Co-transplantation of neural stem cell with endothelial cell for improving of the environment of the injured spinal cord

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ABSTRACT

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The vessel disruption caused by physical impact of spinal cord injury leads to ischemic environment. This condition directly affects to reduce the survival rate of transplanted stem cells, consequentially decreasing the effectiveness of stem cell therapy. In this study, we investigated the affinity between angiogenesis and survival of transplanted neural stem cells in a spinal cord injury model. Hypoxia-specific luciferase-expressing neural stem cells (EpoSV-Luc NSC) were used as a detection system of hypoxic condition caused by spinal cord injury. *In vitro,* co-culture of endothelial cells (EC) improved the survival of neural stem cells and also decreased apoptosis caused by oxidative stress. *In vivo* angiogenesis by co-transplantation of endothelial cells quickly recovered ischemic environment caused by spinal cord injury. As a result, co-transplantation of endothelial cells improved the survival of neural stem cells transplanted into the injured spinal cord.

Key words: spinal cord injury, co-culture, co-transplantation, endothelial cells, hypoxia-specific gene expressing neural stem cells

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I. INTRODUCTION

Spinal cord injury (SCI) gives rise to the disruption of blood vessels, demyelination, axonal degeneration, and scar tissue formation[1]. Recently, therapeutic strategies involving stem cell grafts have emerged as a powerful and promising tool to enhance restoration of lost function [2,3]. However, survival of integrated cells decreases with time after transplantation [4]. The vessel disruption caused by physical impact of spinal cord injury leads to ischemic environment. This condition directly affects to reduce the survival rate of transplanted stem cells [5]. Thus, the formation of new blood vessels is a critical component of the wound healing process [6].

To improve angiogenesis or survival of transplanted cells, a variety of strategies such as co-transplantation or combined therapy with stem cells and therapeutic

genes have been performed in variety disease models. It has been reported that co-culture of endothelial cells with neural stem cells expands neurogenesis of neural stem cells *in vitro*[7], and co-transplantation of endothelial cells supports survival, proliferation, and differentiation of neural stem cells in a cerebral infarction model [8]. However, it is unclear whether the oxygen supply provided by transplantation of endothelial cells directly affects cell survival.

The hypoxia-inducible gene expression system was first introduced as a tool for minimizing side effects caused by the over-expression of genes in normal tissue [9]. In a previous study, we established stable neural stem cell lines (EpoSV-Luc NSCs) expressing hypoxia-inducible genes [10]. In addition, we confirmed that SCI leads to hypoxia, and expression of EpoSV-Luc NSCs show consistent hypoxia-specific gene expression *in vitro* as well as in a spinal cord injury model. Therefore, this system can be used as a detection tool for hypoxia.

In this study, we investigated whether co-transplantation of endothelial cells (EC) improves angiogenesis and if the oxygen supply provided by co-transplantation of ECs increased survival of EpoSV-Luc NSCs. Consequentially, the therapeutic efficacy of stem cell therapy may be improved by increasing cell survival.

II. MATERIALS AND METHODS

Cell culture

Endothelial cells (Human Umbilical Vein Endothelial Cells, HUVEC) were purchased from the American Type Culture Collection (ATCC) and cultured in EGM-2 complete media (Lonza) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed twice a week. Mouse neural stem cells (NSCs) were purchased from ATCC (CRL-2925) and cultured in Dulbecco's-modified Eagle's medium (DMEM/F12, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (100 unit/mL, Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Establishment of stable neural stem cell lines expressing hypoxia-inducible luciferase

The construction of pEpoSV-Luc and establishment of stable cell lines were described previously [10]. EpoSV-Luc plasmid was transfected into NSCs using lipofectamine 2000 (Invitrogen). Transfectants were selected by treatment with Zeocin (200ug/ml) every 2 days. Stable NSC lines expressing intense DsRed were expanded under normoxic or hypoxic conditions for screening.

Co-culture of endothelial cells with stable neural stem cell line expressing hypoxia-inducible luciferase

We confirmed whether co-culture with ECs had an effect on proliferation and survival of EpoSV-Luc NSCs. Briefly, ECs ($6x10^5$ cells) were seeded on 6-well plates and then incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours, EpoSV-Luc NSCs ($3x10^5$ cells) were seeded on 6-well plates with or without an EC monolayer. The next day, cells were incubated under *in vitro* injury conditions (serum starvation and 500uM hydrogen peroxide) for 24 hours.

Flow cytometry

Endothelial cells were confirmed by positive immunostaining for von Willebrand factor (vWF). To confirm whether co-culture with ECs can prevent apoptosis of NSCs under *in vitro* injury, staining using the ApoScan ™ Annexin-V FITC apoptosis detection kit (BioBud, Seoul, Korea) was performed. In brief, cells were harvested after injury using trypsin/EDTA, washed with ice cold PBS, and resuspended in 1X binding buffer. Annexin-V solution (250ng/ul) was added to the cell suspension, and the mixture was incubated for 15 minutes in the dark at room temperature. The stained cells were then washed with ice-cold PBS and analyzed using a FACS Caliber cell sorter and Cell Quest software (Becton-Dickson, Mountain View, CA, USA). Approximately $1x10^4$ cells were analyzed for each sample.

Luciferase imaging

The *In Vivo* Imaging System (IVIS) was used to confirm whether EpoSV-Luc NSCs showed hypoxia- specific luciferase expression. Briefly, EpoSV-Luc NSCs were seeded on multi-well plates and then incubated at 37°C in a humidified atmosphere containing 5% CO_2. The next day, EpoSV-Luc NSCs were maintained under normoxia or hypoxia for 24 hours, and luciferase expression was analyzed. In a different set of experiments, EpoSV-Luc NSCs were maintained under normoxia for 24 hours, transferred to hypoxia for 24 hours, and then returned back to normoxia for 24 hours. Luciferase imaging was performed at each time point.

RT-PCR

Total RNA of NSCs cultured under normoxia or hypoxia was extracted using a RNA extraction kit (QIAGEN, Valencia, CA, USA). After synthesis of cDNA, RT-PCR was performed with forward (5'-CAAATCATTCCGGATACTGCG-3') and reverse (5'-GAATTACACGGCGATCTTTCC-3') primers for luciferase. DNA was amplified by the following steps: 94℃ for 5 minutes; 30 cycles of 94℃ for 30 seconds, 51℃ for 30 seconds, 72℃ for 30 seconds; and extension at 72℃ for 10 minutes.

Western blot assay

After quantitative analysis of protein using the BCA assay, samples were electrophoresed on 8~12% SDS-PAGE gels and transferred to PVDF membranes at 100 V for 90 minutes. Blocking was performed with 3% BSA in PBS containing 0.2% Tween 20 for 1 hour at room temperature. The blots were probed with anti-luciferase (1:2000, Millipore), vWF (1:2000, Abcam), VEGF (1:4000, Millipore), and anti- beta actin (1:10000, Abcam) antibodies. Bands were visualized with suitable horseradish peroxidase-conjugated and ECL Western blotting detection reagents. For quantitative analysis, band intensity was measured with Image J software.

Spinal cord injury and cell transplantation

Adult male Sprague-Dawley rats (250-300 g, Orient Bio, Gyeonggi-do, Korea) were used for the spinal cord injury (SCI) model. All protocols were approved by the Animal Care and Use Committee of Yonsei University College of Medicine. All experiments were performed according to international guidelines on the ethical use of animals, and the number of animals used was minimized. Animals were anesthetized by intraperitoneal injection with Zoletil 50 (10 mg per rat, Virbac, France). After anesthesia, laminectomy was performed at the T9 level. The spinal cord was injured by clip compression for 10 minutes. Experimental animals were divided into four groups as follows: group 1, PBS control (PBS/5ul); group 2, EC ($6x10^5$ cells/3ul) + (PBS/3ul); group 3,

EpoSV-Luc NSCs ($3x10^5$ cells/2ul) + (PBS/3ul); group 4, EpoSV-Luc NSCs ($3x10^5$ cells/2ul) + EC ($6x10^5$ cells/3ul). Cells were injected into the injury epicenter using a 26-gauge Hamilton syringe. Cyclosporine (10mg/kg) was administered to all animals after cell transplantation. Open field locomotor testing was performed using the Basso, Beattie, and Bresnaham (BBB) locomotor scale every week for 5 weeks. Each animal walked freely on testing board, two researchers observed the hind limb movement.

Tissue extraction and immunofluorescence staining

To obtain spinal cord tissue, all rats were sacrificed by heart perfusion with saline (pH 7.4) and 4% paraformaldehyde (PFA, Merck, Germany). After post-fixation in 4% PFA for 24 hours, samples were incubated in 30% sucrose until they sank to the bottom of the vessel. After freezing with OCT compound, tissues were cut into 20-μm-thick sections. Samples were fixed in 4% PFA for 10 minutes at room temperature. After washing with ice-cold PBS, samples were incubated with 0.5% Triton X-100 (Invitrogen, Carlsbad, CA, USA) in PBS for 15 minutes at room temperature. Samples were blocked with normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS for 1 hour at room temperature and incubated with primary antibody as follows: anti-vWF (1:250, Abcam) for 1 hour. After incubation, samples were washed with PBS and incubated with secondary antibody conjugated to fluorescent dye (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour at room

temperature. Samples were washed three times with ice-cold PBS and then covered with a cover slip after drops of Vectashield mounting medium containing 4',6'-diamino-2-phenylindole (DAPI, Vector, Burlingame, CA) were added. Samples were analyzed using an Olympus BX51 fluorescence microscope (Olympus, Japan) and a laser confocal microscope (LSM 700, ZEISS).

Optical density analysis

To measure DsRed or immunostaining-positive pixels, optical density analysis was performed. After imaging using the Olympus BX51 fluorescence microscope (Olympus, Japan) on equal magnification and fluorescence intensity, the optical density of DsRed or immunostaining-positive pixels was measured with Metamorph software.

Statistical analysis

Student's t-test was used to compare data between the two experimental groups. One-way ANOVA was used to compare data among the three experimental groups. Data are expressed as mean ± SD or SE (behavior test). A p-value less than 0.05 was considered statistically significant.

III. RESULTS

Characterization of neural stem cells expressing hypoxia-inducible luciferase

To confirm that hypoxia-inducible luciferase-expressing neural stem cells (EpoSV-Luc NSCs) show hypoxia-specific expression of luciferase, we performed IVIS imaging, RT-PCR, and Western blotting. Luciferase expression in EpoSV-Luc NSCs under hypoxia steadily increased over time. In contrast, EpoSV-Luc NSCs under normoxia maintained low luciferase expression. As a result, luciferase expression in EpoSV-Luc NSCs was clearly changed after 24 hours (Fig. 1A). In addition, the high expression of luciferase in EpoSV-Luc NSCs decreased when EpoSV-Luc NSCs were transferred back from hypoxia to normoxia (Fig. 1B). This *in vitro* result indicates that luciferase expression will also decrease *in vivo* when conditions change from hypoxic to normoxic. Therefore, we suggest that our hypoxia-specific luciferase expressing NSCs can be used as a tool for oxygen sensing.

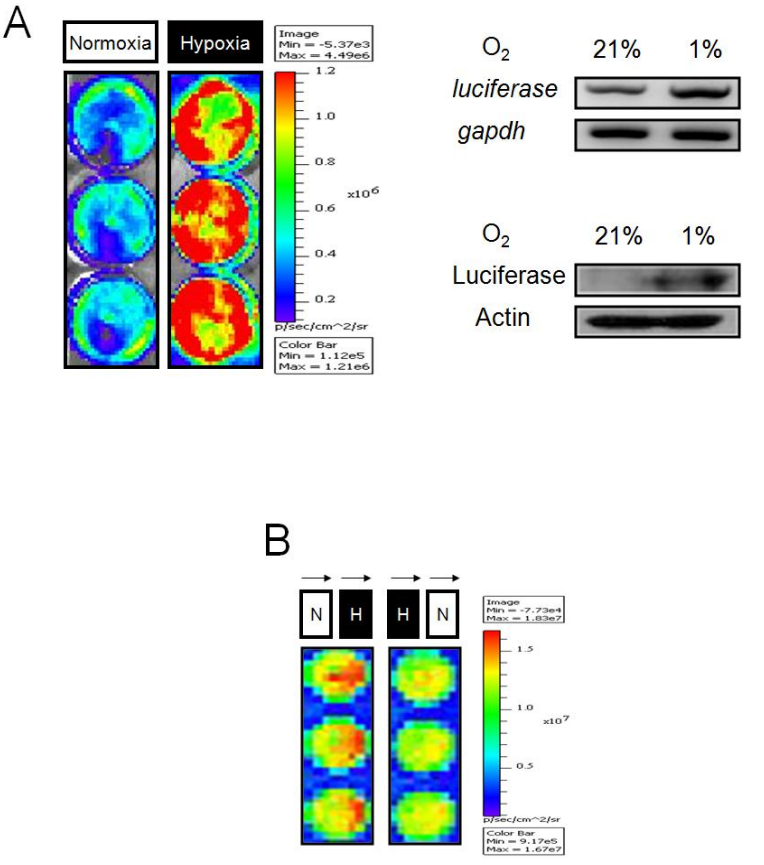


Figure 1 Characterization of hypoxia-inducible luciferase-expressing neural stem cells (A) Hypoxia-specific luciferase expression in EpoSV-Luc NSCs was confirmed by luciferase imaging, RT-PCR, and Western blotting. Luciferase expression in EpoSV-Luc NSCs was clearly different after 24 hours under hypoxia compared with normoxia. (B) Luciferase expression in EpoSV-Luc NSCs was increased under hypoxia. However, the high expression of luciferase under hypoxia was reduced by transition from hypoxia to normoxia.

Characterization of endothelial cells

Endothelial cells were characterized by immunofluorescence staining with von Willebrand factor (vWF) and flow cytometry. Most of the ECs stained positive for vWF (Fig. 2A), and 89% were positive for vWF by flow cytometry (Fig. 2B). Thus, the majority of ECs expressed this characteristic endothelial cell marker. VEGF expression in ECs was also investigated by Western blotting. VEGF was highly expressed in ECs under hypoxia compared with normoxia (Fig. 2C). This *in vitro* result suggests that ECs may also express higher levels of VEGF after injection into an injured spinal cord in a hypoxic environment.

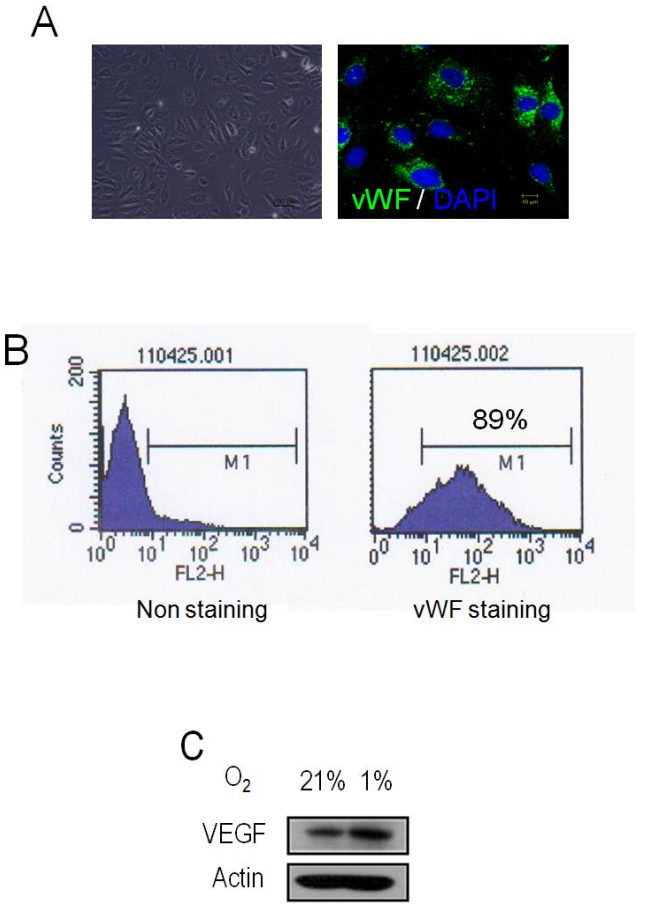


Figure 2 Characterization of endothelial cells Characterization of ECs was confirmed by immunostaining (A) and flow cytometry (B) with von Willebrand factor (vWF). Most of the ECs were positive for vWF. (C) VEGF expression in ECs showed a greater increase under hypoxia compared with normoxia. Scale bar = 100 μm (regular microscopy) and10 μm (fluorescence microscopy).

Co-culture with endothelial cells improves survival of neural stem cells

To confirm whether co-culture with ECs improves the survival of NSCs, NSCs were cultured with or without ECs in serum-starvation media containing hydrogen peroxide (500uM) under normoxia or hypoxia for 24 hours. The NSCs were then stained with Annexin-V (apoptosis marker), and optical density analysis and flow cytometry were performed. The optical density of EpoSV-Luc NSCs expressing DsRed significantly increased in co-cultures of NSCs and ECs compared with NSC cultures alone (Fig. 3B). In addition, the number of Annexin-V-positive NSCs was decreased in co-cultures of NSCs and ECs (Fig. 3C). These results indicate that co-culture with ECs improves the survival of NSCs.

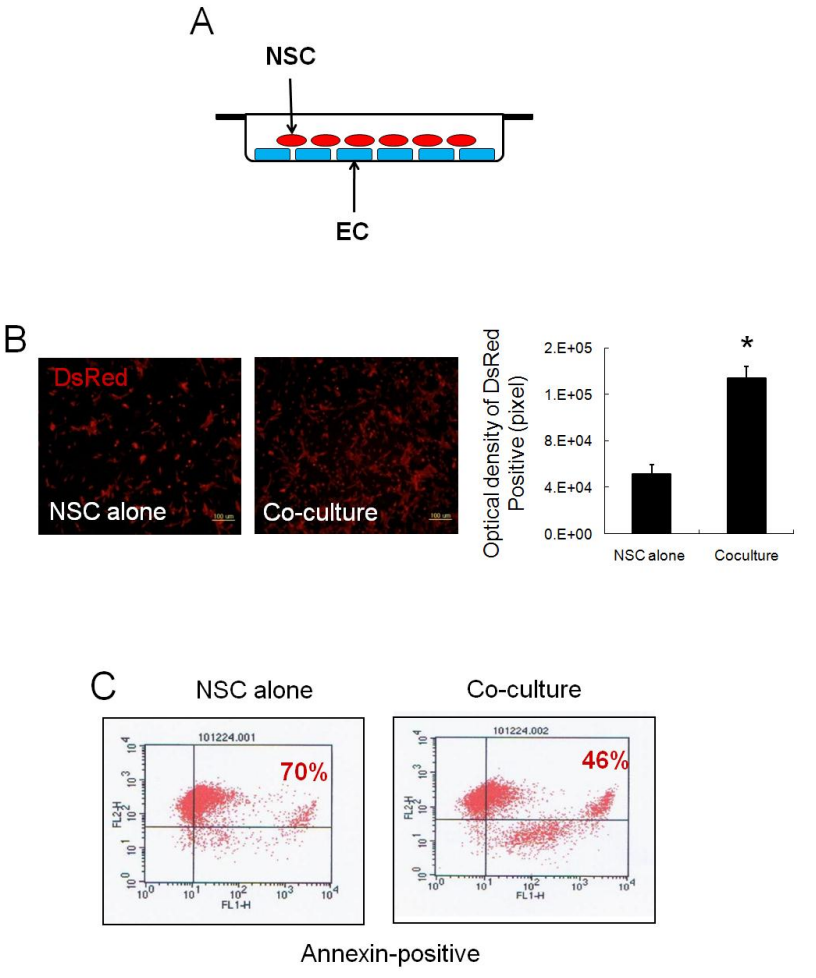


Figure 3. Co-culture with endothelial cells improves the survival of neural stem cells (A) The illustration of co-culture with EC and NSC (B) The optical density of DsRed-expressing NSCs was significantly increased in the co-culture group. (C) In flow cytometry analysis with Annexin-V, the number of NSCs positive for Annexin-V was decreased in the co-culture group. *: significant difference between each group ($p < 0.05$). Data are shown as mean ± SEM.

Spinal cord injury and loss of blood vessels

To confirm whether spinal cord injury (SCI) leads to disruption of blood vessels, fluorescence staining and Western blotting with vWF antibody were performed in injured spinal cord. In fluorescence staining, vWF-positive vessels were observed in normal spinal cord tissue. The vWF-positive vessels were severely destroyed in injured spinal cord tissue (Fig. 4A). To confirm changes in vWF and angiogenic factor expression after spinal cord injury, the expression patterns of vWF and VEGF, which have been established as angiogenic factors, were investigated in normal and injured spinal cord at 1 and 2 weeks post-injury. In Western blot analysis, the expression of vWF was increased in normal spinal cord tissues and gradually decreased in injured spinal cord tissue. The expression of VEGF was also decreased in injured spinal cord tissue (Fig. 4B). EC transplantation was performed to confirm whether transplantation of ECs increases angiogenesis in a spinal cord injury model. In the EC transplanted group, vWF expression was significantly increased, and VEGF expression was also increased at 1 and 2 weeks post- transplantation (Fig. 4C). These results indicate that EC transplantation will improve angiogenesis in spinal cord injury.

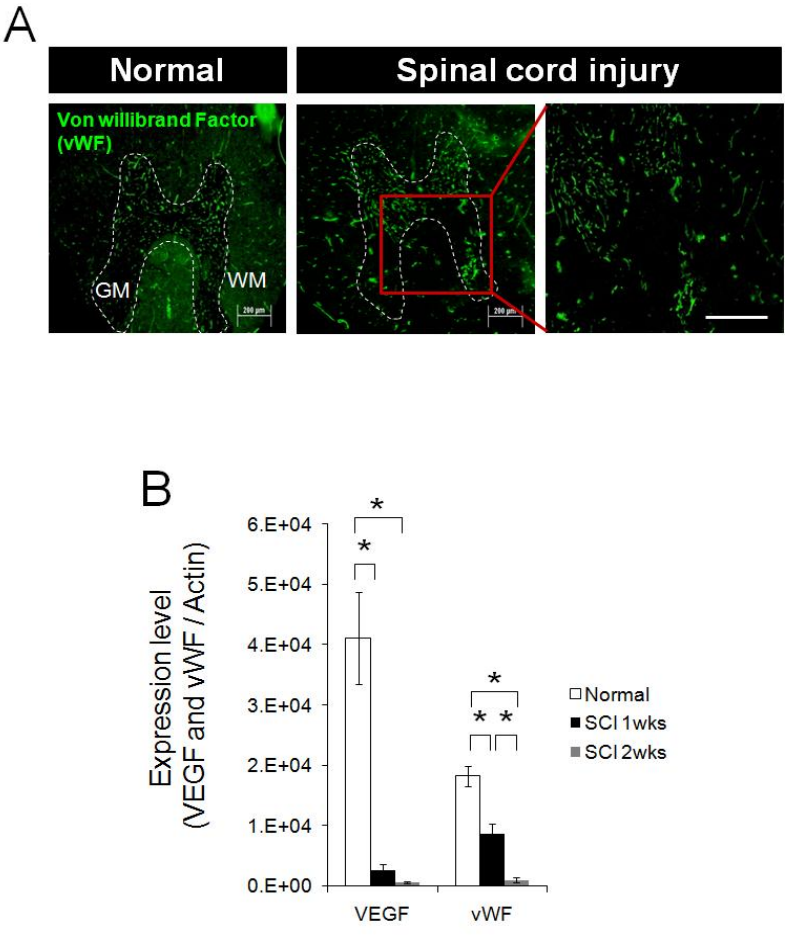
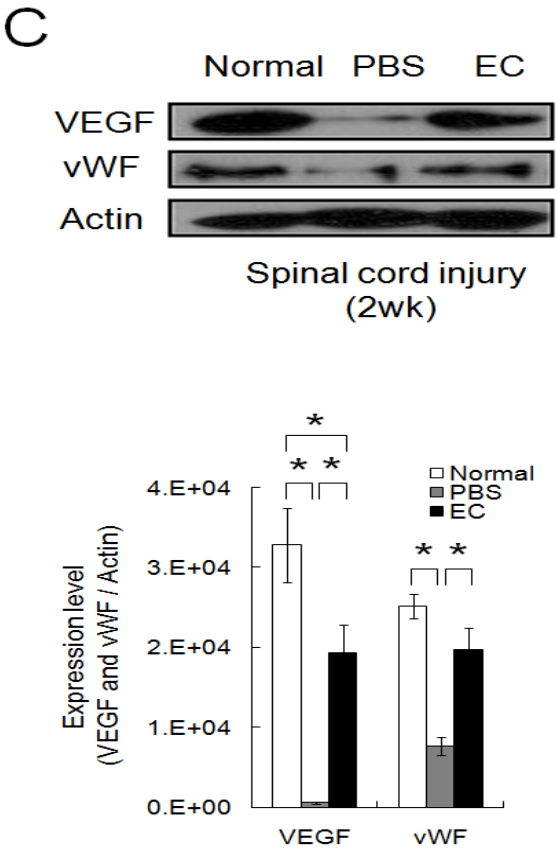


Figure 4. Spinal cord injury and loss of blood vessels (A and B) Spinal cord injury leads to disruption of vWF-positive vessels. Both VEGF and vWF expression patterns were significantly decreased in injured spinal cord tissue.



(C) After spinal cord injury, EC transplantation significantly increased VEGF and vWF expression. Scale bar = 200 μm. *: significant difference between each group ($p < 0.05$). Data are shown as mean ± SEM.

Neural stem cell survival and Angiogenesis

We investigated whether improved angiogenesis by co-transplantation increases the survival of transplanted NSCs at 1 and 2 weeks post-transplantation. The numbers of transplanted NSCs and vWF-positive pixels were highly increased in the co-transplanted group (Fig. 5A). The optical densities of DsRed and vWF-positive pixels were also significantly increased in the co-transplanted group compared with the NSC-transplanted group (Fig. 5B). These results indicate that co-transplantation with ECs improves new vessel formation as well as survival of NSCs.

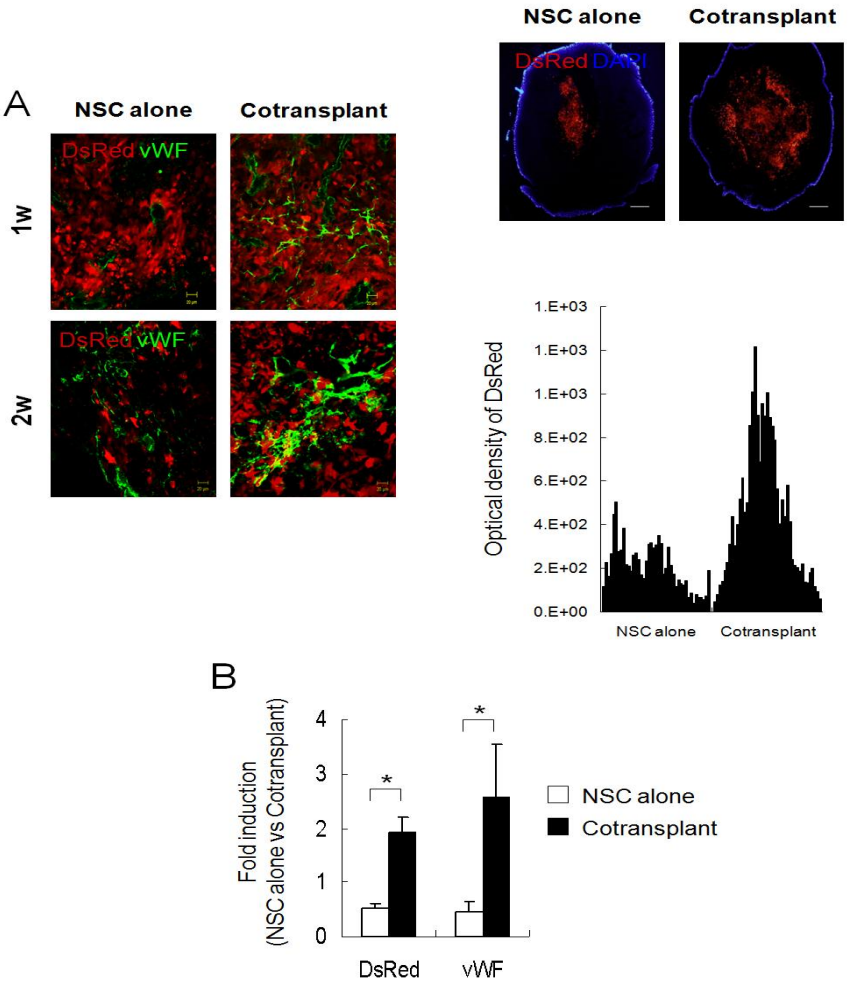


Figure 5 Neural stem cell survival and Angiogenesis (A) The numbers of von Willebrand factor (vWF) positive vessels and DsRed-tagged NSCs were increased in the co-transplanted group. (B) The optical densities of vWF-positive and DsRed-expressing NSCs were significantly increased in the co-transplanted group. Data are shown as mean ± SEM. Scale bar = 20 μm (DsRed, vWF), 200 μm (DsRed, DAPI)

Luciferase expression in spinal cord

To confirm whether high VEGF and vWF expression after EC transplantation improves angiogenesis, the level of luciferase expression in EpoSV-Luc NSCs transplanted into injured spinal cord was investigated at 1 and 2 weeks post-transplantation (high luciferase expression represented hypoxia). Luciferase expression was significantly decreased in the co-transplanted group compared with the NSC-transplanted group (Fig. 6). This result indicates that co-transplantation with ECs not only improves angiogenesis after spinal cord injury, but also changes the hypoxic environment caused by spinal cord injury in normoxic conditions.

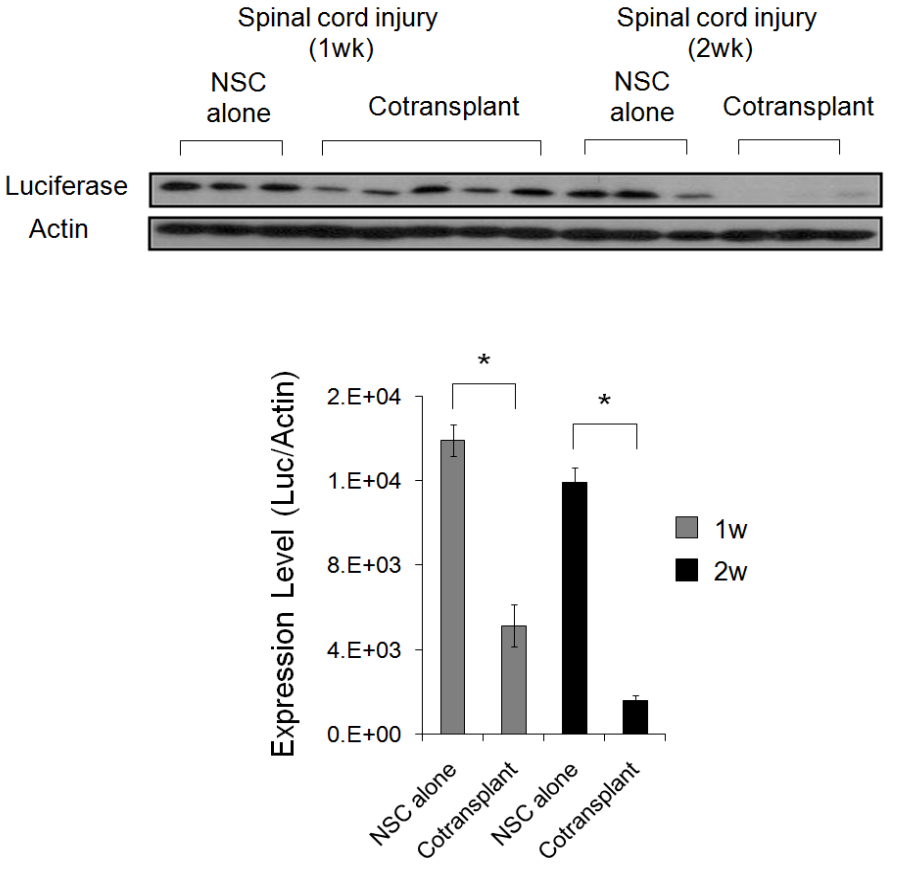


Figure 6 Luciferase expression in spinal cord Low luciferase expression indicates that that the tissue environment was recovered from hypoxic to normoxic conditions. *: significant difference between each group ($p < 0.05$). Data are shown as mean ± SEM.

Co-transplantation of endothelial cells with neural stem cells improve the functional recovery

Open field locomotor testing was performed for 5 weeks after transplantation (Fig. 7). Functional recovery was significantly improved in the cell-transplanted groups compared with the PBS control group. The functional recovery was significantly improved in co-transplanted group compare with NSC-transplanted group.

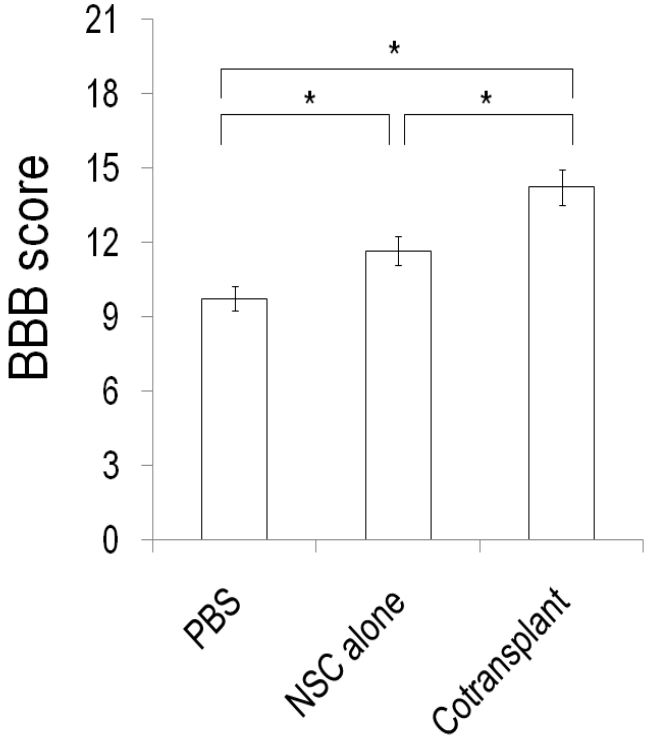


Figure 7 Co-transplantation of endothelial cells with neural stem cells improves functional recovery Functional recovery of the co-transplanted group was significantly improved compared with that of the NSC-transplanted group at 5 weeks post-transplantation. *: significant difference between each group ($p < 0.05$).

IV. DISCUSSION

In this study, we investigated that angiogenesis by co-transplantation of endothelial cells (EC) recovers tissue hypoxia caused by spinal cord injury. Further we examined that hypoxia-inducible luciferase expression system capable of detecting the hypoxic environment.

The endothelial cells used in this study were strongly positive for the EC marker vWF. In addition, transplantation of ECs increased the expression of VEGF, a known angiogenic factor, as well as vWF. However, it is not surprising that transplantation of ECs increased vWF expression since the ECs were strongly positive for vWF in the characterization experiment (Fig. 2). Therefore, we needed to investigate whether functional angiogenesis was improved by the transplanted ECs.

It is known that endothelial cells affect the self-renewal of neural stem cells *in vitro* [7]. In addition, it has been reported that co-transplantation of endothelial cells improves angiogenesis as well as the survival of neural progenitor cells in cerebral infarction and spinal cord injury models[8,11]. However, it is unclear whether the oxygen supply originated from the transplanted endothelial cells. In a previous study, we established hypoxia-specific luciferase-expressing neural stem cells (EpoSV-Luc NSCs) and confirmed that EpoSV-Luc NSCs

consistently show hypoxia-specific luciferase expression *in vitro* as well as *in vivo* [10]. Thus, we thought that these EpoSV-Luc NSCs could be used as a tool for detection of hypoxic environments when co-transplanted with ECs into an injured spinal cord.

In the present study, we confirmed hypoxia-specific luciferase expression in NSCs using a variety of experimental methods such as RT-PCR to detect mRNA levels and Western blotting and luciferase imaging to detect protein levels. In particular, it is interesting that high luciferase expression in EpoSV-Luc NSCs maintained under hypoxia can be reduced by transition to normoxia. This characteristic of EpoSV-Luc NSCs was useful for the experimental design of our *in vivo* study.

In the spinal cord injury model, co-transplantation of ECs significantly increased VEGF and vWF expression compared with NSCs alone. Interestingly, luciferase expression in NSCs was significantly decreased in the co-transplantation group. This indicates that the hypoxic environment caused by spinal cord injury was recovered to normoxic conditions by co-transplantation of ECs. We also investigated whether functional angiogenesis could be improved within 1 or 2 weeks post-transplantation with ECs. It has been reported that transplantation of endothelial cells improved functional angiogenesis within 1 or 2 weeks after transplantation in a variety of disease models [12-14]. Moreover, we confirmed that

the *in vitro* level of luciferase expression was dependent on the oxygen concentration; therefore, luciferase expression was not an indication of cell number. In addition, the number of surviving NSCs in an injured spinal cord was significantly increased in the co-transplantation group. Therefore, we suggest that the reduced luciferase expression in the co-transplantation group indicates normoxic recovery rather than decreasing cell number. As a result, the co-transplantation of ECs improved new vessel formation, and the hypoxic environment was rapidly recovered. Furthermore, the number of surviving NSCs was significantly increased in the vWF-positive area. Thus, the oxygen supply provided by new vessel formation might be increased by survival of NSCs in the sub- acute phase after injury.

It is known that VEGF plays a major role in a variety of functions such as neuroprotection, neurogenesis, and angiogenesis [15-23]. We confirmed that *in vitro* endothelial cells express higher levels of VEGF under hypoxia compared with normoxia; consequently, transplanted ECs may express higher amounts of VEGF in an injured spinal cord. Thus, we suggest that enhanced VEGF expression might contribute to angiogenesis.

In behavior tests, all of the cell-transplanted groups showed a significant improvement in functional recovery compared to the PBS control group. Among the cell-transplanted groups, a significant difference in functional

recovery was observed between the co-transplanted and NSC-transplanted groups. We think that the formation of new vessels by transplantation of ECs in the acute phase may have resulted in the difference in functional recovery between the co-transplanted and NSC-transplanted groups.

V. CONCLUSION

In this study, we investigated whether co-transplantation of endothelial cells improves angiogenesis and survival rate of co-transplanted neural stem cells. After spinal cord injury, the angiogenesis and oxygen supply provided by co-transplantation of endothelial cells increased the survival of NSCs transplanted into an injured spinal cord. We suggest that angiogenesis is important for recovering hypoxic environment caused by spinal cord injury, and providing an enhanced tissue environment might contribute to increased cell survival.

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ABSTRACT (IN KOREAN)

신경줄기세포와 혈관세포의 합동 이식이 척수 손상 환경의 향상에 미치는 효과

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척수손상으로 인한 혈관의 손상은 조직 허혈성 환경을 일으킨다. 이러한 허혈성 환경은 이식된 줄기세포의 생존을 감소시키며, 결과적으로 줄기세포치료의 효율을 감소시킨다. 본 연구에서는 척수손상동물에 이식된 신경줄기세포의 생존과 신생혈관 사이의 관계에 대하여 조사하였다. 척수손상으로 인해 야기된 허혈성 환경을 탐침하기 위한 도구로서 허혈성환경 특이적으로 luciferase 를 과발현하는 신경줄기세포를 사용하였다. 시험관조건에서, 혈관세포의 합동배양은 신경줄기세포의 생존을 증가시켰을 뿐만 아니라 세포사멸을 감소시켰다. 척수손상동물모델에서, 혈관세포와의 합동이식에 의한 신생혈관생성은 척수손상으로 야기된 허혈성 환경을 빠르게 회복시켰다. 결과적으로 혈관세포와의 합동이식은 손상된 척수조직으로 이식된 신경줄기세포의 생존을 향상시켰다.

핵심되는 말: 척수손상, 합동배양, 합동이식, 혈관세포, 허혈환경 특이적 유전자 발현 신경줄기세포