as low signal background. In this research, we examined the supportive effect of implanted neural stem cells on three different types of biocompatible scaffolds (hydrogel, matrigel, PLLA) in corticectomized rat model. By using optical imaging system, we evaluated suvival and proliferation pattern of transplanted F3-effluc cells *in vitro* and *in vivo*.

Ⅱ. MATERIALS AND METHODS

Production of scaffolds

Hydrogel was synthesized as gelatin-polyethylene glycol-tyramine (GPT) hydrogel. GPT hydrogel was developed as an injectable material for tissue regeneration and drug delivery [22]. GPT conjugates were synthesized by coupling TA-labeled PEG (PNC-PEG-TA) and gelatin backbone. Briefly, PNC-PEG-PNC was synthesized, by reacting hydroxyl groups of the PEG with p-nitrophenyl chloroformate (PNC) and then Triamcinolone acetonide (TA) was reacted with PNC-PEG-PNC and gelatin subsequently to produce GPT polymer. Matrigel (BD matrigel^{TM} membrane matrix, BD science, USA) is a form of basement membrane that is isolated from the Engelbreth-Holm-Swarm (EHS) sarcoma. This contains microenvironment protein including laminin (60%), collagen IV (30%), and entactin (8%), and various growth factors including epidermal growth factor (EGF), insulin-like growth factor (IGF-1), and fibroblast growth factor (FGF). Poly-L lactic acid (PLLA) is types of fibrous scaffold fabricated by the wet spinning technique. The 6% PLLA solution prepared in 9:1 (v/v) methylene chloride/acetone was loaded into a syringe, and the polymer fiber with

the flow rate (0.9 and 1.1 mL/h) was formed in the bath. For cell seeding, PLLA scaffolds were sterilized with 70% alcohol overnight, and were rinsed using phosphate buffered saline (PBS). To make incorporated cells dispersed in whole PLLA scaffold, the cell/scaffold complex was incubated in a CO_2 incubator for 2 hr and complete cell medium was added.

Cell culture and effluc virus infection

HB1.F3, human neural stem cells derived from 15 week fetal telencephalon periventricular layer was maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Grand Island, NY) with 10 U/ml penicillin and 10 μg/ml streptomycin (Invitrogen, Grand Island, NY) in the humidified incubator at 37 °C. For *in vivo* visualization of grafted stem cells, F3 cells were genetically engineered using retroviral vector. The backbone of retroviral MSCV DNA vector contains the enhanced firefly luciferase coding gene modified by codon optimization technique and Thy1.1 (CD90.1) linked with internal ribosome entry site (IRES) regulated by cytomegalovirus (CMV) promoter in 5'-long terminal repeat (LTR) region. For retrovirus production, the viral polyproteins (gag, pol, and env) were transfected to 293FT packaging cells. The harvested viral supernatant

was infected to the F3 cells with 10 mM polybrene to prevent electrostatic repulsion between virus and cellular membrane. F3 cells infected with enhanced firefly luciferase gene (F3-effluc) were separated by magnetic-activated cell sorting (MACS) technique (Miltenyi Biotech Ltd, Bisley, Surrey, UK) using monoclonal anti-CD90.1-microbeads. The purity of magnetically separated CD90.1+ F3-effluc cells was examined by fluorescence-activated cell sorting (FACS) analysis (BD Immunocytometry System, CA) using the monoclonal antibody anti-CD90.1-fluorescein isothiocyanate (FITC).

In vitro bioluminescence assay

1×10^4 F3-effluc cells were incorporated into each scaffold (hydrogel, matrigel, PLLA) on 96 well plate. F3-effluc mixed with each scaffold was pre-incubated for 1 hr for stable adhesion, and complete medium was added into each well. D-luciferin (0.1 ml at 3 μg/μl) was directly treated into cell/scaffold mixture. Simultaneous treatment of D-luciferin substrate in each group was carried out using multipipette for 96 well high throughput plate. The luciferase intensity was measured using a microplate luminometer (TR717; Applied Biosystems, Carlsbad, CA).

Establishment of corticectomized rat model

Male Sprague Dawley rats (age 7 weeks) were divided into four groups: (1) cell only injected group (n=5), (2) cell/hydrogel scaffold group (n=5), (3) cell/matrigel scaffold group (n=4), (4) cell/PLLA scaffold group (n=3). Although animals were started from 6 numbers of animals in each group, several mice were died from severely ablated brain cortex or long anesthesia for *in vivo* imaging during total experimental period. Rats were intramuscularly anesthetized with Zoletil (30mg/kg, i.p) and Xylazine (10 mg/kg, i.p). Anesthetized rats were then secured in a stereotatic frame, and scalp was incised along midline. To remove the left side of motor cortex, we drilled a small hole on the skull using hand drill machine. The left side of skull at the area of the motor cortex was removed (size of craniectomy: 15 mm^2). The coordinates of the three points from the bregma were 4 mm rostral/1 mm lateral (coordinate A=+4, +1), 2 mm caudal/1 mm lateral (coordinate B = -2, +1), and 4 mm rostral/6 mm lateral (coordinate C=+4, +6). The exposed dura over the motor cortex was sharply removed with a surgical blade. Motor cortex was clearly removed using a surgical blade, and electrocauterization was used to prevent bleeding during removal of motor cortex area. The resection region was filled

with Gelfoam (Pharmacia and Upjohn, Kalamazoo, MI) to keep the resected motor cortex space, and the incised skin area was sutured. All animal experiments were approved by Seoul National University Hospital Animal Care and Use Committee (IACUC NO.12-0317).

Behavioral test

For the limb placement test, the motor function of each rat was evaluated before surgery, and then after surgery. Limb placement test is modified version described by Ryck group, which has been widely used for functional scoring in stroke models [23,24]. Animals were habituated to handling before the test. The test is comprised of three limb-placing tasks that assess the motor integration of fore-limb and hind-limb responses to proprioceptive and tactile stimulations. The test consisted of three domains: (1) proprioception, (2) whisker tactile and (3) visual forward. Behavioral test was carried out in the semi-dark and silent experimental room to minimize the impact on other environmental stimulation. Proprioceptive fore-limb or hind-limb observation test was performed on the experimental table by gently pulling down the fore-limb or hind-limb of corticectomized rats to examine the degree of re-retraction pattern of fore-limb or hind-limb. The scores of fore-limb or hind-limb test were calculated as follows; 0 point for normal lifting, 1,2,3 points for abnormal lifting as the number

of stretching. Whisker tactile observation test was also performed to verify the sensory function from whisker's stimulus. Whisker tactile study was tested to examine whether fore-limb of rat is stretched out to the table when rat's whisker approached less than 2 mm from the surface of table. Each animal was tested three times, and the scores of whisker tactile study were calculated as follows; 0 point for normal lifting, 1,2,3 points for abnormal lifting as the number of stretching. 'Visual forward' is the observation of forelimb flexion by an animal while its tail is held up. The stretch of the forelimbs toward the table was evaluated and scored: normal stretch = 0 points and abnormal flexion = 1 point.

In vivo **bioluminescence images in cell/scaffold-implanted corticectomized rat model.**

1×10^6 F3-effluc cells (without or with each scaffold) were transplanted into resected area of the motor cortex of the rat brain. In case of the hydrogel scaffold group, the prepared each cell pellet was firstly mixed with GPT solution containing HRP. and loaded into insulin syringe The two separated insulin syringes filled with either GPT solution dissolved in HRP (25 μg/ml) or H_2O_2 containing solution (8 μg/ml) were simultaneously injected into ablated motor cortex area. In case of matrigel group, F3-effluc cells were mixed with matrigel

liquid on ice and then rapidly transplanted into resected area of left motor cortex. And in case of PLLA scaffold group, PLLA scaffold was incubated with 70% ethanol for 1 hour, and PLLA scaffold was washed using PBS three times. After F3-effluc cells were transferred into PLLA scaffold, and incubated for 1 hr, F3-effluc/PLLA complex was transplanted into the cortical resection area. The cell/scaffold implanted rats underwent immunosuppression by daily receiving cyclosporine A (10 mg/kg) via intraperitoneal injection. To acquire the bioluminescence image, the mice were anesthetized with 2% isoflurane in at low rates of O_2 gas flow (1 L/min) via the nose cone by placing the rat inside of induction chamber. D-luciferin was locally injected into ablated brain area at a dosage of 150 mg/kg. The bioluminescence images were acquired using an IVIS-100 equipped with a high sensitive CCD-based camera (Caliper lifescience, Hopkinton, MA). Bioluminescence light emission was collected for 1 min and region of interest (ROI) signal intensity was drawn in each representative area, and expressed as photons/second/cm^2/steradian. IVIS-100 bioluminescence image parameter mode for binning and f/stop was set up as 2 (binning), 1 (f/stop), respectively.

Immunohistochemistry analysis

After transplanted F3-effluc cells within scaffold were monitored

during 10 days, corticectomized rats were anesthetized and perfused with PBS and then 4% paraformaldehyde. After isolated brain was frozen-sectioned, they were immersed in 30% sucrose solutions for dehydration. The brain section was rinsed using PBS and stained with hematoxylin for 1 min at room temperature. The mounted sections were dipped with 70% ethanol for destaining, and counter-stained with aqueous eosin for 1 min. Finally, the sections were dehydrated using ethanol (70% to 100%) and passed through xylene and then mounted. The histological sections of the brain were cut in the axial plane at 20 μm thickness in a freezing microtome. Brain sections were rinsed with 0.5% PBS blocked in normal horse serum plus Triton-X100 (Sigma, Saint Louis, MO). Brain sections were then incubated overnight at 4 °C with primary antibodies. Primary antibodies were used as follows: rabbit anti-Tuj-1 (1:200, Sigma, Saint Louis, MO), anti-nestin (1:200, Sigma, Saint Louis, MO) at 4 °C for overnight. Then, the samples were treated with secondary antibodies, Alexa Fluor 488-conjugated anti-rabbit and anti-goat antibodies (Invitrogen, Grand Island, and N.Y) for 1 hr. Brain sections were then counterstained with the nuclear marker DAPI (4', 6-diamidino-2-phenylindole). Fluorescence signal was detected by confocal laser scanning microscopy (Olympus confocal microscope, Fluoview 1000, Tokyo, Japan).

Ⅲ. RESULTS

Characteristics of individual hydrogel, matrigel, and PLLA scaffolds

The 1800 Pa GPT hydrogel that is gel type of in situ enzyme-triggered crosslinking was synthesized at the H_2O_2 concentration (0.0038%, w/w). When the solution containing HRP was rapidly mixed with other solution containing H_2O_2, the gelation of GPT hydrogel began to be formed (Fig. 1A). Matrigel has red-pink colors that contain phenol red. The liquid form of matrigel matrix on ice will start to form a gel above 10 ºC, and matrix will become gelation rapidly at 37 °C (Fig. 1B). PLLA microfibers (50-100 μm in diameter) were prepared using a wet-spinning method. These fibers were randomly stacked to form 3D matrices with large pore size (Fig. 1C). The PLLA microfibrous 3-D scaffold presented a highly interconnected structure in which the pore size is relatively large (400 μm). A roughened surface was created to induce increased cell attachment.

Linear correlation of luciferase activity with F3-effluc cells on cell number or time-dependent manner

Retrovirus-mediated infection of F3, human neural stem cells with enhanced firefly luciferase gene (F3-effluc) was previously conducted to visualize the transplanted F3-effluc cells [25]. The luciferase signals of F3-effluc cells were continuously increased as the cell number increased (Fig. 2A). Also, to confirm that the luciferase gene is not silenced during cell proliferation, initial F3-effluc cell number of 0.2×10^4 was used to make cells maintained until 6 days in 6 well plates. The luciferase signals of F3-effluc cells were constantly increased until 6 days, indicating that luciferase gene was stably expressed in F3-effluc cells without any gene silencing (Fig. 2B).

Morphological feature of F3-effluc cells within the three different types of scaffolds

To observe a distinct feature in proliferation ability of F3-effluc cells encapsulated within individual scaffolds over time, small amount of F3-effluc (0.2×10^4) cells were mixed with each scaffold in 24 well plate. The hydrogel containing its elastic property of 1800 Pa was used to have similar mechanical strength with brain tissue that has approximately less than 1000 Pa elasticity. We also used the matrigel scaffold containing high concentration of growth factors including nerve growth factor (NGF). Phase contrast microscopic results showed the round shape of F3-effluc within hydrogel or matrigel matrix on day

0 (Fig. 3A). From day 1, evenly distributed F3-effluc cells began to be highly proliferated on 3D environment, showing partial spindle morphology inside each gel condition (Fig. 3A). One day after F3-effluc cells were loaded into pre-wet PLLA scaffold, The scanning electron microscopy (SEM) results showed that the cells were robustly attached onto each macrofiber of PLLA scaffold with formation of interconnected cell network (Fig. 3B).

Vigorous cell proliferation within hydrogel, matrigel, and PLLA scaffold

To investigate that F3-effluc cells incorporated within different types of scaffold show the different cell proliferation pattern, we prepared the cell attachment into three different types of scaffolds in 96 well plate. When the same number of F3-effluc cells were loaded into each scaffold, F3-effluc cells were significantly proliferated from 4 days (Fig. 4). The luciferase signals were constantly increased in F3-effluc cells within three different scaffolds until 12 days, showing highest luciferase intensity in F3-effluc cells incorporated within PLLA and matrigel, compared to F3-effluc within hydrogel.

Limb replacement behavior test to validate abnormal motor function in corticectomized rat model

In this study, we introduced the motor cortex-ablated rat as a brain injury model for cell/scaffold transplantation. The motor cortex area with the established precise coordinates was clearly ablated in rat brain (Fig. 5A). Seven days after establishing the corticectomized rat model, we performed four different limb placement tests (proprioception fore-limb or hind-limb, whisker tactile, and visual forward). The animals that underwent sugical resection of the motor cortex showed motor weakness at one postoperative week. Aberrant behavioral movement by keeping stepping down their fore-limb or hind-limb on the table was clearly observed in corticectomized rats (Fig. 5B). No any significant response in whisker tactile test was observed in corticectomized rat model. In visual forward test, we found abnormal stretch of one fore-limb in corticectomized rats. Total limb placement score showed high abnormality with the impaired motor function, compared to normal rat (Fig. 5C).

In vivo bioluminescence imaging of transplanted F3-effluc cells within three different scaffolds in corticectomized rat model

To compare which types of scaffolds show potential effect of cell viability, on post-operative day 7, 1×10^6 F3-cells encapsulated with hydrogel, matrigel, and PLLA scaffold were engrafted into the cortex-removed area in rat brain. As soon as cell/scaffold complex was

transplanted into corticectomized rats, *in vivo* time course bioluminescence images were acquired at 0, 1, 2, 4, 6, 8 and 10 days. The cell/scaffold complex was well inoculated to ablated cortex region, and luciferase signal intensities in four different groups were almost similar on day 0. The implanted cell proliferation in using three different scaffolds was gradually increased in corticectomized rat brain, compared to the only cell implantation group. In the F3-effluc only-implanted group, the cell viability only sustained until 6 days, and the bioluminescence signals disappeared from 8 days. However, prolonged viability of F3-effluc cells supported with all of three different scaffolds (hydrogel, matrigel, and PLLA) was clearly found in ablated cortex area (Fig. 6A). In particular, F3-effluc cells coated with matrigel or PLLA scaffold were highly proliferated until 10 days, showing robust bioluminescent signal in the brain region, compared to cell/hydrogel implantation group. For the quantitative analysis, photon counts in region of interest (ROI) was measured from *in vivo* bioluminescence data (Fig. 6B). Comsistent with *in vivo* bioluminescence imaging results, dramatic change in bioluminescent intensity between cell only group and cell/scaffold implantation group from 6 days was observed, showing constant increase of cell proliferation in the groups of cell/matrigel or cell/PLLA scaffold.

Histological analysis of the transplanted F3-effluc within three different scaffolds isolated from corticectomized rat brain

After the serial image acquisition until 10 days, the rats were perfused with paraformaldehyde solution, and whole brain was isolated from corticectomized rats. The fixed brain showed ablated cortex region seemed to be filled with cell/scaffold complex-shaped mold in matrigel or PLLA group (Fig. 7A). The 20 μm brain sections were immunostained using two different specific antibodies for intermediate filament nestin protein as neural stem cell marker, and early neuronal marker, beta III tubulin (TuJ-1). The hematoxylin & eosin (H&E) staining results demonstrate that F3-effluc cells were highly proliferative within matrigel matrix, showing compactly distributed cell population, compared to cells within hydrogel or cell only group (Fig. 7B). The high amounts of cell population were also maintained around microfiber of PLLA scaffold. Immunohistochemistry results showed that a large number of implanted F3-effluc cells still existed within matrigel or PLLA scaffold and nestin expression was co-localized with DiI fluorescence dye-labeled F3-effluc cells (Fig. 7C). In contrast, only a few implanted F3-effluc cells were observed in free-scaffold group or hydrogel scaffold group. In addition, Tuj-1 specific immunostaining results showed that early neuronal differentiation in the implanted F3-effluc cells encapsulated with matrigel or PLLA scaffold was markedly

generated within about 2 weeks (Fig. 7D).

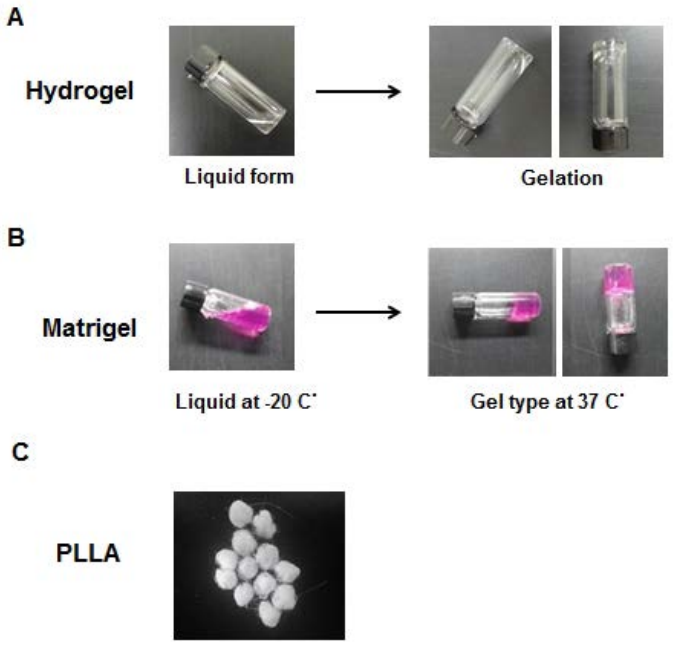


FIGURE 1. Photographic images of GPT hydrogel, matrigel, and PLLA scaffold. (A) Representation of enzymatic crosslinking of GPT conjugates. The GPT hydrogel were rapidly formed by the enzyme-mediated coupling reaction using horseradish peroxidase (HRP) and H_2O_2. (B) Red-pink colored matrigel was maintained as a liquid form on -20 °C. The liquid form of matrigel matrix starts to become gel rapidly at 37 °C. (C) The microfibrous type of 3D poly-L lactic acid (PLLA) scaffold was designed using a wet-spinning technique.

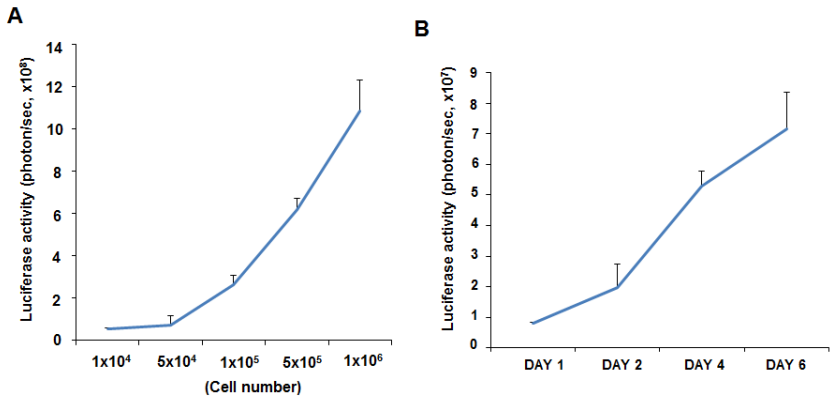


FIGURE 2. Stably expressing luciferase reporter gene of F3-effluc cells in cell number or time dependent manner. (A) The F3-effluc cells (n=3) were cultured in 24 well plate, and the luciferase intensity from F3-effluc cell lysates was calculated using luminometer. (B) 0.2×10^4 F3-effluc cells was seeded into 6 well plate, and luciferase signals were acquired until 6 days. The luciferase intensity in F3-effluc cells was constantly increased as the days went by.

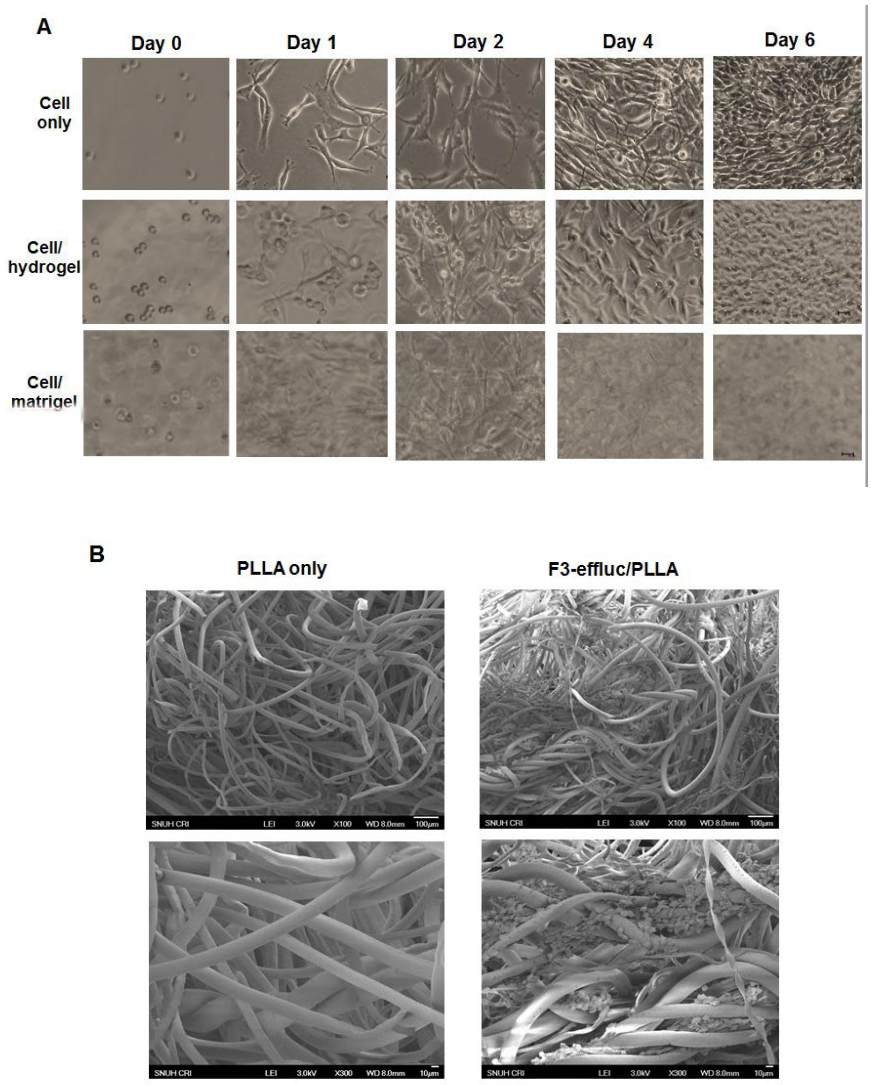


FIGURE 3. Mophological feature of the F3-effluc cells incorporated within hydrogel, matrigel, PLLA scaffold. (A) Each of F3-effluc/hydrogel or matrigel mixture (10 ul) was loaded into the bottom of 24 well plate. Morphological changes on 3D gel condition were observed until 6 days. F3-effluc cells mixed with hydrogel and

matrigel were vigorously proliferated until 6 days. Scale bar: 10 μm. (B) F3-effluc cells were loaded into pre-wet PLLA scaffold, and the scanning electron microscopic (SEM) images were acquired in PLLA only or F3-effluc/PLLA. Most of F3-effluc cells were stably incorporated onto the each microfiber of PLLA scaffold. Scale bar in upper panel is 100 μm, and scale bar in lower panel is 10 μm.

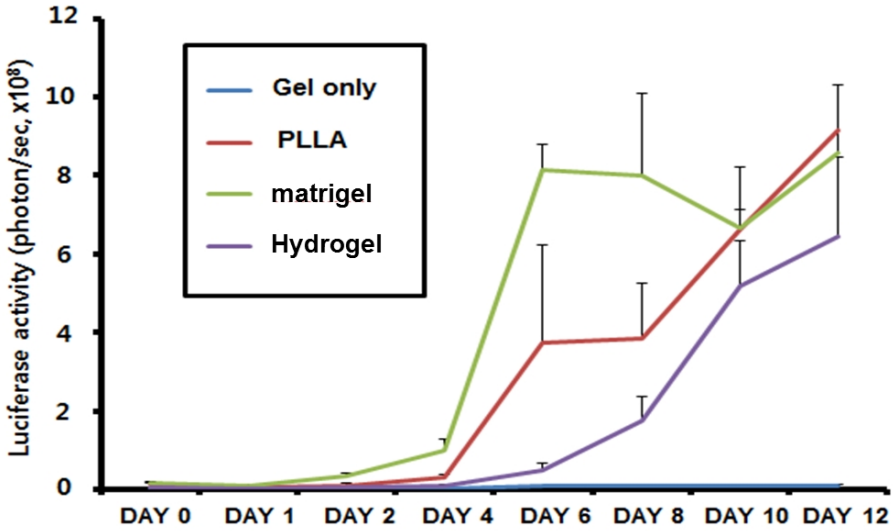


FIGURE 4. Vigorous proliferation of F3-effluc cell growth encapsulated within individual scaffolds in day dependent manner. The same number of F3-effluc cells were detached, and incorporated into hydrogel, matrigel, and PLLA scaffold. D-luciferin substrate was treated into individual cell/scaffold group to observe the luciferase signal until 12 days. It was shown that F3-effluc cells were highly proliferated within three different scaffolds until 12 days.

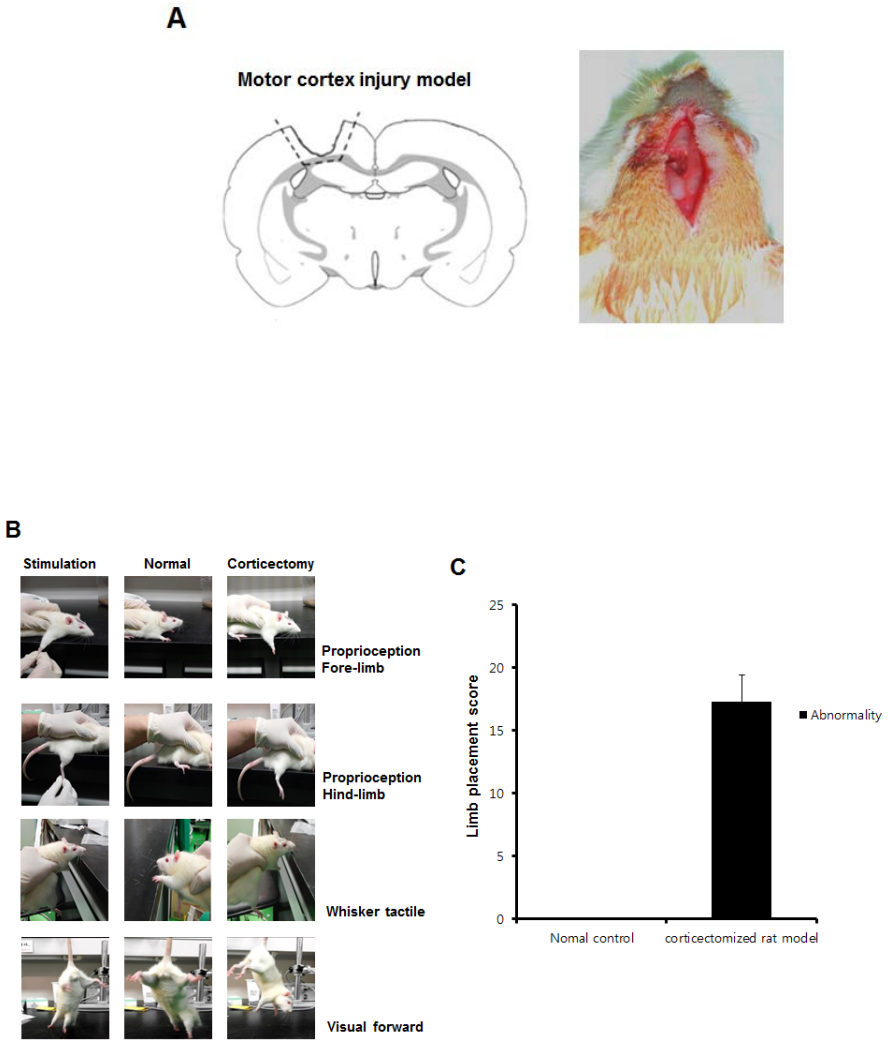


FIGURE 5. The establishment of motor cortex-ablated corticectomized rat model (A) The motor cortex removed as the determined coordinates are shown in rat brain. (B) Limb placement test was performed in normal or corticectomized rats. The component of

limb placement test includes fore-limb or hind-limb proprioception, whisker tactile and visual forward. Abnormal behavior was observed in corticectomized rat model, compared to normal rats. (C) High abnormality was calculated via total limb placement score, compared to normal rat.

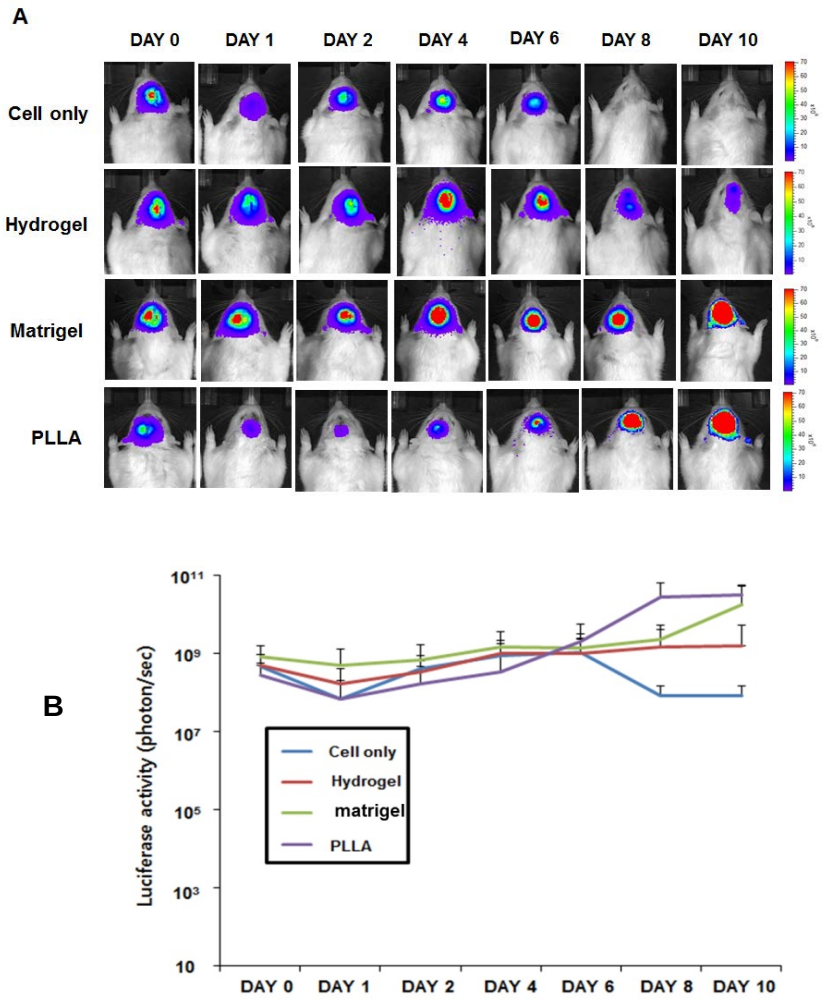
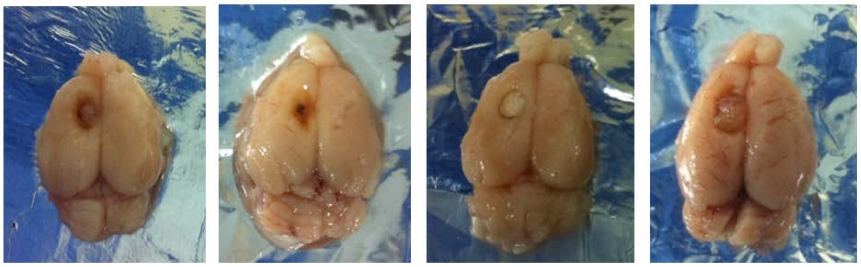


FIGURE 6. ***In vivo*** **bioluminescence visualization to evaluate the different proliferation rate among three different scaffold embedding cells in corticectomized rats.** (A) Control group (F3-effluc cell only), F3-effluc/hydrogel, F3-effluc/matrigel, and F3-effluc/PLLA scaffolds were transplanted into the resected motor cortex area of the rat brain. Bioluminescence images were acquired until 14 days on the post-operative day 7. The proliferation of implanted F3-

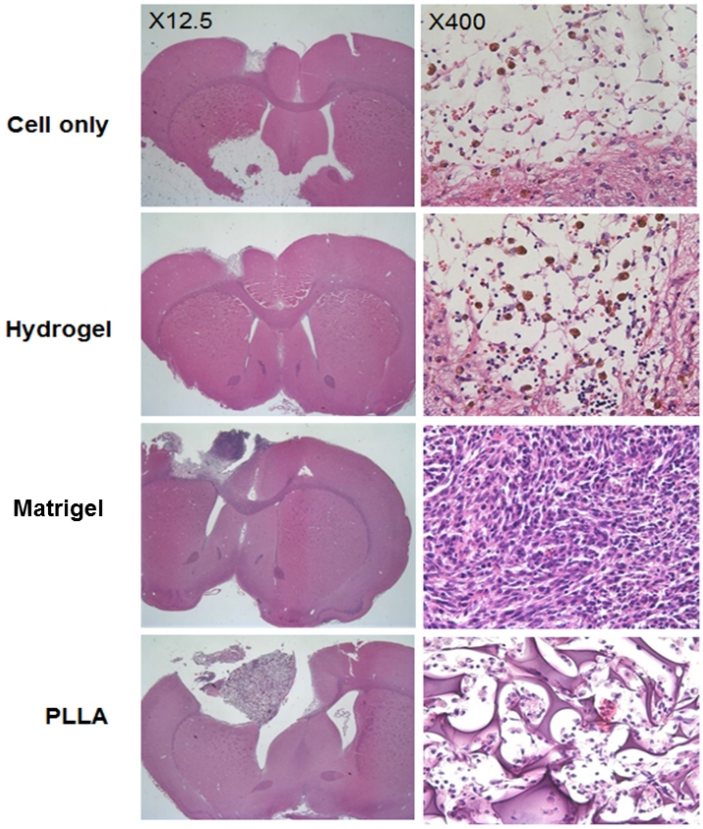
effluc cells within matrigel or PLLA scaffold was gradually increased until 10 days. (B) Quantitative region of interest (ROI) analysis showed continuously increasing cell proliferation within matrigel or PLLA scaffold, compared to cell-only implantation group.

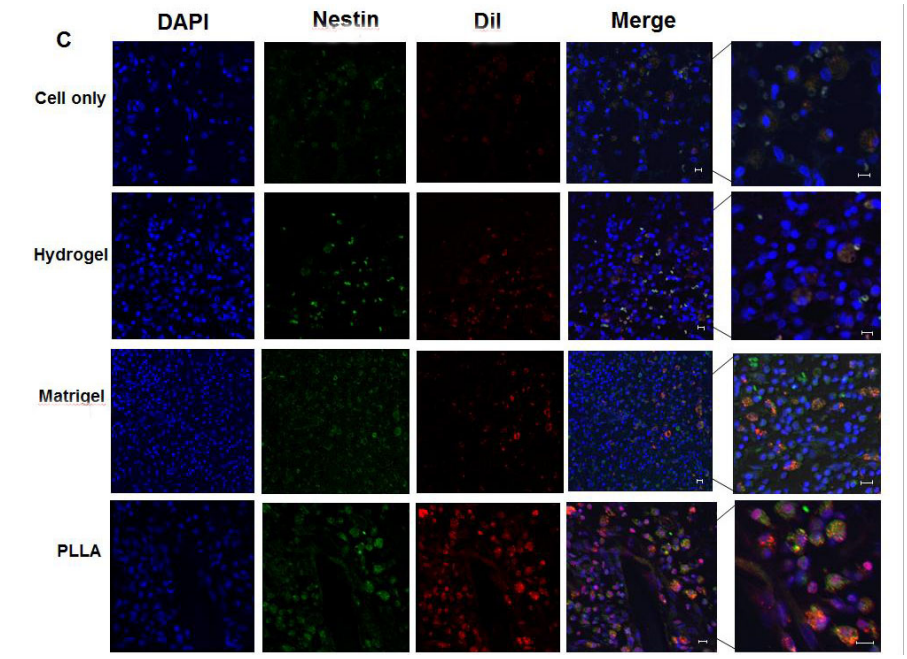
A

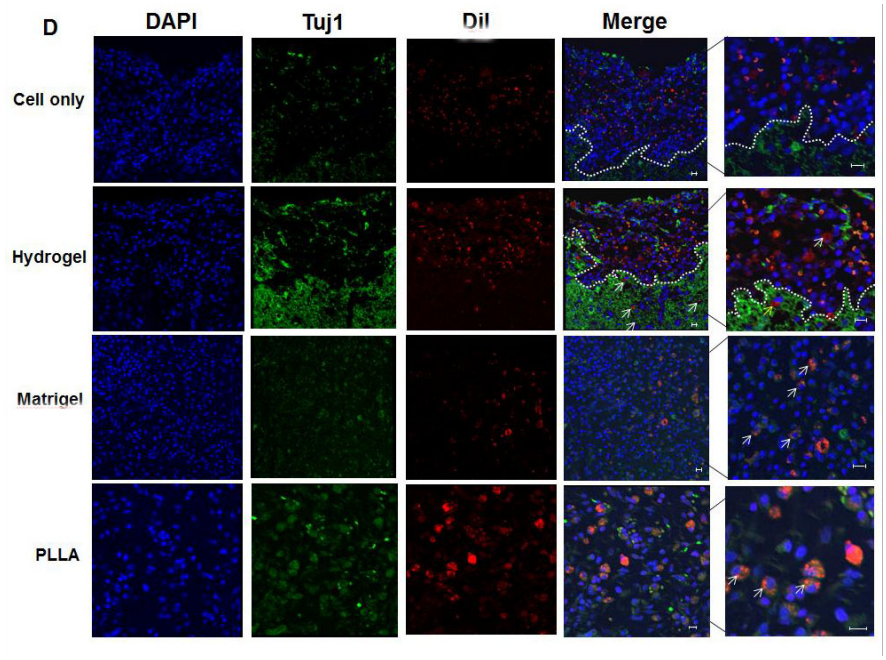


Control Hydrogel PLLA Matrigel

B




C
DAPI
Nestin
DiI
Merge
Cell only
Hydrogel
Matrigel
PLLA


D
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Tuj1
DiI
Merge
Cell only
Hydrogel
Matrigel
PLLA

FIGURE 7. Immunohistochemistry analysis of the isolated F3-effluc/each scaffold complex in the corticectomized rat brain. Each of brain was isolated 10 days after *in vivo* bioluminescence acquisition. (A) The cell/scaffold complex mold was maintained in the ablated cortex area of fixed brain s in matrigel or PLLA group (Fig. 7A). (B) Hematoxylin and eosin (H&E) staining was conducted in the 4 different groups to observe the whole cell population within the each scaffold. The specific antibodies to nestin (C) or Tuj-1 (D) protein was used to observe the maintenance of neural stem cell or early neural differentiation, respectively. Large amount of nestin positive F3-effluc within matrigel or PLLA scaffold was co-localized with DiI-labeled F3-effluc cells. The implanted F3-effluc cells within matrigel or PLLA scaffold was enough to induce differentiation into early neuronal cells in corticectomized brain within about 2 weeks. The lower panels at dotted line represent host brain tissue. White arrow indicates double stained (DiI and Tuj1) F3-effluc cells. Yellow arrow indicates infiltrated F3-effluc cells. All scale bars are 10 μm.

Table 1. Comparison of biocompatible scaffolds on pros and cons

Biocompatible scaffolds	Properties	Pros	Cons
Hydrogel	High water contents Enzyme-mediated gelation Use as therapeutic delivery vehicle	Tissue mimetic from stiffness Injectable system	Low mechanical Strength Batch variation
Matrigel	Temperature sensitive gelation Various growth factors: (EGF,FGF,NGF,PDGF ,IGF-1,TGF-β)	Complex tissue-like structures	Feasible immune responses
Poly-L lactic acid (PLLA)	Control of pore structure	Good tensile strength FDA approved	Biologically inactive *in vivo*

Ⅳ. DISCUSSION

Neural stem cell niches including 3D microenvironment, vasculature, and surrounding neuronal or glial cells have been recognized as an important component to maintain self-renewal and control the tissue-specific differentiation. Biocompatible tissue engineering using artificial materials for optimized microenvironment can potentially control the spatial and temporal progression of regenerative processes in CNS injury-related disease. A various types of biocompatible scaffolds have served as bio artificial 3D microenvironment to support cell adhesion and proliferation. Although rapid advances for potential scaffold-based cell therapy has contributed to enhancing therapeutic potential in numerous degenerative disorders, the choice of proper scaffold is critical for optimal therapeutic strategy.

In this study, among a well-known biocompatible scaffold; hydrogel, matrigel, and PLLA, we evaluated which types of scaffolds play an effective roles in promoting the implanted cell growth and proliferation *in vivo*, and verified these viability of scaffold-encapsulated neural stem cells by non-invasive bioluminescence

imaging techniques. The hydrogel we used in this study was in situ crosslinking GPT hydrogel that gelation process is rapidly formed under enzyme-mediated reaction using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) [22]. Out of the different hydrogel possessing various types of mechanical elasticity, we choice the 1800 Pa hydrogel that similar elasticity with the intact brain tissue [17]. A number of recent studies highlight the importance of hydrogel as a powerful *in vivo* scaffold or delivery carrier for treatment of central nervous system (CNS) disorder [16,26]. The matrigel used is temperature sensitive gel type composed of highly organized structure containing extracellular matrix proteins including 60% laminin and 30% collagen and high contents of growth factors. In the case of PLLA scaffold, the 3D microfibrous structure of PLLA scaffold was fabricated by wetspinning techniques with high pore size. Usefulness of PLLA scaffold to mimick the extracellular matrix has been proven, in terms of therapeutic application by efficiently promoting the cell differentiation in injury model such as bone defects [27,28]. Hydrogel, matrigel, and PLLA scaffolds have various advantages and limitations for biomedical application (Table 1). By considering individual characteristics of each scaffold, the optimal condition of scaffold-based cell therapy can be determined for better therapeutic effect in a variety of disease models.

Our result showed constant increase in proliferation of F3-effluc cells within the hydrogel, matrigel, and PLLA scaffold in 96 well plate at 4 days after cells were mixed with individual scaffolds. Phase contrast microscopy or SEM results revealed that well established cell seeding techniques allowed cells to stably adhere to each scaffold and to robustly proliferate within scaffold. We successfully monitored the viability and proliferation pattern of implanted neural stem cells within individual scaffolds, and compared how long implanted cells on different types of scaffolds survive in motor cortex removed rat brain model. When F3-effluc/scaffolds were transplanted into resected motor cortex area in rat brain, significantly prolonged viability of implanted neural stem cells within matrigel or PLLA scaffold was found until 10 days. Matrigel containing many types of growth factor would promote the cell proliferation in injured brain tissue. PLLA scaffold comprised of microfibrous structure may also provide implanted cells with proper microenvironment in the ablated brain region after cells are stably adhered onto microfiber network. In contrast, cell-only implantation group exhibited rapid decrease in cell survival although the immunosuppressor was daily injected. The decreased cell survival in cell only implantation group would be possibly due to the necrotic or inflammatory response created from ablated brain cortex. In hydrogel-encapsulating cell group, although cell survival sustained more than

cell only group, there were decreased survival from 6 days compared to other scaffolds. We speculated that soft type of 1800 Pa hydrogel that has biodegradation property may rapidly and gradually degraded, and cell survival would be also decreased in injured cortex area. Interestingly, in cell/hydrogel implantation group, several implanted F3-effluc cells were infiltrated into host brain tissue, compared to cell-only implantation group. We also confirmed the highly expressed early neural marker of scaffold-coated transplanted stem cells, which demonstrated that the implanted F3-effluc cells within all of scaffolds were enough to induce neuronal lineage in resected motor cortex area. In the case of functional recovery study based on behavioral test, the motor cortex-ablated rat tends to relatively show fast natural recovery pattern even in normal rat, and it would be difficult to explain the precise recovery by the effect of each scaffold. Therefore, in this study, we did not include the behavioral evaluation study for therapeutic effect in the rat bearing the implanted neural stem cells encapsulated within each scaffold.

In the study regarding the treatment of traumatic brain injury, biomimetic gel types of scaffolds could play a critical role as an ideal biomaterial, providing numerous advantages. The function of the gel type scaffold which can reduce the spread of stem cells from the injection site in the early period is to support the maintenance of strong

interaction between the implanted stem cells themselves. In addition, the gel type scaffold incorporating the given cells can make the injured brain space filled up completely. In the case of microfibrous PLLA scaffold, this scaffold is suited for treatment of the externally injured brain tissue model owing to appropriate structure of the sold type of scaffold suitable for inserting into damaged space of brain tissue.

Biocompatible scaffold-based stem cell therapy is becoming important therapeutic strategy because the engineered scaffold can control the implanted stem cell self-renewal or cell fate specification [17]. Various types of bioscaffolds have been intensively designed for preclinical and clinical application. In this situation, our image-guided approach will help to evaluate the supportive function of the developed bioscaffolds in various diseases.

Ⅴ. REFERENCE

1. Martínez-Morales PL, Revilla A, Ocaña I, González C, Sainz P, McGuire D, et al. Progress in stem cell therapy for major human neurological disorders. Stem Cell Rev. 2013;9:685-99. 2. Politis M, Lindvall O. Clinical application of stem cell therapy in Parkinson's disease.

BMC Med. 2012;4:10:1.

3. Richardson RM, Singh A, Sun D, Fillmore HL, Dietrich DW, Bullock MR. Stem cell biology in traumatic brain injury: effects of injury and strategies for repair. J Neurosurg. 2010;112:1125-38.

4. Jain KK. Cell therapy for CNS trauma. Mol Biotechnol. 2009;42:367-76.

5. Stefano Pluchino, Neural stem cells and their use as therapeutic tool in neurological disorders, Brain Research 2005;48:211-9.

6. Karam JP, Muscari C, Montero-Menei CN. Combining adult stem cells and polymeric devices for tissue engineering in infarcted myocardium. Biomaterials. 2012;33:5683-95.

7. Subramanian A, Krishnan UM, Sethuraman S. Development of biomaterial scaffold for nerve tissue engineering: Biomaterial mediated neural regeneration, Journal of Biomedical Science 2009;16:108.

8. Tam RY, Fuehrmann T, Mitrousis N, Shoichet MS. Regenerative Therapies for Central Nervous System Diseases: a Biomaterials Approach. Neuropsychopharmacology. 2013 E-pub. 9. Delcroix GJ, Schiller PC, Benoit JP, Montero-Menei CN. Adult cell therapy for brain neuronal damages and the role of tissue engineering. Biomaterials. 2010;31:2105-20.

10. Galler KM, Cavender AC, Koeklue U, Suggs LJ, Schmalz G, D'Souza RN. Bioengineering of dental stem cells in a PEGylated fibrin gel. Regen Med. 2011;6:191-200.

11. Guazzini S, Petrini M. Trombi L, D'Alessandro D, Pacini S, Fiorentino B, et al. Good manufacturing practice-grade fibrin gel is useful as a scaffold for human mesenchymal stromal cells and supports in vitro osteogenic differentiation. Transfusion. 2008;48:2246-51.

12. Yang F, Murugan R, Wang S, Ramakrishna S. Electrospinning of nano/micro scale poly(L-lactic acid) aligned fibers and their potential in neural tissue engineering, Biomaterials 2005;26:2603-10.

13. Jung MR, Shim IK, Kim ES, Park YJ, Yang YI, Lee SK, et al. Controlled release of cell-permeable gene complex from poly(L-lactide) scaffold for enhanced stem cell tissue engineering, J Control Release. 2011;152:294-302.

14. Lin CC, Anseth KS. PEG hydrogels for the controlled release of biomolecules in regenerative medicine.Pharm Res. 2009;26:631-43.

15. Jabbari E. Bioconjugation of hydrogels for tissue engineering. Curr Opin Biotechnol. 2011;22:655-60.

16. Pakulska MM, Ballios BG, Shoichet MS. Injectable hydrogels for central nervous system therapy. Biomed Mater. 2012;7:024101.

17. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell. 2006;126:677-89.

18. Benton G, Kleinman HK, George J, Arnaoutova I. Multiple uses of basement membrane-like matrix (BME/Matrigel) in vitro and in vivo with cancer cells. Int J Cancer. 2011;128:1751-7.

19. Dickinson LE, Kusuma S, Gerecht S. Reconstructing the differentiation niche of embryonic stem cells using biomaterials. Macromol Biosci. 2011;11:36-49.

20. Izal I, Aranda P, Sanz-Ramos P, Ripalda P, Mora G, Granero-Moltó F, et al. Culture of human bone marrow-derived mesenchymal stem cells on of poly(L-lactic acid) scaffolds: potential application for the tissue engineering of cartilage. Knee Surg Sports Traumatol Arthrosc. 2013;21:1737-50.

21. Lu H, Feng Z, Gu Z, Liu C. Growth of outgrowth endothelial cells on aligned PLLA nanofibrous scaffolds. J Mater Sci Mater Med. 2009;20:1937-44.

22. KM Park, KS Ko, YK Joung, H Shin, KD Park. In situ cross-linkable gelatin poly(ethy lene glycol)−tyramine hydrogel viaenzyme-

mediated reaction for tissue regenerative medicine. J. Mater. Chem., 2011;21:13180-87.

23. De Ryck M, Van Reempts J, Borgers M, Wauquier A, Janssen PA. Photochemical stroke model: flunarizine prevents sensorimotor deficits after neocortical infarcts in rats, Stroke. 1989;20:1383-1390.

24. De Ryck M, Van Reempts J, Duytschaever H, Van Deuren B, Clincke G. Neocortical localization of tactile/proprioceptive limb placing reactions in the rat, Brain Research 1992;573:44–60.

25. Im HJ, Hwang do W, Lee HK, Jang J, Lee S, Youn H, et al. In Vivo Visualization and Monitoring of Viable Neural Stem Cells Using Noninvasive Bioluminescence Imaging in the 6-Hydroxydopamine-Induced Mouse Model of Parkinson Disease, Molecular Imaging, 2013;12:224-34.

26. Wang Y, Cooke MJ, Morshead CM, Shoichet MS. Hydrogel delivery of erythropoietin to the brain for endogenous stem cell stimulation after stroke injury. Biomaterials. 2012;33:2681-92.

27. Ghaedi M, Soleimani M, Shabani I, Duan Y, Lotfi AS. Hepatic differentiation from human mesenchymal stem cells on a novel nanofiber scaffold. Cell Mol Biol Lett. 2012;17:89-106.

28. Schofer MD, Roessler PP, Schaefer J, Theisen C, Schlimme S, Heverhagen JT, Voelker M, Dersch R, Agarwal S, Fuchs-Winkelmann S, Paletta JR. Electrospun PLLA nanofiber scaffolds and their use in

combination with BMP-2 for reconstruction of bone defects. PLoS One. 2011;6:e25462.1- e25462.9.

국문초록

목적: 외상성 뇌 질환은 외부의 충격으로 뇌를 이루는 세포가 손상되기 때문에 세포치료를 기반으로 한 연구가 활발히 진행 중이다. 하지만 이식줄기세포의 낮은 생존률과 증식률은 치료 효율을 떨어뜨리는 핵심적 요소가 되고 있다. 현재 이식세포의 생존률을 높이기 위해서 생체적합성 지지체를 이용한 세포치료의 연구도 활발히 이루어지고 있다. 본 연구에서는 널리 알려진 생체 적합성 지지체중 하이드로젤, 메트리젤, PLLA 지지체내 이입된 줄기세포의 생존률을 외상성 뇌질환 모델에서 광학 영상기술을 이용하여 비교하였다.

연구방법: Luciferase발현 레트로바이러스가 도입한 인간유래 신경줄기세포를 확립하고 메트리젤, 하이드로젤, PLLA 지지체와 섞은 후 신경줄기세포의 생존률을 날짜에 따라 확인하였다. HRP효소를 기반으로 젤화가 진행되는 하이드로젤, 온도 민감성인 메트리젤, 마이크로fiber로 구성된 PLLA지지체를 준비하였다. PLLA 지지체는 세포이입 24시간전에 70% 에탄올에 담궈 소독시킨 상태에서 PBS로 씻어낸 후 사용하였다. 운동영역을 담당하는 대뇌피질이 제거된 랫드 동물 모델을 확립하였고, 쥐의 뇌 운동영역은 브레그마(bragma)를 중심으로 세 좌표 (A= +4, +1, B= −2, + 1 C= +4, +6)로 이뤄진 삼각 좌표의 형태로 대뇌 피질을 절개하였다. 운동영역 뇌 손상 동물모델의 확립을 평가하기 위해 행동평가 (limb place ment test)를 수행하여 동물 모델이 적절하게 만들어 졌는지를 확인하였다. 면역 억제제를 매일 복강 내 주사하여 면역활성이

유도되지 않도록 하였다. 생체 내 영상은 IVIS-100 광학 영상 장비를 이용하여 영상을 획득하였다.

결과: *in vitro* 결과에서는 F3-effluc세포 수 및 날짜에 따라 luciferase활성이 증가하였다. F3-effluc세포와 하이드로젤, 메트리젤, PLLA와 반응시킨 후 24 well 플레이트에 loading 하였을 때, 12일까지 세포의 증식이 점점 증가하였다. 특히, 세포/메트리젤, 세포/PLLA그룹에서 높은 증식률을 관찰할 수 있었다. 외상 성 뇌 질환 동물 모델에서 비정상적인 운동행동 장애를 관찰할 수 있었다. 확립된 동물 외상성 모델을 이용하여 준비된 세포그룹, 세포/하이드로젤, 세포/메트리젤, 세포/PLLA지지체를 이식하여 10일동안 luciferase영상을 확인하였을 때 메트리젤과 PLLA지지체내 이입된 F3-effluc세포가 날짜가 지남에 따라 점점 증가하였고 10일째 가장 강한 luciferse활성을 관찰할 수 있었다. 반면 세포만 이식한 그룹에서는 8일째 영상이 관찰되지 않았고, hydrogel그룹에서는 8일째부터 영상이 감소하기 시작하였다. 뇌를 적출하여 신경줄기세포 마커인 nestin과 신경분화마커인 Tuj-1에 대한 항체를 이용하여 면역염색을 실시하였다. 메트리젤, PLLA내 이입된 많은 수의 F3-effluc세포가 nestin 양성으로 확인되었고, 다른 일부는 신경세포로 분화가 되는 것을 관찰할 수 있었다.

결론: 이번 연구에서는 세가지 대표적 생체적합성 지지체인 하이드로젤, 메트리젤, PLLA 각각의 지지체내에 이입된 신경줄기세포의 생존 및 증식률을 광학분자영 상기술을 이용하여 뇌

손상 모델에서 지지체의 지지효능을 평가하였다. 이번 연구는 세포 치료 분야에서 유망하게 쓰여질 대표적인 지지체들의 효능을 동물질환모델에서 직접 평가함으로서 차후 다양한 지지체후보군들을 뇌손상치료에 응용하는데 도움을 줄 것이라 예상한다.