

rule out the possibility that inhibition of other signaling pathways contributed to the cell fate determination in our experiment.

At this point, how the down-regulation of Notch signaling is involved in the maturation of GFAP-positive astrocytes remains uncertain. However, the transient activation of Notch signaling that occurs during the commitment and/or early phase of astroglial differentiation might be sufficient to induce the transcriptional activation of the mouse GFAP gene by excluding the co-repressor N-CoR from the RBP-J complex that binds to the mouse GFAP gene promoter (Hermanson et al., 2002). Although the precise mechanism responsible for the suppressed maturation of astroglia as a result of the persistent activation of Notch signaling must be further clarified, the activation of signaling pathways other than Notch signaling (e.g., the JAK/STAT pathway) is likely to play an important role in the maturation of GFAP-positive astrocytes (Bonni et al., 1997; Nakashima et al., 1999).

Conclusions and Perspectives: the complexity of the role of Notch signaling in CNS development

In our present study, we developed new reporter systems using wild-type and RBP-J binding site-disrupted *Hes1* promoters and the fluorescent protein Venus/dVenus. Using these reporter genes, we examined the live monitoring of RBP-J-mediated Notch activation in developing mouse brain and neural stem cells. Notch activation selectively occurred in the radial glia during forebrain development, in neurosphere-initiating cells. Thus, the combination of our reporter system and gain-of/loss-of function studies clearly demonstrated the complexity of Notch signaling in CNS development, and our reporter system may be a potentially useful tool for future studies designed to elucidate the physiological roles and activation mechanisms of Notch signaling. Naturally, the *Hes1*-promoter-dependent reporter system developed in the present study would be a versatile tool for the live monitoring of in situ Notch signaling during development, stem cell maintenance, and the oncogenesis of various organs.

Acknowledgments

We are grateful to Drs. Takuya Shimazaki, Kazunobu Sawamoto, Yukiko Gotoh, and Ola Hermanson for their critical reading of the manuscript and to Hiroko Kouike for her assistance with the FACS analysis. We also thank Drs. Tasuku Honjo for providing the TP-1 luciferase plasmid, Raphael Kopan and Ryoichiro Kageyama for the *Hes1*p-luciferase plasmids, Masato Nakafuku for the Notch1 expression vectors, and Roger Tsien for the mRFP construct. This work was supported by grants to H.O. from the Japanese Ministry of Education, Culture, Sports, Science and Technology and the Japan Science and Technology Corporation (CREST) and by a grant-in-aid to Keio

University from the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.08.003.

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The FASEB Journal express article 10.1096/fj.05-4082fje. Published online September 1, 2005.

In vivo imaging of engrafted neural stem cells: its application in evaluating the optimal timing of transplantation for spinal cord injury

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ABSTRACT

Neural stem/progenitor cells (NSPCs) hold promise in neural tissue replacement therapy after spinal cord injury. However, understanding the survival time of grafted NSPCs and determining the extent of migration away from transplantation sites are essential for optimizing treatment regimens. Here, we used *in vivo* bioluminescence imaging to noninvasively assess the survival and residence time of transplanted NSPCs at the injury sites in living animals, and we used histologic analyses to assess cell integration and morphology. Third-generation lentiviral vectors enabled efficient transduction and stable expression of both luciferase and a variant of green fluorescent protein in primary cultured NSPCs. Signals from these cells were detectable for up to 10 months or more after transplantation into the injured spinal cords of C57BL/6J mice. Histological and functional data supported the imaging data and suggest that the timing of NSPC transplantation may be a key determinant of the fates and function of integrated cells since cell survival and migration depended on the time of transplantation relative to injury. Optimization of cell therapies can be greatly accelerated and refined by imaging, and the methods in the present study can be widely applied to various research fields of regeneration medicine, including transplantation study.

Key words: bioluminescence imaging • neural stem/progenitor cells • delayed transplantation

Neural stem/progenitor cells (NSPCs) are essential for the development of the central nervous system (CNS) during embryonic development as well as in the ongoing processes of neurogenesis that occur in specialized areas of the adult mammalian brain (e.g., the olfactory bulb and hippocampus) (1). The establishment of a clonogenic expansion method for NSPCs by neurosphere formation (2) enabled experimental definition of the NSPCs population and bulk harvesting of these cells in culture (3, 4). Due to their characteristic migratory ability toward sites of tissue damage, self-renewal capability, and multilineage-differentiation potential, NSPCs are considered to have significant therapeutic potential for several pathologic conditions of the CNS. Although the precise mechanism of symptomatic improvement remains to be elucidated, several studies have shown the effectiveness of NSPC transplantation in the treatment of Parkinson's disease, multiple sclerosis, brain ischemia, and trauma of CNS, including spinal cord injury (SCI) (5–11). The development of NSPC transplantation protocols for tissue regeneration and repair would benefit greatly from *in vivo* assays that can rapidly assess the extent of cell survival and localization of transplanted cells. At present, these measures rely on histological analyses and require serial sacrifices of animals. As such, real-time data pertaining to cell migration and survival over time cannot be assessed. Among the various imaging modalities that have been developed, several offer opportunities in this type of investigation. Magnetic resonance imaging (MRI) and fluorescence imaging provide means of tracking transplanted cells *in vivo* (12–14) but can be constrained by sensitivity and/or retention of the label (15–17). To circumvent the problem of signal loss due to dilution with cell division, it is desirable to integrate a reporter gene into the cells before transplantation. One such approach that has been used for cell tracking and survival studies uses cells labeled with genes encoding light-emitting enzymes and low light imaging instruments; this is referred to as *in vivo* bioluminescence imaging (BLI) (18).

BLI has been shown to be a useful tool for tumor, immune, and hematopoietic cell tracking studies (19–23). In this method, transplanted cells stably expressing luciferase can be detected *in vivo* through the tissue of live animals using ultrasensitive cooled charged-coupled device (CCD) cameras after administration of luciferin, the luciferase substrate. Since this reaction depends on ATP and oxygen, it is a metabolic indicator where only living cells release photons. However, long-term tracking of primary cultured cells, including NSPCs, has been limited by the relative difficulty in obtaining a high level of sustained reporter gene expression, including luciferase.

To address this limitation, we used a third-generation self-inactivating lentiviral vector system based on human immunodeficiency virus type 1 (HIV-1) (24). This viral vector offers safe, high titer transduction and efficient integration into cells irrespective of their state of division (25, 26). Here we used the vector to achieve efficient delivery and stable expression of both luciferase and green fluorescent protein (GFP) in primary cultured NSPCs in order to monitor transplanted cells in the injured spinal cord. To determine the optimal timing of transplantation for SCI, we transplanted transfected NSPCs at different times relative to injury and observed differences in survival, migration, and differentiation of NSPCs.

MATERIALS AND METHODS

Lentiviral vector expressing the luciferase reporter gene

The third-generation self-inactivating HIV-1-based lentivirus vector, pCS II -EF-MCS-IRES2-Venus, has been described previously (24). The vector contains elongation factor (EF)-1 α

promotor; internal ribosomal entry site (IRES) followed by *Venus*, which is a variant of GFP, an improved version of yellow fluorescent protein as described previously (27); and a woodchuck hepatitis virus posttranscriptional regulatory element (PRE). The luciferase gene fragment was excised from the pGL3-Basic vector (Promega, Madison, WI) and cloned into pCS II-EF-MCS-IRES2-Venus at the *Bam*H1 site (Fig. 1A). Twenty-four hours before transfection, 293T cells were seeded in poly-L-lysine-coated T175 flask. The cells were transfected using lipofection protocol of the FuGENE6 transfection reagent (Roche, Indianapolis, IN). Two days after transfection, the conditioned medium was collected and the virus was concentrated by centrifugation at 21,000 rpm for 2 h at 4°C. The pelleted virus was resuspended and stored frozen at -80°C. The titer of concentrated virus was 1×10^8 to 2×10^8 transducing units per milliliter (TU/ml) when assayed on 293T cells, and infectivity was determined by GFP expression as analyzed on FACS calibur (Becton-Dickinson, Franklin, Lakes, NJ).

NSPC primary cultures

The methods for the culture and expansion of NSPCs have been described previously (2). In brief, the striatum of C57BL/6J mouse on embryonic day 14 was dissociated using a fire-polished glass pipette, and the dissociated cells were then collected by centrifugation and resuspended in separate culture medium. The culture medium consists of DMEM/F12 supplemented with the hormone mixture as described previously (2), and human recombinant fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) (20 ng/ml each) were added every 2 days. The cells formed floating cell clusters (neurospheres) within 2–3 days. The concentrated viruses were added into the culture medium to infect primary NSPCs (multiplicity of infection, MOI=1.0). After being propagated with two passages, neurospheres were used for in vivo BLI or dissociated into single cells and plated onto poly-L-ornithine-coated cover slips at a density of 1×10^5 cells/ml for in vitro assay. Both the untreated NSPCs and virally transduced NSPCs were allowed to differentiate for 7 days and were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for immunohistochemistry.

SCI model

Adult female C57BL/6J mice (20–22 g) were anesthetized via an i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After laminectomy at the 10th thoracic spinal vertebrae (T10), the dorsal surface of dura matter was exposed and SCI was induced using a commercially available SCI device (IH impactor, Precision Systems and Instrumentation, Lexington, KY) as described previously (28). This device creates a reliable contusion injury by rapidly applying a force-defined impact (60 kdyn) with a stainless steel-tipped impounder. Motor function of the hind limbs was evaluated by the locomotor rating test on the Basso-Beattie-Bresnahan (BBB) scale as described previously (29, 30) until 6 wk after injury. All procedures were approved by the ethics committee of Keio University, which were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD)

Transplantation

For in vivo BLI, transplantation of lentivirally transduced NSPCs was performed by using a glass micropipette and a stereotaxic injector (KDS 310, Muromachi-kikai, Tokyo, Japan). The tip of the micropipette was inserted into the epicenter of the injured spinal cord, and 2 μ l of the suspension of NSPCs was injected at a rate of 0.5 μ l/min. The number of viable cells in the

suspensions was determined by cell counting using Trypan blue dye exclusion, and cell density was adjusted to an approximate range of 1.25×10^7 to 2.5×10^8 cells/ml to examine the correlation between cell numbers and bioluminescent signal. To examine the survival rate of NSPCs in the injured spinal cord, we performed transplantation of NSPCs (5×10^5) immediately after injury (acute transplantation, $n=8$) or at 9 days after injury (delayed transplantation, $n=8$). Instead of NSPCs, the culture medium without growth factors was injected into the lesion site at 9 days after injury in the control group ($n=8$).

Immunohistochemistry

Animals were anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS at 6 wk after injury. The spinal cords were removed, embedded in OCT compound, and sagittally sectioned at 20 μ m on a cryostat. Both cultured cells and tissue sections were stained with primary antibodies, including anti-GFP (1:500; MBL, Woburn, MA), anti-Nestin (1:200; Chemicon, Temecula, CA), anti-luciferase (1:100; Rockland Immunochemicals, Gilbertsville, PA), anti-glial fibrillary acid protein (GFAP) (1:1000; DAKO, Glostrup, Denmark), anti- β III tubulin (Tuj-1) (1:200; Sigma, St. Louis, MO), anti-APC CC-1 (1:200; Oncogene, Cambridge, MA), anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (1:200; Sigma), and anti-Hu (a gift from Dr. Robert Darnell, The Rockefeller University). Nuclear counterstaining was obtained with Hoechst 33342 (Molecular Probes, Eugene, OR). Images were obtained by fluorescence microscopy (Axioskop 2 plus; Carl Zeiss, Munich, Germany) or confocal microscopy (LSM510; Carl Zeiss). To quantify the proportion of each phenotype in vivo, we selected five representative midsagittal sections and captured five regions within 500 μ m rostral and caudal to the lesion epicenter randomly at $\times 200$ magnification. GFP-positive engrafted cells as well as each phenotypic marker-positive cells were counted in each section.

BLI

Xenogen-IVIS 100 cooled CCD optical macroscopic imaging system (SC BioScience, Tokyo, Japan) (31) was used for BLI. Signal intensity of NSPCs was determined by plating cells at various densities and imaging immediately after D-luciferin (150 μ g/ml) was added. Signals are reported as photons/cell/s. The integration time was fixed at 5 min duration for each image. For in vivo imaging, mice were given an i.p. injection of D-luciferin (150 mg/kg body weight), and serial images were acquired from 15–40 min after the administration until the maximum intensity was obtained with the field-of-view set at 7.2 cm. We found this time window to be optimal since the signal intensity peaked at 15 min after administration followed by a plateau of 20 min (data not shown). All images were analyzed with Igor (WaveMetrics, Lake Oswego, OR) and Living Image software (Xenogen, Alameda, CA), and optical signal intensity was expressed as photon flux, in units of photons/s/cm²/steradian. Each image was displayed as a false color photon count image superimposed on a grayscale anatomic image. To quantify the measured light, we defined regions of interest (ROI) over the cell-implanted area and examined all values with the same ROI.

Statistical analysis

Statistical analysis was performed with a Student's *t* test. For the open field score, repeated measure ANOVA, Kruskal-Wallis test, and post hoc Bonferroni/Dunn test were used. Values are reported as mean \pm SE. In all statistical analyses, significance was accepted at $P < 0.05$.

RESULTS

Imaging of lentivirally transduced NSPCs

NSPCs were harvested and labeled with luciferase and GFP reporter genes via lentiviral transduction. To examine the sensitivity of BLI, we used the CCD-based macroscopic detector to detect luminescence intensity of these cells at various cell numbers (ranging from 10^2 to 10^6 cells per well) in the presence of D-luciferin (150 $\mu\text{g/ml}$). Quantitative analysis of bioluminescence revealed that luminescence intensity was clearly in direct proportion to cell numbers in vitro (Fig. 1B). A population of 100 cells was sufficient to produce a signal that was significantly above background.

Additionally, the intense luminescence of NSPCs enabled us to detect a single neurosphere with the CCD microscope (Fig. 1C–E), and sufficient fluorescence and bioluminescence were detected from these cells with fluorescence microscopy (Fig. 1F–H). FACS caliber analysis revealed that >70% of these cells were positive for GFP (data not shown).

To confirm that lentiviral transduction had not altered the properties of NSPCs, we performed immunostaining and differentiation assays on labeled and untreated NSPCs. Fluorescence microscopy and immunostaining with anti-luciferase antibody confirmed the expression of luciferase and GFP in lentivirally transduced NSPCs (Fig. 2A, 2B). Both untreated and lentivirus-transfected NSPCs were allowed to differentiate for 7 days without growth factors in vitro. There were no differences in cell viability, in the pattern of phenotypes after differentiation, or in the ability to differentiate into neurons, astrocytes, and oligodendrocytes; some of them were positive for Nestin (32), a marker of NSPCs (Fig. 2C–G). Additionally, the stable expression of both luciferase and GFP in transduced NSPCs was maintained after differentiation (data not shown). These results demonstrated the ability of lentiviral vectors to confer high levels of specific gene expression while preserving the character of NSPCs.

In vivo imaging of transplanted cells

Since the signal from the labeled NSPCs appeared to be sufficient for in vivo cell tracking and viability studies, 5×10^5 of labeled NSPCs were stereotactically transplanted into the intact spinal cord of adult C57BL/6J mouse and cell distribution and signal intensity were assessed using BLI. On the exposed spinal cord, an intense focal spot of bioluminescence was observed at the transplanted site (Fig. 3A), indicating transplantation and placement of the cells into the spinal cord and not the surrounding tissue; this was confirmed by excising the spinal cord and observing its bioluminescence (Fig. 3B). To examine whether the number of the transplanted cells correlated with the intensity of bioluminescent signal in vivo, we performed transplants with different numbers of NSPCs (ranging from 25,000 to 500,000) at the level of T10 and assessed the signal intensity by BLI. The results indicated that the signal intensity was proportional to the number of transplanted cells; this was despite the use of mice with dark fur and skin, C57BL/6J (Fig. 3C, 3D), which had been believed inappropriate for optical imaging due to absorbance of the signals.

Viability of engrafted NSPCs in injured spinal cord

The great advantage of BLI is the capability to quantify only living cells since the luciferin-luciferase reaction depends on oxygen and ATP. To examine the engrafted cell viability in the injured spinal cord, we induced contusion injury at T10 level of adult mice and then transplanted 5×10^5 NSPCs in 2 μ l medium at the lesion site. The accuracy of transplantation could be confirmed immediately after transplantation using BLI, and the average signal intensity was $4.60 \pm 0.45 \times 10^6$ photons/mouse/s in 16 mice. Images were obtained daily for 1 wk and then weekly over a 6-wk period. To investigate whether there is a relation between the timing of transplantation and grafted cell viability, we performed transplant operation either immediately (acute transplantation) or at 9 days after contusion injury (delayed transplantation). Analysis of both acute and delayed transplantation groups revealed drastic reductions in signal intensity within the first 4 days after transplantation, which was followed by a relatively stable bioluminescent signal for 6 wk (Fig. 4A). Quantitative comparison between the two groups revealed that there were no apparent differences in photon emission, suggesting that cell viability was comparable in acute and delayed transplantation (Fig. 4B, 4C). In mice that were not killed at the end of the experimental period, the bioluminescent signal could be followed for up to 10 months after transplantation in both groups (data not shown).

Integrated cell morphology in acute and delayed transplantation

Although no significant differences in cell viability were observed between the acute and delayed transplantation, there were apparent differences in the location and morphology of the integrated cells. SCI in C57BL/6J mice results in large connective tissue scars at the lesion epicenter dependent on the degree of crush injury (33) compared with cavity formation that is usually observed in the injured spinal cords of rats (29) or primates (34). The significant difference that was observed between the acute and delayed transplantation groups was that in the acute group NSPCs were mainly distributed within the scar area, while in the delayed group the NSPCs were found around the scar (Fig. 5A and 5B). Moreover, immunostaining and confocal microscopic images revealed that these integrated cells within the scar area of the acute group were GFAP-positive, and their morphology suggested that they contributed to the formation of an astrocytic glial scar (Fig. 5C–E, Supplemental Fig. 1). There was a small percentage of neurons (1.27%) and oligodendrocytes (0.99%) in the acute group (Table 1). In contrast, some of the grafted cells in the delayed group appeared to be single cells that expressed the oligodendrocyte marker APC (Fig. 5F–H), or the neuronal marker Hu (Fig. 5I–K, Supplemental Fig. 2) (Table 1). These results suggested that the microenvironment of injured spinal cord had a distinct influence on the differentiation of transplanted NSPCs.

Migratory cells were reflected in the configuration of bioluminescence

In the course of tracking luminescent signals of grafted NSPCs for 6 wk, the luminescent configuration changed from a round to an elliptic shape in mice of the delayed transplantation group (Fig. 6A). NSPCs transplanted into lesion epicenter (Fig. 6, *) migrated away caudally while extending the neurites, and there was a correspondence between histological and in vivo BLI (Fig. 6B). Migratory cells were mainly found along the white matter of the host spinal cord, and the confocal microscopic image revealed that some of these cells were Hu-positive neurons (Fig. 6C–E).

Transplanted NSPCs partially promoted functional recovery

The histological assays suggested that the timing of transplantation may also affect functional recovery. Here we used a standardized open-field measure of locomotor function: the BBB score to assess function in acute and delayed transplantation. In both treated groups, partial prompt recovery of hind-limb movement was observed within 1 wk after transplantation and was followed by a period of gradual recovery. While improved recovery was found in both acute and delayed transplantation groups compared with the medium-injected control group, the mice in the delayed transplantation group showed a tendency of long-lasting recovery, and the only statistically significant difference was between the control group and the delayed transplantation group with the post hoc Bonferroni/Dunn test (Fig. 7).

DISCUSSION

The present study is the first to use molecular imaging tools to monitor primary NSPCs and their accurate viability after transplantation into injured spinal cords in living animals. Although the feasibility and usefulness of *in vivo* BLI have been demonstrated in several studies, the conventional application of BLI was to track grafted cells whose signal intensity would increase with time, such as tumor cells or established cell lines, and long-term tracking of primary cultured NSPCs has been relatively difficult because unlike immortalized or proliferative cells, a large percentage of cells were lost within a short time period after transplantation. Furthermore, it has been difficult for primary cells to obtain high levels of sustained reporter gene expression compared with cell lines. To overcome this limitation and to follow primary cells *in vivo*, we used third-generation lentiviral vectors to transfer the luciferase and GFP genes to NSPCs. The transduction efficiency was high in the present study, owing to the ability to concentrate the vectors to high titers using their modified envelop glycoprotein from vesicular stomatitis virus (VSV-G). Their broad host range and their ability to infect nondividing cells and to integrate make these vectors ideal for gene transfer into NSPCs (24, 35). Transduced NSPCs demonstrated sustained expression of luciferase while maintaining the characteristic features of NSPCs (Fig. 2). Their bioluminescent signals appeared as a focal spot after transplantation into injured spinal cords of mice. By combining two novel tools, the third-generation lentiviral vectors and *in vivo* BLI, we could successfully monitor primary NSPCs transplanted into injured spinal cords, even with the dark skin of C57BL/6J mice, over long periods of time.

In the research of cell therapy, the accuracy of transplantation is a fundamental premise and especially significant when using small animals such as mice. Here we used BLI as a novel tool for confirming successful transplantation. The standard error of the mean in the signal value was <10% when 5×10^5 NSPCs were transplanted into injured spinal cords of 16 mice, which was an acceptable value for experimentation.

One of the advantages of BLI is to demonstrate the migration of grafted cells in living animals as Tang et al. (36) and Kim et al. (37) have previously reported. Here we demonstrate the stability of signals from NSPCs that integrated at the lesion epicenter in the acute transplantation group (Fig. 4A), and histology confirmed that the cells remained and participated in the formation of a glial scar at the scar area (Fig. 5A). In the delayed transplantation groups, NSPCs integrated around the scar area and differentiated into migrating neurons, and the images from BLI corresponded with the histological images (Fig. 6).

Signals in BLI have been shown to correlate with cell viability in a number of models (18, 23, 38). In the viability studies, we observed significant reductions in signal intensity over the first 4 days posttransplantation. This was followed by a period of relatively stable signal (6 wk), which was observed in both the acute and delayed transplantation groups (Fig. 5A). These data suggested that ~80% of NSPCs were lost within 4 days of transplantation and the remaining 15–20% of transplanted NSPCs survived over 6 wk (Fig. 5C). Several other studies have reported that few NSPCs integrated into the injured spinal cord and participated in the glial scar if transplanted at the time of acute injury (9, 39). Despite our expectation of a better survival rate in the delayed transplantation group, the results showed no apparent differences between the two groups. Histological examination confirmed the presence and integration of transplanted cells in both groups, with differences in location and differentiated cell type. Discrepancies between other previous reports (9) and the present study may be due to differences in the animals and models of SCI. In the contusive SCI of C57BL/6J mice, a large connective tissue scar typically forms at the lesion epicenter (33) and transplanted cells were mainly contained within this scar in this study. In contrast, in similar transplantation experiments in other animals, including rats and primates, large cavities are formed at the lesion epicenter that result in tissue necrosis, and the survival of cells transplanted into the lesion site in the acute phase was low.

Histological examination in our model revealed that the morphology of integrated cells was clearly different depending on the timing of transplantation (Fig. 5 and Table 1). These phenotypic differences may be due to differences in the microenvironment since the environment changes dramatically during this time. In the acute phase after injury, a transient and dramatic increase in chemical mediators and cytokines, for example, IL-1 β , IL-6, CNTF, and TNF- α , are observed around the injured site (40), and they are likely to influence the differentiation of transplanted NSPCs. IL-6, CNTF, and BMPs induce NSPCs to undergo astrocytic differentiation selectively both in vivo and in vitro (30, 41, 42), and the majority of acutely transplanted NSPCs formed a glial scar at the lesion epicenter. NSPCs transplanted in the delayed phase appeared to differentiate into neurons and oligodendrocytes in addition to astrocytes (Table 1, Fig. 5 and 6).

We also evaluated the recovery of locomotor function for 6 wk and examined the correlation between the functional recovery and cell viability estimated by luminescent intensity. Better recovery was found in NSPC transplanted groups compared with the medium injected control group (Fig. 7), and significant differences were observed in motor function scores at 6 wk and signal intensity at 4 days or 6 wk, as the result of Spearman's correlation coefficient by rank test. The fact that there was no statistically significant difference between the acute and delayed transplantation groups with post hoc test suggests that the greatest contribution of NSPCs to functional recovery may be the secretion of neurotrophic factors (4, 43). However, considering that the only significant difference shown was between the control and delayed transplantation groups, and that the delayed transplantation group showed a tendency of long-lasting recovery, the morphological difference revealed between the acute and delayed groups may be significant. Although additional studies will be needed to determine the precise mechanism of functional recovery by NSPC transplantation, delayed transplantation is considered to also be more practical from the aspect of clinical applications.

The methods in the present study can be widely applied to transplantation research and the study of primary cultured cells in the context of living animal models. Image guidance in this study offered the opportunity to monitor engraftment and migration, which were used to select the times for tissue sampling. Here, we used this approach to demonstrate the differences between

early and delayed cell transplantation, and the functional data suggest that delayed transplantation could have significant clinical efficacy.

ACKNOWLEDGMENTS

This work was supported by grants from the Japan Science and Technology Agency, Core Research for Evolutional Science and Technology, a Grant-in-Aid for the 21st century COE program, Keio Gijuku Academic Development Funds, National Grant-in-Aid for the Establishment of High-Tech Research Center, and the National Institutes of Health (R24 CA 92862 to CHC).

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Received April 13, 2005; accepted July 15, 2005.

Table 1**The percentages of differentiated cell types derived from the donor cells in vivo^a**

	GFAP	APC	Hu
Acute TP	80.7 ± 3.7	0.99 ± 0.4	1.27 ± 0.5
Delayed TP	61.0 ± 6.1	8.33 ± 1.1	11.8 ± 1.2

^aThe percentage of Hu/GFP double-positive neurons or APC/GFP double-positive oligodendrocytes among the GFP positive cells was ten times greater in the delayed transplantation (TP) group than that of the acute transplantation group.

Fig. 1

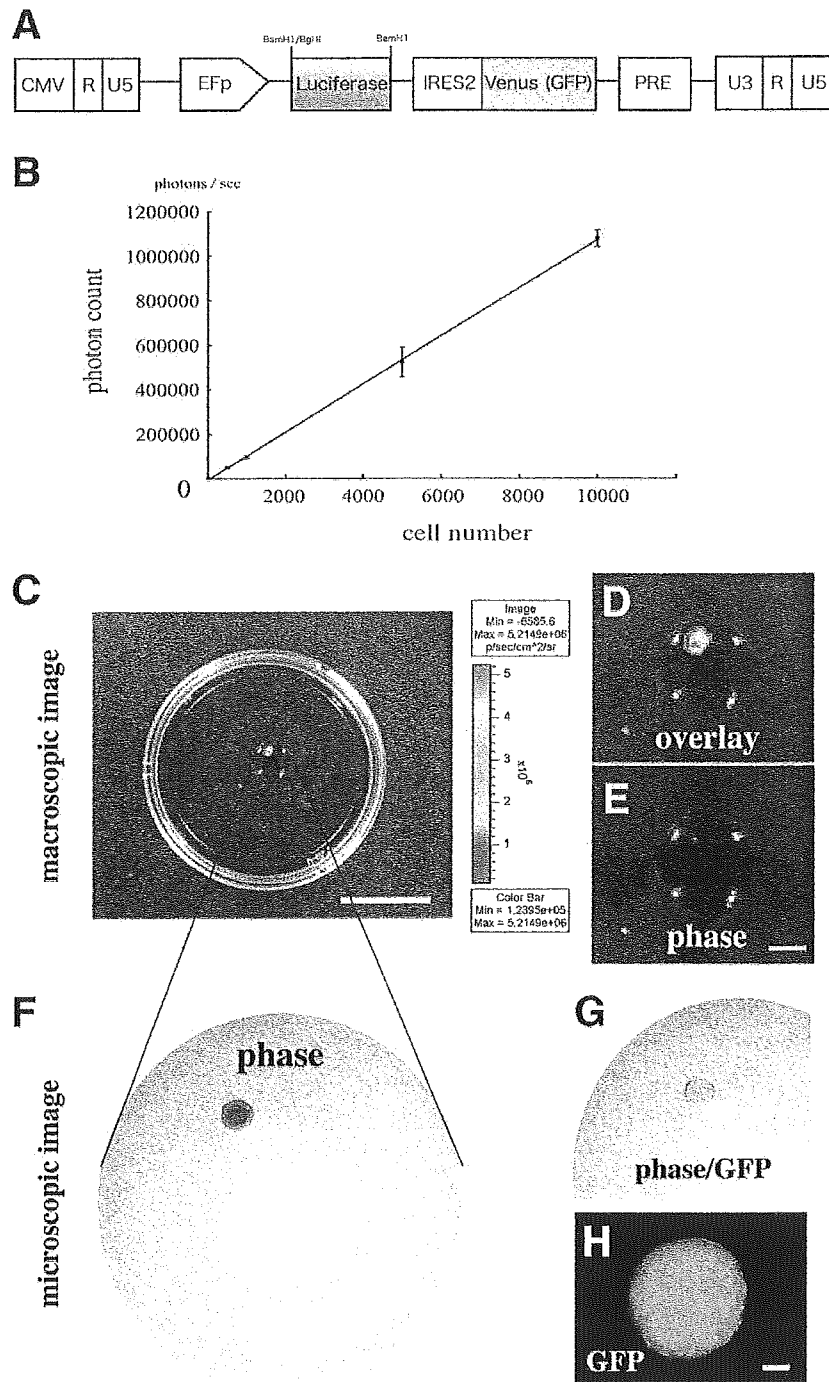


Figure 1. Sufficient expression of luminescence and fluorescence in the lentivirally transfected NSPCs. **A)** Lentiviral construct encoding a dual function luciferase and GFP bicistronic reporter gene connected via an internal ribosomal entry site (IRES). **B)** The correlation between photon counts and numbers of NSPCs expressing luciferase transduced by lentivirus. **C–H)** Macroscopic and microscopic images of a single neurosphere of transfected NSPCs in a drop of the medium. A single neurosphere expressed sufficient luminescence as well as fluorescence to be detected by the CCD macroscope or fluorescent microscope. Values are means \pm SE ($n=8$). Scale bar: 15 mm (**C**), 2 mm (**E**), and 50 μ m (**H**).

Fig. 2

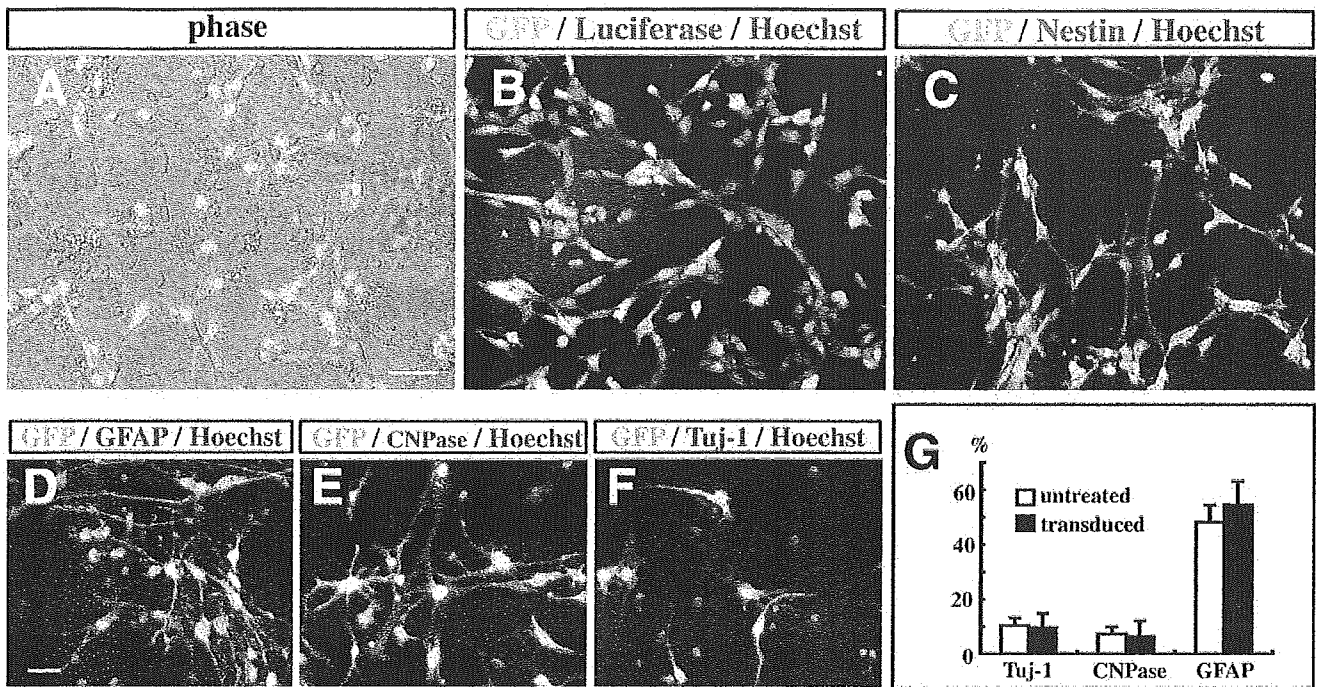


Figure 2. Lentiviral transduction did not influence the character of NSPCs. *A, B*) Fluorescence microscopy and immunostaining with anti-luciferase antibody confirmed the expression of GFP and luciferase after differentiation. Immunohistochemistry showed that derivatives of NSPCs were reactive for nestin (*C*), GFAP (*D*), CNPase (*E*), and Tuj-1 (*F*). *G*) The proportion of differentiated phenotypes in the lentivirally transduced NSPCs was identical to that of untreated NSPCs. Values are means \pm SE ($n=3$). Scale bar: 50 μ m (*A*) and 25 μ m (*D*).

Fig. 3

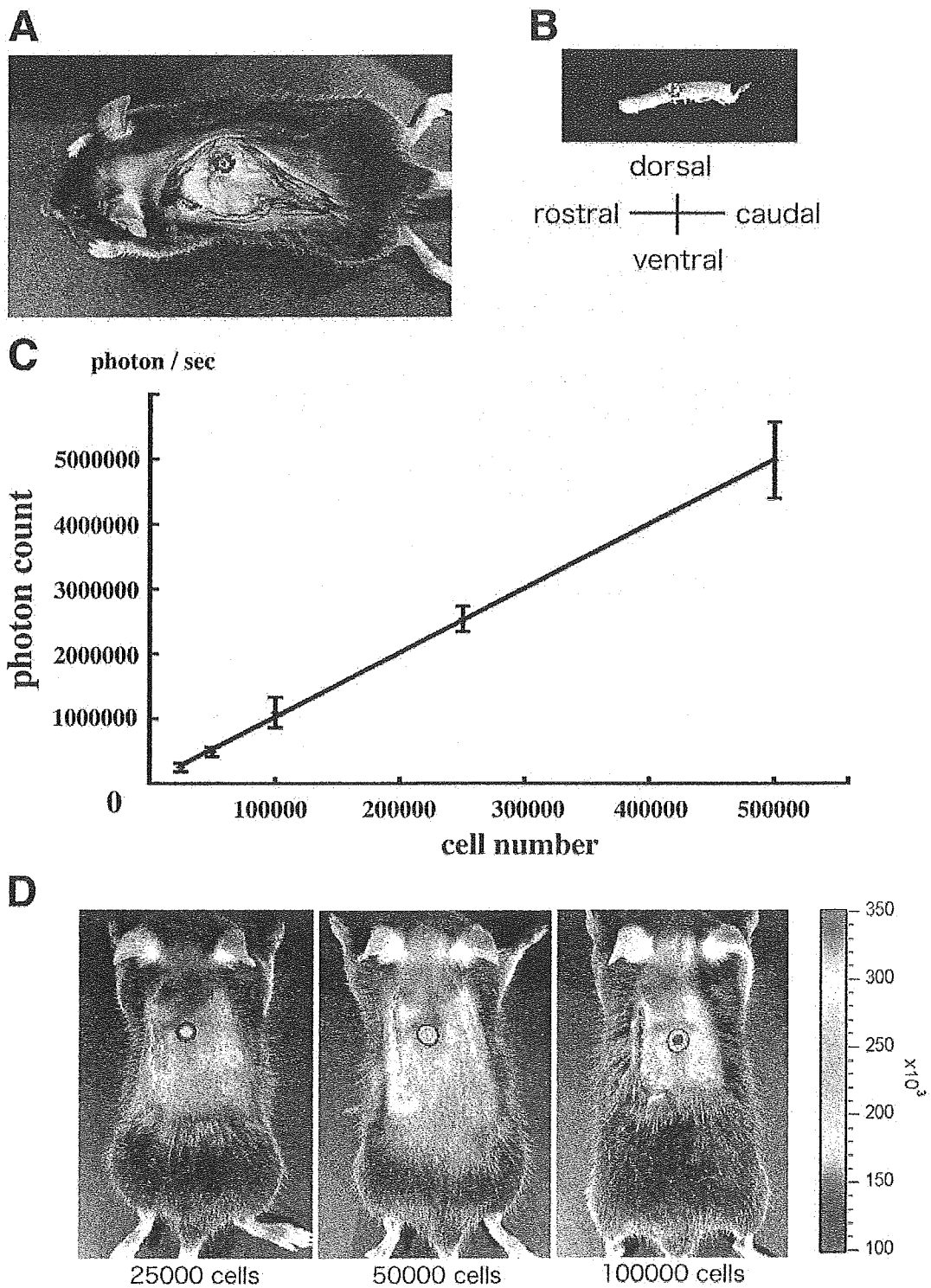


Figure 3. In vivo and ex vivo images of transplanted luciferase-expressing NSPCs into the intact spinal cord of mice. Accurate transplantation into spinal cord without leaking could be confirmed by the in vivo images (*A*) and ex vivo images (*B*) of exposed spinal cord. *C, D*) The correlation between grafted cell numbers and luminescent intensity was also confirmed in vivo. Values are means \pm SE ($n=4$).

Fig. 4

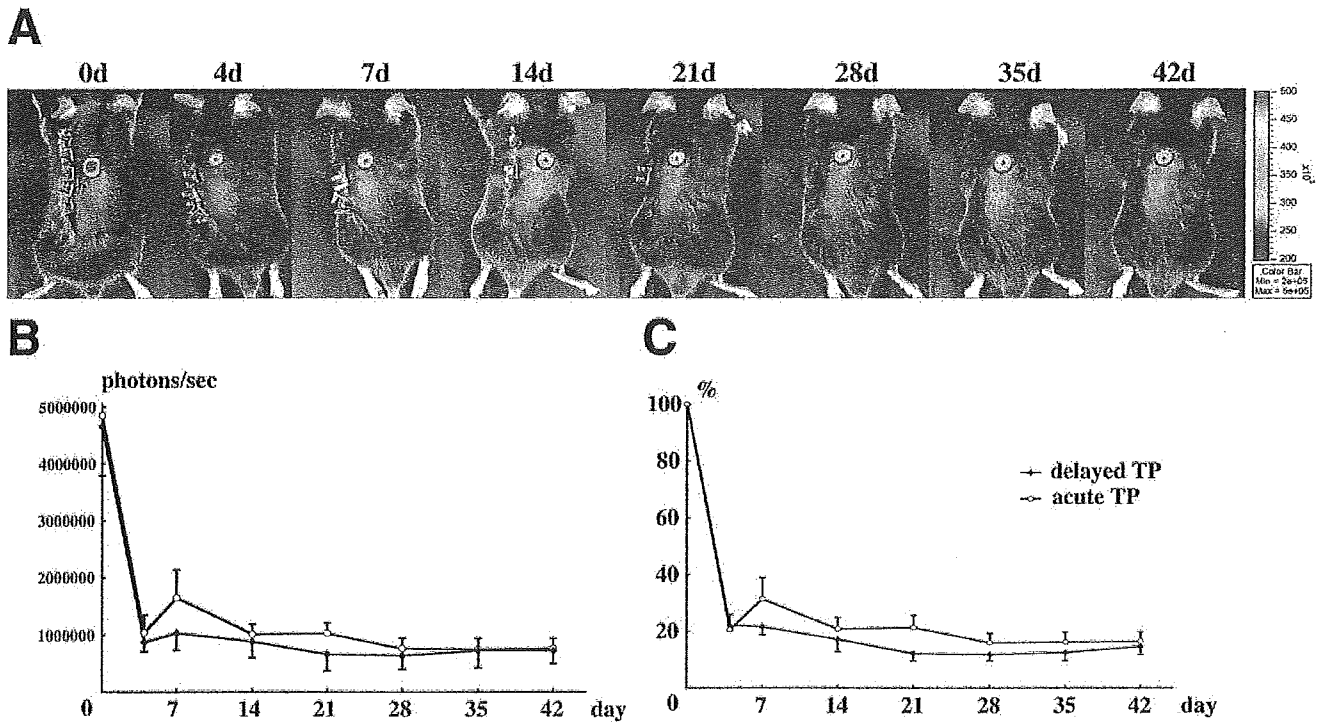


Figure 4. The time course of viability of transplanted NSPCs for SCI. **A)** Images of a representative mouse that received acute transplantation (TP) of luciferase-expressing NSPCs confirmed long-term cell viability. Drastic reductions in signal intensity within the first 4 days after transplantation and then relatively stable bioluminescent signals for the following 6 wk were observed in both acute and delayed transplantation groups. There were no differences between acute and delayed transplantation groups in both value of signal intensity (**B**) and the rate to initial value (**C**) at each time point. Values are means \pm SE ($n=8$).