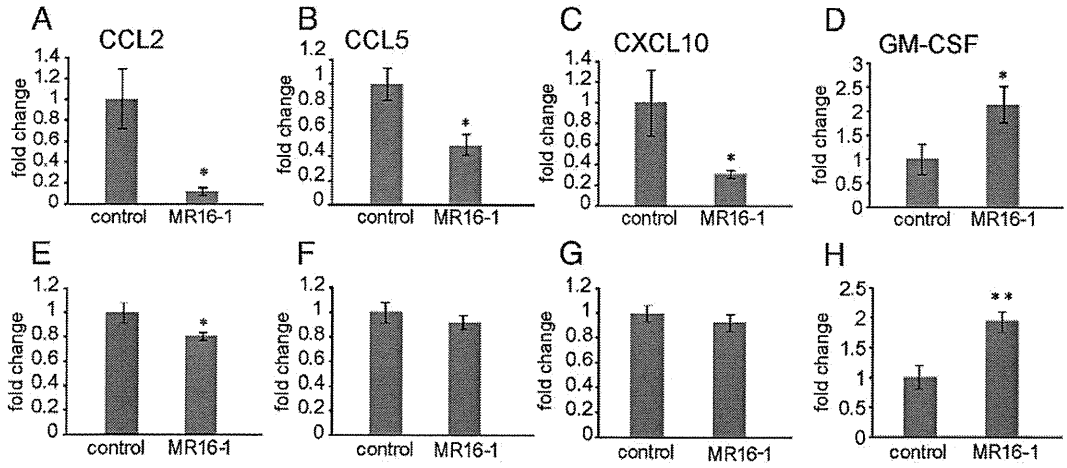

Fig. 3. MR16-1 decreases the expression level of macrophage-recruiting chemokines, while increasing that of GM-CSF. A–D: The CCL2 (A), CCL5 (B), CXCL10 (C), and GM-CSF (D) mRNA expression levels in the spinal cord tissue 12 h after injury were determined using quantitative RT-PCR. Macrophage-recruiting chemokines had significantly decreased, while the expression of GM-CSF, a known mitogen for microglia, had increased. E–H: The protein levels of the CCL2(E), CCL5(F), CXCL10(G) and GM-CSF(H) 24 h after injury were determined by western blot analysis. The protein level of CCL2 (E) was significantly decreased, whereas the GM-CSF (H) level was significantly increased. There was a tendency for the protein levels of CCL5 (F) and CXCL10 (G) to decrease, although the change was not statistically significant. Values are means ± SEM. *$P<0.05$.

fibers was observed in the MR16-1-treated group than in the control group (Figs. 6M–O).

Discussion

IL-6 is a pro-inflammatory cytokine that triggers secondary injury in the pathophysiology of SCI. IL-6 binds to soluble and membrane-bound IL-6-receptor to form a complexed ligand for gp130, the common signal transducer of IL-6 and its related cytokines. MR16-1 is a neutralizing antibody for IL-6-receptor that competitively inhibits its binding to IL-6, thereby blocking IL-6-receptor-mediated cell signaling. We previously reported that the systemic administration of MR16-1 decreases the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in the injured spinal cord, demonstrating that this treatment potently affects the IL-6/JAK/STAT3 signaling pathway. Subsequently, we showed that MR16-1 administration after SCI reduces the number of inflammatory cells present 2 weeks after injury and decreases the amount of reactive astrogliosis, leading to improved functional recovery (Okada et al., 2004). Our present study extends these findings, showing that MR16-1 treatment alters the nature of the inflammatory response after SCI.

Here we found that the temporary inhibition of IL-6 signaling by MR16-1 treatment caused a significant reduction in the macrophage/microglia accumulation at 14 dpi, but not at 4 or 7 dpi. We had expected MR16-1 to have an anti-inflammatory effect, because IL-6 is a pro-inflammatory cytokine, so it was unclear why the immediate administration of MR16-1 affected only the late phase of the inflammatory response after SCI. We hypothesize that the response was delayed because the effects of MR16-1 treatment were owed to changes in the nature of the inflammatory response, rather than to the immediate effects of inhibiting the IL-6 signal.

Previous studies showed that functional recovery after SCI is affected by the properties of the inflammatory response, which are determined by the cell types involved and their state of activation (Gris et al., 2004; Popovich et al., 1999; Rapalino et al., 1998; Saville et al., 2004; Schwartz et al., 1999). Hematogenous macrophages and microglia are the major players in the inflammatory pathology of SCI, and their characteristics have therefore been of major interest. Of the two, hematogenous macrophages are regarded as more detrimental, because removing them or preventing their infiltration into the injured tissue reduces the degree of secondary injury and improves functional recovery (Gris et al., 2004; Popovich et al., 1999). In contrast, microglia are believed to be relatively beneficial for spinal cord repair, owing to their higher phagocytotic activity and expression of various neurotrophic factors (Lalancette-Hebert et al., 2007; Schilling et al., 2005). The state of cell activation also affects the nature of the inflammation; the implantation of bone marrow-derived macrophages previously stimulated by co-incubation with peripheral nerve or skin improves spinal cord repair (Bomstein et al., 2003; Rapalino et al., 1998).

Because IL-6 has a leading role in recruiting macrophages during inflammation, we hypothesized that MR16-1 treatment would decrease the infiltration of hematogenous macrophages. Therefore, we focused on the balance between the types of inflammatory cells, i.e., the hematogenous macrophages and microglia. Flow cytometric analysis revealed that the proportion of infiltrated $CD45^{high}$ hematogenous macrophages decreased markedly following MR16-1 treatment, with the result that resident microglia replaced hematogenous macrophages as the major inflammatory cell type at the lesion site. Furthermore, we performed quantitative analyses in chimeric mice bearing transplanted EGFP-expressing, highly purified HSCs. These analyses showed that, besides the reduced infiltration of hematogenous macrophages following MR16-1 treatment, the number of resident microglia increased, contributing to the shift in the major inflammatory cell type. The increase in the number of BrdU+ microglia by MR16-1 indicated that the higher number of microglia might have resulted from their increased proliferation (Suppl. Fig. 1).

Two mechanisms appear to mediate this phenomenon. First, the number of hematogenous macrophages is reduced because fewer are recruited from the blood pool. Various chemokines are known to mediate macrophage infiltration; in particular, CCL2, CCL5, and CXCL10 have a demonstrated role in recruiting macrophages following CNS injury (Babcock et al., 2003; Ghirnikar et al., 1998; Glass et al., 2001; Liu et al., 2001). Here we observed decreased expression levels of CCL2, CCL5, and CXCL10 following MR16-1-treatment, which could account for the reduced infiltration of hematogenous macrophages. This agrees with the observation that IL-6 is a major player in directing chemokine-mediated macrophage infiltration during inflammation (Hurst et al., 2001; Romano et al., 1997).

In contrast to hematogenous macrophage recruitment, microglial proliferation is mainly regulated by colony-stimulating factors (CSFs) (Giulian and Ingeman, 1988; Lee et al., 1994), and the local increase in GM-CSF that we observed could have stimulated their proliferation, resulting in their increased numbers at the lesion site (Lee et al., 1994). Since IL-6, like other cytokines, interacts with various other

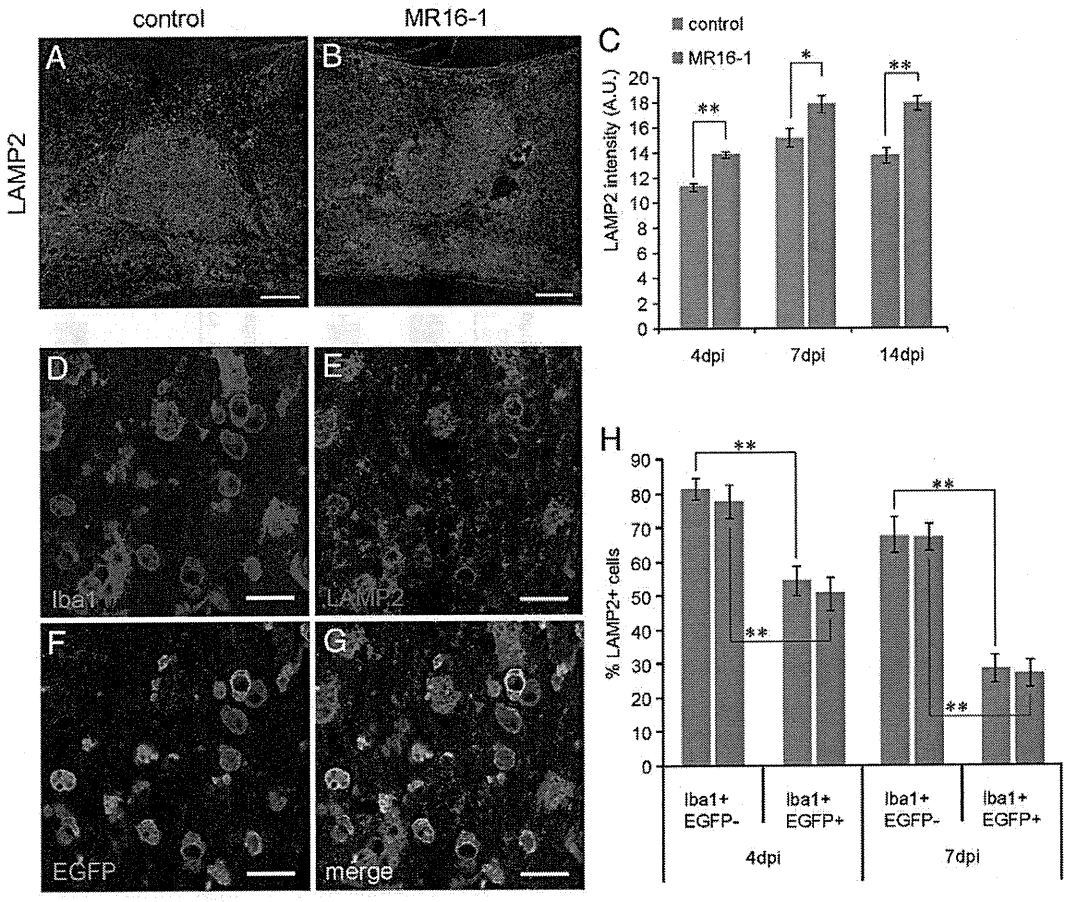

Fig. 4. MR16-1 enhances the phagocytotic activity in the injured spinal cord. A–C: Phagocytosis, indicated by LAMP2 expression, was increased by MR16-1-treatment. D–H: Quantification of the LAMP2$^+$ inflammatory subsets in injured chimeric mice indicated that the microglia had significantly higher phagocytotic activity than the hematogenous macrophages. MR16-1-treatment did not affect the phagocytotic activity of either cell subpopulation. Values are means ± SEM. *$P<0.05$. Scale bars = 200 μm in A, B; 20 μm in D–G.

cytokines or neurotrophic factors, and since previous reports show that the inhibition of IL-6 signaling drastically alters the expression profiles of various cytokines (Matsumura et al., 1999; Romano et al., 1997), the temporary inhibition of IL-6 signaling may, by altering the chemical milieu, underlie the shift in the dominant inflammatory cell type following MR16-1 treatment.

Given the characteristics of hematogenous macrophages vs. those of resident microglia, the MR16-1-induced switch in the central player in post-SCI inflammation should be beneficial. The evidence bears out this prediction. In the injured spinal cord, broad destruction of the blood–spinal cord barrier (BSCB) permits the infiltration of hematogenous macrophages into the lesion. If the infiltrating neutrophils and macrophages are depleted or blocked, tissue sparing improves, as does axonal regeneration/sprouting (Gris et al., 2004; Popovich et al., 1999; Saville et al., 2004). The cytotoxicity of hematogenous macrophages may be due to their increased NO production in the injured CNS (Ponomarev et al., 2007). In addition, a recent report showed that direct physical interactions between activated hematogenous macrophages and axons causes the axons to retract; microglia have a similar effect, but it is much weaker (Horn et al., 2008).

Despite the detrimental effects of hematogenous macrophages, the accumulation of inflammatory cells is considered to be critical for the repair process, because these cells clear away tissue debris and release neurotrophic factors. Previous studies demonstrated that the resident microglia play an active role in repairing the injured CNS, through their relatively high phagocytotic activity (Schilling et al., 2005) and by releasing various neuroprotective cytokines or neurotrophic factors (Lalancette-Hebert et al., 2007; Lambertsen et al., 2009). In fact, the tissue debris within the injured spinal cord contains myelin-derived axonal growth inhibitory factors (Bregman et al., 1995; Merkler et al., 2001) that hinder the repair process after SCI, so clearing away this debris is prerequisite for axonal re-growth.

In this study, MR16-1 treatment led to an increased accumulation of microglia, which expressed higher levels of the phagocytic markers LAMP2 and Mac2 than did the hematogenous macrophages. Furthermore, the Oil red O-stained intracellular area of microglial cells was greater than that of hematogenous macrophages. These data indicate that the microglia had higher phagocytotic activity than the hematogenous macrophages, and this activity was enhanced by the MR16-1 treatment.

The enhanced phagocytosis by MR16-1 treatment, along with attenuation of injury, resulted in decreased Oil red O staining (indicating reduced deposition of myelin) and decreased immunostaining for the axonal growth inhibitor Nogo-A, at the chronic phase of post-SCI inflammation. These effects could have contributed to the formation of a permissive environment for the regeneration or sprouting of neuronal fibers. In fact, the process of spinal cord repair seemed to be enhanced, given the increase in the RT-97$^+$ fibers and 5HT$^+$ serotonergic fibers between 14 and 42 dpi. The higher density of RT-97 fibers in the penumbral area in the treated group, and the lack of RT-97+ fibers at the center of the injury site in both groups, may indicate that enhanced sprouting contributed to the increase in 5HT+ fibers caudal to the lesion site (Supplemental Fig. 2).

Taken together, it is possible that the immediate administration of MR16-1 affected only the late phase of inflammation, because the change in cytokine profile by MR16-1 enhanced the participation of microglia in the inflammatory process, resulting in a tissue-protective inflammation. Our results with LFB, Oil red O, and immunostaining for

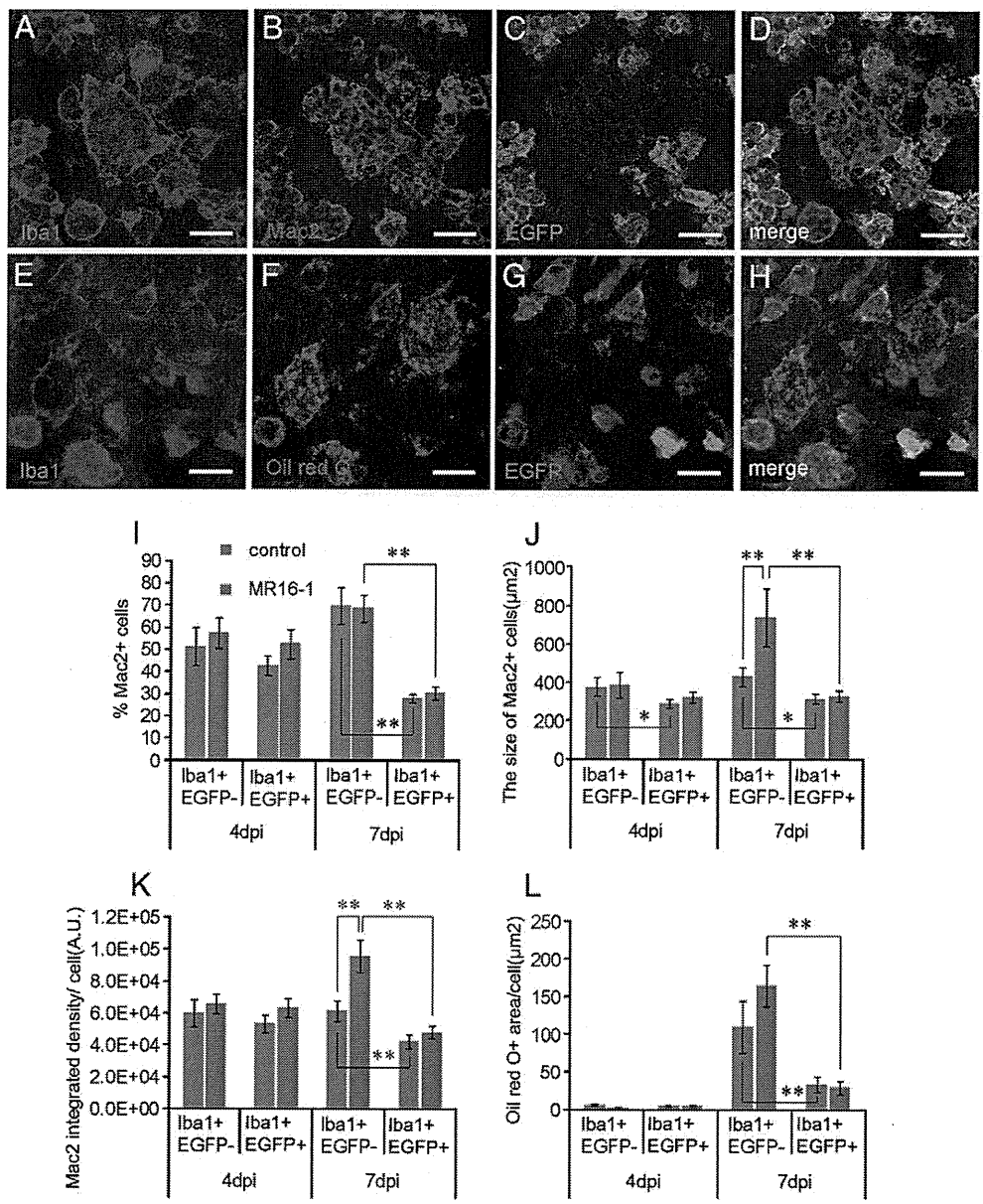

Fig. 5. Microglia have higher phagocytic activity against myelin debris than hematogenous macrophages. A–D: Mac2 expression was observed in the cells at the lesion epicenter. A portion of the macrophages/microglia expressed Mac2 in their cytoplasm. E–H: Oil red O^+ particles were observed in the cell body of macrophages/microglia at the lesion epicenter. I: The cytoplasmic expression of Mac2 was greater in $Iba1^+EGFP^-$ resident microglia than in $Iba1^+EGFP^+$ hematogenous macrophages. J, K: The average size (J) and Mac2-integrated density (K) at 7 dpi of each $Iba1^+EGFP^-$ resident microglial cell were significantly greater than those of $Iba1^+EGFP^+$ hematogenous macrophages, and were increased by MR16-1 treatment. The intracellular Oil red O-stained area was significantly greater in the $Iba1^+EGFP^-$ resident microglia than in the $Iba1^+EGFP^+$ hematogenous macrophages, and this tendency was increased at 7 dpi by MR16-1 treatment (L). Values are means ± SEM. $^*P<0.05$. $^{**}P<0.01$. Scale bars = 20 μm in A–H.

Nogo-A, which showed reduced injury and deposition of debris, support this idea. This series of immune responses takes place over a long time, which is consistent with the effects of MR16-1 treatment appearing only at the late phase.

However, despite the marked improvement in the inflammatory response obtained with MR16-1 treatment, skepticism about the therapeutic effects of IL-6 signal inhibition remains, because several studies have shown that IL-6 signaling has neuroprotective functions after CNS trauma. For example, Penkowa et al. showed that the overexpression of IL-6 results in decreased oxidative stress and apoptosis, leading to faster tissue repair after brain injury (Penkowa et al., 2003), and IL-6-deficient mice show a slower rate of recovery and healing after brain injury than wild-type mice (Swartz et al., 2001).

Although our results may seem to conflict with these reports, we believe that the apparent discrepancies are owing to the context-dependent pleiotropic actions of IL-6. Our present data revealed that the therapeutic effect of MR16-1 was achieved by inhibiting the excessive infiltration of hematogenous macrophages through the damaged BSCB, during the acute phase of SCI. This idea is corroborated by previous reports demonstrating that the overexpression of IL-6 family cytokines during the acute phase of SCI significantly increases inflammatory cell accumulation, resulting in greater damage (Kerr and Patterson, 2004; Lacroix et al., 2002). On the other hand, IL-6 can enhance spinal cord repair by modifying the migration of reactive astrocytes (Okada et al., 2006) or enhancing axonal re-growth (Miao et al., 2006), and IL-6's temporary inhibition may have little effect on these functions, because astrocyte migration and axonal re-growth are rather long-term processes. Although numerous studies have shown that the recruitment of inflammatory cells aggravates secondary injury after SCI (Ghirnikar et al., 2001; Hausmann, 2003;

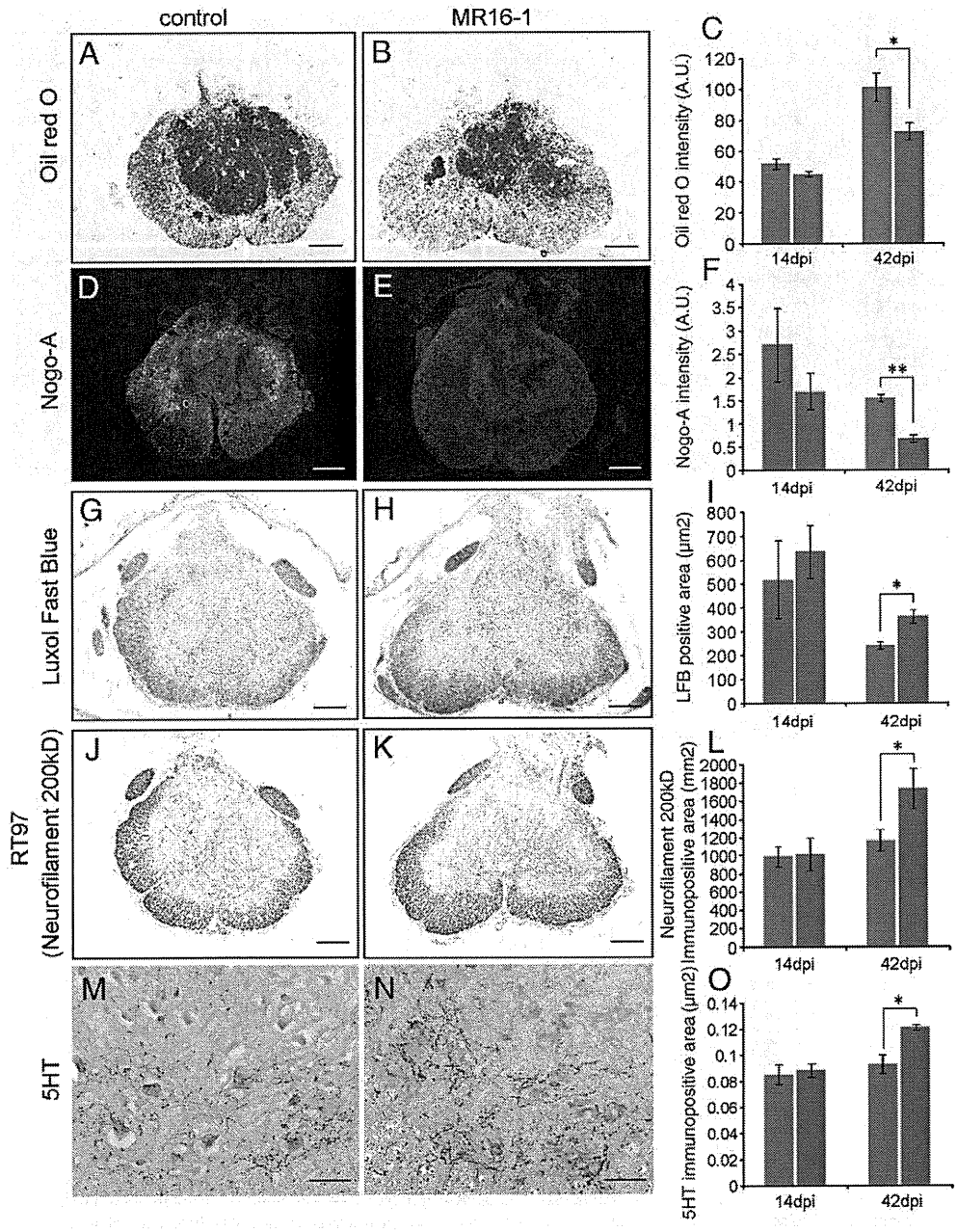

Fig. 6. MR16-1 treatment improves repair of the spinal cord. A–C: MR16-1 treatment improved the clearance of myelin debris, as seen by a significant reduction in the Oil red O-stained area. D–F: The deposition of Nogo-A was decreased in the MR16-1-treated group. G–I: The area of Luxol Fast Blue-stained tissue, which represents spared myelin sheath, was significantly increased by the MR16-1 treatment. J–L: RT-97^{+} (Neurofilament 200kD) fibers at the lesion epicenter were significantly increased in the MR16-1-treated animals at 42 dpi. M–O: 5HT-positive fibers at the ventral horn. 5HT-immunostaining of the area 2 mm caudal to the lesion revealed a significant increase in 5HT^{+} fibers at 42 dpi by the MR16-1-treatment. *$P<0.05$. Scale bars = 200 μm in A, B, D, E, G, H; 50 μm in J, K.

Saville et al., 2004), inflammation itself is important for spinal cord repair (Donnelly and Popovich, 2008), and excessive suppression of the inflammatory response can be detrimental. In the present study, we showed that anti-IL-6-receptor antibody treatment at the acute stage of injury switches the main subpopulation of inflammatory cells from hematogenous macrophages to microglia, which does not simply suppress inflammation: rather, it changes the characteristics of the post-traumatic inflammation to promote spinal cord repair.

Given that a humanized antibody for the human IL-6 receptor (MRA; tocilizumab) is already in clinical use (Choy et al., 2002; Nishimoto et al., 2000; Sato et al., 1993), the present data support the potential application of the anti-IL-6-receptor antibody for the treatment of SCI. However, in most clinical situations, it is not possible to administer an antibody immediately after SCI. Further investigation to determine the therapeutic time window will be needed before this treatment can be tried clinically. Nevertheless, these findings shed light on IL-6's role in the pathology of SCI, and suggest a new approach for SCI treatment, in which the characteristics of inflammation are adjusted to support spinal cord repair, by modifying the cytokine-mediated cellular response.

Author contributions

M.M., M.N., Y.T., M.L. and H.O. designed the research; M.M., O.Y., A. I., T.I., F.RM. and O.T. performed the in vivo experiments; S.M. and Y.M. generated the chimeric mice; Y.O. supplied the anti-IL-6 receptor antibody and analyzed the antibody results; M.M., M.N., S.O., F.RM. and H.K. analyzed the data; M.M., M.N. and H.O. wrote the paper; M.N. and H.O. supervised all the experiments.

Acknowledgments

This work was supported by grants from the Project for the Realization of Regenerative Medicine from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan to H.O.; the General Insurance Association of Japan to M.N., A.I. and Y.T.; and a Grant-in-Aid for the Global COE Program from MEXT to Keio University. This work was also supported by grants from the Grant of Orthopaedics and Traumatology Foundation, Inc. No. 0178 to A.I. We are grateful to Professor Claude Bernard at Monash University for his critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2010.04.020.

References

Babcock, A.A., Kuziel, W.A., Rivest, S., Owens, T., 2003. Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. J. Neurosci. 23, 7922–7930.

Bomstein, Y., Marder, J.B., Vitner, K., Smirnov, I., Lisaey, G., Butovsky, O., Fulga, V., Yoles, E., 2003. Features of skin-coincubated macrophages that promote recovery from spinal cord injury. J. Neuroimmunol. 142, 10–16.

Bregman, B.S., Kunkel-Bagden, E., Schnell, L., Dai, H.N., Gao, D., Schwab, M.E., 1995. Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378, 498–501.

Cafferty, W.B., Gardiner, N.J., Das, P., Qiu, J., McMahon, S.B., Thompson, S.W., 2004. Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice. J. Neurosci. 24, 4432–4443.

Choy, E.H., Isenberg, D.A., Garrood, T., Farrow, S., Ioannou, Y., Bird, H., Cheung, N., Williams, B., Hazleman, B., Price, R., Yoshizaki, K., Nishimoto, N., Kishimoto, T., Panayi, G.S., 2002. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum. 46, 3143–3150.

Donnelly, D.J., Popovich, P.G., 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp. Neurol. 209, 378–388.

Gensel, J.C., Nakamura, S., Guan, Z., van Rooijen, N., Ankeny, D.P., Popovich, P.G., 2009. Macrophages promote axon regeneration with concurrent neurotoxicity. J. Neurosci. 29, 3956–3968.

Ghirnikar, R.S., Lee, Y.L., Li, J.D., Eng, L.F., 1998. Chemokine inhibition in rat stab wound brain injury using antisense oligodeoxynucleotides. Neurosci. Lett. 247, 21–24.

Ghirnikar, R.S., Lee, Y.L., Eng, L.F., 2001. Chemokine antagonist infusion promotes axonal sparing after spinal cord contusion injury in rat. J. Neurosci. Res. 64, 582–589.

Giulian, D., Ingeman, J.E., 1988. Colony-stimulating factors as promoters of ameboid microglia. J. Neurosci. 8, 4707–4717.

Glass, W.G., Liu, M.T., Kuziel, W.A., Lane, T.E., 2001. Reduced macrophage infiltration and demyelination in mice lacking the chemokine receptor CCR5 following infection with a neurotropic coronavirus. Virology 288, 8–17.

Gris, D., Marsh, D.R., Oatway, M.A., Chen, Y., Hamilton, E.F., Dekaban, G.A., Weaver, L.C., 2004. Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. J. Neurosci. 24, 4043–4051.

Hashimoto, M., Nitta, A., Fukumitsu, H., Nomoto, H., Shen, L., Furukawa, S., 2005. Involvement of glial cell line-derived neurotrophic factor in activation processes of rodent macrophages. J. Neurosci. Res. 79, 476–487.

Hausmann, O.N., 2003. Post-traumatic inflammation following spinal cord injury. Spinal Cord 41, 369–378.

Horn, K.P., Busch, S.A., Hawthorne, A.L., van Rooijen, N., Silver, J., 2008. Another barrier to regeneration in the CNS: activated macrophages induce extensive retraction of dystrophic axons through direct physical interactions. J. Neurosci. 28, 9330–9341.

Hurst, S.M., Wilkinson, T.S., McLoughlin, R.M., Jones, S., Horiuchi, S., Yamamoto, N., Rose-John, S., Fuller, G.M., Topley, N., Jones, S.A., 2001. Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. Immunity 14, 705–714.

Kawamoto, S., Niwa, H., Tashiro, F., Sano, S., Kondoh, G., Takeda, J., Tabayashi, K., Miyazaki, J., 2000. A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. FEBS Lett. 470, 263–268.

Kerr, B.J., Patterson, P.H., 2004. Potent pro-inflammatory actions of leukemia inhibitory factor in the spinal cord of the adult mouse. Exp. Neurol. 188, 391–407.

Klusman, I., Schwab, M.E., 1997. Effects of pro-inflammatory cytokines in experimental spinal cord injury. Brain Res. 762, 173–184.

Koide, Y., Morikawa, S., Mabuchi, Y., Muguruma, Y., Hiratsu, E., Hasegawa, K., Kobayashi, M., Ando, K., Kinjo, K., Okano, H., Matsuzaki, Y., 2007. Two distinct stem cell lineages in murine bone marrow. Stem Cells 25, 1213–1221.

Koopman, R., Schaart, G., Hesselink, M.K., 2001. Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. Histochem. Cell Biol. 116, 63–68.

Lacroix, S., Chang, L., Rose-John, S., Tuszynski, M.H., 2002. Delivery of hyper-interleukin-6 to the injured spinal cord increases neutrophil and macrophage infiltration and inhibits axonal growth. J. Comp. Neurol. 454, 213–228.

Lalancette-Hebert, M., Gowing, G., Simard, A., Weng, Y.C., Kriz, J., 2007. Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. J. Neurosci. 27, 2596–2605.

Lambertsen, K.L., Clausen, B.H., Babcock, A.A., Gregersen, R., Fenger, C., Nielsen, H.H., Haugaard, L.S., Wirenfeldt, M., Nielsen, M., Dagnaes-Hansen, F., Bluethmann, H., Faergeman, N.J., Meldgaard, M., Deierborg, T., Finsen, B., 2009. Microglia protect neurons against ischemia by synthesis of tumor necrosis factor. J. Neurosci. 29, 1319–1330.

Lee, S.C., Liu, W., Brosnan, C.F., Dickson, D.W., 1994. GM-CSF promotes proliferation of human fetal and adult microglia in primary cultures. Glia 12, 309–318.

Liu, M.T., Keirstead, H.S., Lane, T.E., 2001. Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. J. Immunol. 167, 4091–4097.

Matsumura, M., Banba, N., Motohashi, S., Hattori, Y., 1999. Interleukin-6 and transforming growth factor-beta regulate the expression of monocyte chemoattractant protein-1 and colony-stimulating factors in human thyroid follicular cells. Life Sci. 65, PL129–PL135.

Matsuzaki, Y., Kinjo, K., Mulligan, R.C., Okano, H., 2004. Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. Immunity 20, 87–93.

McTigue, D.M., Popovich, P.G., Morgan, T.E., Stokes, B.T., 2000. Localization of transforming growth factor-beta1 and receptor mRNA after experimental spinal cord injury. Exp. Neurol. 163, 220–230.

Merkler, D., Metz, G.A., Raineteau, O., Dietz, V., Schwab, M.E., Fouad, K., 2001. Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-A. J. Neurosci. 21, 3665–3673.

Miao, T., Wu, D., Zhang, Y., Bo, X., Subang, M.C., Wang, P., Richardson, P.M., 2006. Suppressor of cytokine signaling-3 suppresses the ability of activated signal transducer and activator of transcription-3 to stimulate neurite growth in rat primary sensory neurons. J. Neurosci. 26, 9512–9519.

Mildenberger, M., Beach, T.G., McGeer, E.G., Ludgate, C.M., 1990. An animal model of prophylactic cranial irradiation: histologic effects at acute, early and delayed stages. Int. J. Radiat. Oncol. Biol. Phys. 18, 1051–1060.

Nesic, O., Xu, G.Y., McAdoo, D., High, K.W., Hulsebosch, C., Perez-Pol, R., 2001. IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury. J. Neurotrauma 18, 947–956.

Nishimoto, N., Sasai, M., Shima, Y., Nakagawa, M., Matsumoto, T., Shirai, T., Kishimoto, T., Yoshizaki, K., 2000. Improvement in Castleman's disease by humanized anti-interleukin-6 receptor antibody therapy. Blood 95, 56–61.

Okada, S., Nakamura, M., Mikami, Y., Shimazaki, T., Mihara, M., Ohsugi, Y., Iwamoto, Y., Yoshizaki, K., Kishimoto, T., Toyama, Y., Okano, H., 2004. Blockade of interleukin-6 receptor suppresses reactive astrogliosis and ameliorates functional recovery in experimental spinal cord injury. J. Neurosci. Res. 76, 265–276.

Okada, S., Nakamura, M., Katoh, H., Miyao, T., Shimazaki, T., Ishii, K., Yamane, J., Yoshimura, A., Iwamoto, Y., Toyama, Y., Okano, H., 2006. Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. Nat. Med. 12, 829–834.

Okazaki, M., Yamada, Y., Nishimoto, N., Yoshizaki, K., Mihara, M., 2002. Characterization of anti-mouse interleukin-6 receptor antibody. Immunol. Lett. 84, 231–240.

Penkowa, M., Giralt, M., Lago, N., Camats, J., Carrasco, J., Hernandez, J., Molinero, A., Campbell, I.L., Hidalgo, J., 2003. Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. Exp. Neurol. 181, 130–148.

Ponomarev, E.D., Maresz, K., Tan, Y., Dittel, B.N., 2007. CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. J. Neurosci. 27, 10714–10721.

Popovich, P.G., Guan, Z., Wei, P., Huitinga, I., van Rooijen, N., Stokes, B.T., 1999. Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Exp. Neurol. 158, 351–365.

Popovich, P.G., Guan, Z., McGaughy, V., Fisher, L., Hickey, W.F., Basso, D.M., 2002. The neuropathological and behavioral consequences of intraspinal microglial/macrophage activation. J. Neuropathol. Exp. Neurol. 61, 623–633.

Rapalino, O., Lazarov-Spiegler, O., Agranov, E., Velan, G.J., Yoles, E., Fraidakis, M., Solomon, A., Gepstein, R., Katz, A., Belkin, M., Hadani, M., Schwartz, M., 1998. Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. Nat. Med. 4, 814–821.

Rinner, W.A., Bauer, J., Schmidts, M., Lassmann, H., Hickey, W.F., 1995. Resident microglia and hematogenous macrophages as phagocytes in adoptively transferred experimental autoimmune encephalomyelitis: an investigation using rat radiation bone marrow chimeras. Glia 14, 257–266.

Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., van Hinsbergh, V., Sozzani, S., Bussolino, F., Poli, V., Ciliberto, G., Mantovani, A., 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity 6, 315–325.

Rotshenker, S., Reichert, F., Gitik, M., Haklai, R., Elad-Sfadia, G., Kloog, Y., 2008. Galectin-3/MAC-2, Ras and PI3K activate complement receptor-3 and scavenger receptor-AI/II mediated myelin phagocytosis in microglia. Glia 56, 1607–1613.

Sato, K., Tsuchiya, M., Saldanha, J., Koishihara, Y., Ohsugi, Y., Kishimoto, T., Bendig, M.M., 1993. Reshaping a human antibody to inhibit the interleukin 6-dependent tumor cell growth. Cancer Res. 53, 851–856.

Saville, L.R., Pospisil, C.H., Mawhinney, L.A., Bao, F., Simedrea, F.C., Peters, A.A., O'Connell, P.J., Weaver, L.C., Dekaban, G.A., 2004. A monoclonal antibody to CD11d reduces the inflammatory infiltrate into the injured spinal cord: a potential neuroprotective treatment. J. Neuroimmunol. 156, 42–57.

Schilling, M., Besselmann, M., Muller, M., Strecker, J.K., Ringelstein, E.B., Kiefer, R., 2005. Predominant phagocytic activity of resident microglia over hematogenous macrophages following transient focal cerebral ischemia: an investigation using green fluorescent protein transgenic bone marrow chimeric mice. Exp. Neurol. 196, 290–297.

Schwartz, M., Lazarov-Spiegler, O., Rapalino, O., Agranov, I., Velan, G., Hadani, M., 1999. Potential repair of rat spinal cord injuries using stimulated homologous macrophages. Neurosurgery 44, 1041–1045 discussion 1045-1046.

Sedgwick, J.D., Schwender, S., Imrich, H., Dorries, R., Butcher, G.W., ter Meulen, V., 1991. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proc. Natl. Acad. Sci. U. S. A. 88, 7438–7442.

Sharma, H.S., Winkler, T., Stalberg, E., Gordh, T., Alm, P., Westman, J., 2003. Topical application of TNF-alpha antiserum attenuates spinal cord trauma induced edema formation, microvascular permeability disturbances and cell injury in the rat. Acta Neurochir. Suppl. 86, 407–413.

Steinmetz, M.P., Horn, K.P., Tom, V.J., Miller, J.H., Busch, S.A., Nair, D., Silver, D.J., Silver, J., 2005. Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with the degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord. J. Neurosci. 25, 8066–8076.

Swartz, K.R., Liu, F., Sewell, D., Schochet, T., Campbell, I., Sandor, M., Fabry, Z., 2001. Interleukin-6 promotes post-traumatic healing in the central nervous system. Brain Res. 896, 86–95.

Tamura, T., Udagawa, N., Takahashi, N., Miyaura, C., Tanaka, S., Yamada, Y., Koishihara, Y., Ohsugi, Y., Kumaki, K., Taga, T., et al., 1993. Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. Proc. Natl. Acad. Sci. U. S. A. 90, 11924–11928.

Tuna, M., Polat, S., Erman, T., Ildan, F., Gocer, A.I., Tuna, N., Tamer, L., Kaya, M., Cetinalp, E., 2001. Effect of anti-rat interleukin-6 antibody after spinal cord injury in the rat: inducible nitric oxide synthase expression, sodium- and potassium-activated, magnesium-dependent adenosine-5′-triphosphatase and superoxide dismutase activation, and ultrastructural changes. J. Neurosurg. 95, 64–73.

Turrin, N.P., Plante, M.M., Lessard, M., Rivest, S., 2007. Irradiation does not compromise or exacerbate the innate immune response in the brains of mice that were transplanted with bone marrow stem cells. Stem Cells 25, 3165–3172.

Van Wagoner, N.J., Benveniste, E.N., 1999. Interleukin-6 expression and regulation in astrocytes. J. Neuroimmunol. 100, 124–139.

Cell Transplantation, Vol. 20, pp. 727–739, 2011

0963-6897/11 $90.00 + .00
DOI: 10.3727/096368910X536554
E-ISSN 1555-3892
www.cognizantcommunication.com

Comparative Study of Methods for Administering Neural Stem/Progenitor Cells to Treat Spinal Cord Injury in Mice

Yuichiro Takahashi,*† Osahiko Tsuji,* Gentaro Kumagai,‡ Chikako Miyauchi Hara,† Hirotaka James Okano,† Atsushi Miyawaki,§¶ Yoshiaki Toyama,* Hideyuki Okano,† and Masaya Nakamura*

*Department of Orthopaedic Surgery, School of Medicine, Keio University, Tokyo, Japan
†Department of Physiology, School of Medicine, Keio University, Tokyo, Japan
‡Department of Orthopaedic Surgery, School of Medicine, Hirosaki University, Aomori, Japan
§Laboratory for Cell Function and Dynamics, Advanced Technology Development Group, Brain Science Institute, RIKEN, Saitama, Japan
¶Life Function and Dynamics, ERATO, JST, Saitama, Japan

To investigate potential cures for spinal cord injury (SCI), several researchers have transplanted neural stem/progenitor cells (NS/PCs) into the injured spinal cord by different procedures, including intralesional (IL), intrathecal (IT), and intravenous (IV) injection. However, there are no reports quantifying or comparing the number of cells successfully transplanted to the lesion site by each procedure in vivo. The purpose of the present study was to determine the optimal method of cell transplantation to the SCI site in terms of grafted cell survival and safety. For this purpose, we developed mouse NS/PCs that expressed a novel Venus-luciferase fusion protein that enabled us to detect a minimum of 1,000 grafted cells in vivo by bioluminescence imaging (BLI). After inducing contusive SCI at the T10 level in mice, NS/PCs were transplanted into the injured animals three different ways: by IL, IT, or IV injection. Six weeks after the transplantation, BLI analysis showed that in the IL group, the luminescence intensity of the grafted cells had decreased to about 10% of its initial level, and appeared at the site of injury. In the IT group, the luminescence of the grafted cells, which was distributed throughout the entire subarachnoid space immediately after transplantation, was detected at the injured site 1 week later, and by 6 weeks had gradually decreased to about 0.3% of its initial level. In the IV group, no grafted cells were detected at the site of injury, but all of these mice showed luminescence in the bilateral chest, suggesting pulmonary embolism. In addition, one third of these mice died immediately after the IV injection. In terms of grafted cell survival and safety, we conclude that the IL application of NS/PCs is the most effective and feasible method for transplanting NS/PCs into the SCI site.

Key words: Spinal cord injury (SCI); Transplantation; Neural stem/progenitor cells (NS/PCs); Bioluminescence imaging (BLI)

INTRODUCTION

Because the adult central nervous system (CNS) has little potential for regeneration, spinal cord injury (SCI) usually results in severe damage, leading to paraplegia, tetraplegia, or worse. Several strategies have been used to develop new therapies that would allow patients to regain the use of their paralyzed limbs. Because cell transplantation is one of the potential therapies, various kinds of cells have been used as the transplantation source for SCI (14,18,32–34,37,38,46). Among them, neural stem/progenitor cell (NS/PC) transplantation is considered one of the most promising, because the transplantation of NS/PCs into rodent SCI models (13,15,27,42,44,48) and of human neural stem/progenitor cells (NS/PCs) into a marmoset SCI model (28) have been shown to promote functional recovery.

From the viewpoint of clinical trials, it is critical to determine how best to apply cells to the injured spinal cord. Three application methods for cell transplantation for SCI are used: intralesional (IL), intrathecal (IT), and intravenous (IV). Some previous studies have sought to

Received February 18, 2010; final acceptance September 24, 2010. Online prepub date: November 5, 2010.
Address correspondence to Masaya Nakamura, Department of Orthopaedic Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan. Tel: +81-3-3353-1211; Fax: +81-3353-6597; E-mail: masa@sc.itc.keio.ac.jp *or* Hideyuki Okano, Department of Physiology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan. Tel: +81-3-5363-3747; Fax: +81-3-3357-5445; E-mail: hidokano@sc.itc.keio.ac.jp

compare different cell transplantation methods for treating SCI. For example, the IL application of NS/PCs was reported to be more effective than the IT application (43), and the IV application of NS/PCs more effective than the IL application (10). However, these reports are not directly comparable, because they did not use the same number of cells for the initial transplants. Previous reports using other kinds of cells for transplantation showed that the IL application was more effective than IT or IV for introducing cells to the SCI site (7,18,67), and that IT was more effective than IV (6,54). However, the number of cells transplanted by each method was not quantified or compared for the different methods in vivo, and the safety of the different methods was not compared. Moreover, some reports did not use uniform numbers of cells for the initial transplantation or examine the grafted cells in vivo over the long term. Therefore, it has remained unclear which application of NS/PCs is best for injured spinal cord in terms of cell survival and safety.

In the present study, to determine the best way to transplant NS/PCs into the SCI site, we applied NS/PCs to the mouse injured spinal cord by IL, IT, or IV injection, and quantitatively examined the survival and distribution of the grafted cells, as well as the complications associated with NS/PC transplantation, such as tumor formation and pulmonary embolism. For this purpose, we developed a new reporter gene for bioluminescence imaging (BLI), which enabled us to quantify the bioluminescent signals of small numbers of cells in vivo over time.

MATERIALS AND METHODS

Lentiviral Vector Expressing the ffLuc Reporter Gene

A novel fusion HIV-1-based lentiviral vector, expressing ffLuc under the EF1α promoter (see Fig. 1A) (Hara et al., in preparation), was used in this study. ffLuc is composed of Venus and Luc2: Venus is a kind of yellow fluorescence protein (40), Luc2 is firefly (*Photinus pyralis*)-derived luciferase. The luciferase gene fragment was excised from the pGL4-Basic vector (Promega, Madison, WI). This vector enabled the detection of grafted cells as strong bioluminescent signals from ffLuc in live SCI mice and as fluorescent signals in spinal cord sections. Twenty-four hours before the transfection, 293T cells were seeded into poly-L-lysine-coated T175 flasks. The cells were transfected using the lipofection protocol for the FuGENE6 transfection reagent (Roche, Indianapolis, IN). Three days after transfection, the conditioned medium was collected, and the virus was concentrated by centrifugation at 25,000 rpm for 1.5 h at 4°C. The pelleted viral particles were resuspended and stored frozen at −80°C. The titer of the concentrated virus was 1×10^8 to 2×10^8 transducing units per milliliter (TU/ml) when assayed using 293T cells, and the infectivity was determined by fluorescence expression as analyzed on a FACS Calibur (Becton-Dickinson, Franklin, Lakes, NJ).

NS/PC Labeling and Differentiation Assay

NS/PCs were cultured as reported previously (55). In brief, the striatum of a C57BL/6J mouse on embryonic day 14 was dissociated using a fire-polished glass pipette, and the dissociated cells were collected by centrifugation and resuspended in culture medium. The culture medium consisted of DMEM/F12 supplemented with a hormone mixture as described previously (55). 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 virus was added to the culture medium to infect primary NS/PCs (multiplicity of infection, MOI = 1.0). After being propagated for two passages, the neurospheres were used for in vivo BLI or dissociated into single cells and plated onto poly-L-ornithine-coated coverslips at a density of 1×10^5 cells/ml for in vitro analysis. The virally transduced NS/PCs were allowed to differentiate for 5 days and were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for immunocytochemistry.

SCI Model

Adult female C57BL/6J mice (20–22 g, $n = 20$; Clea, Tokyo, Japan) were anesthetized with an IP injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After laminectomy at the 10th thoracic spinal vertebra (T10), the dorsal surface of the 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 (47). This device creates a reliable contusion injury by rapidly applying a force-defined impact (60 kdyn) with a stainless steel-tipped impactor. All procedures were approved by the ethics committee of Keio University, and were in accordance with the Guide for the Care and Use of Laboratory Animals.

NS/PC Transplantation

The lentivirally transduced NS/PCs were transplanted into each mouse immediately after SCI using one of the three different methods: IL cell transplantation (IL group, $n = 5$), IT cell transplantation (IT group, $n = 5$), and IV cell transplantation (IV group, $n = 10$). For the IL group, the tip of a glass micropipette was inserted into the epicenter of the injured spinal cord, and the NS/PCs were

injected through it. For the IT group, a laminectomy was performed at the L3 level, and the NS/PCs were injected into the subarachnoid space at the same level, through a 32-gauge catheter. For the IV group, the NS/PCs were injected into the tail vein or femoral vein through a 32-gauge catheter. For all the applications, the number and concentration of the transplanted cells were the same (5×10^5 cells/2 μl), and the NS/PCs were injected at a rate of 1 μl/min with a Hamilton syringe (25 μl) and stereotaxic microinjector (KDS 310, Muromachi-kikai, Tokyo, Japan). The number of viable cells in the suspensions was determined by cell counting using trypan blue dye and by the signal intensity of the NS/PCs determined by BLI prior to transplantation.

Bioluminescence Imaging

The Xenogen-IVIS 100 cooled CCD optical macroscopic imaging system (SC BioScience, Tokyo, Japan) (56) was used for BLI. Both in vitro and in vivo BLI was performed as previously reported (47). In brief, the signal intensity of the lentivirally transduced NS/PCs in vitro was examined on cells plated at various densities (approximate range 1×10^2 to 10^6 cells/10 μl) and the bioluminescent images were captured immediately after the addition of D-luciferin [D-(−)-2-(6′-hydroxy-2′-benzothiazolyl) thiazone-4-carboxylic acid] (15 mg/ml). In vivo BLIs were captured 15 min after the IP injection of D-luciferin (0.3 mg/g body weight) with the field-of-view set at 10 cm, because the photon count was most stable with its peak intensity between 10 and 30 min after the IP injection of D-luciferin. The integration time was fixed at 5 min for each image. All images were analyzed with the Igor (WaveMetrics, Lake Oswego, OR) and Living Image software (Xenogen, Alameda, CA), and the optical signal intensity was expressed as photon-flux (photon count), in units of photons/s/cm^2/steradian. Each image was displayed as a pseudocolored photon count image superimposed on a gray scale anatomic image. To quantify the measured light, we defined regions of interest (ROI) over the cell-implanted area and examined all values in the same ROI.

Immunohistochemistry

Animals were anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS 6 weeks after the transplantation. The spinal cord was removed, embedded in OCT compound, frozen in liquid nitrogen, and sagittally sectioned at 30 μm on a cryostat. For immunofluorescence staining, cultured cells or tissue sections were stained with primary antibodies, including anti-green fluorescence protein (GFP) (1:500; MBL, Woburn, MA), anti-glial fibrillary acid protein (GFAP) (1:500; AIC, Long Beach, CA), anti-β-III-tubulin (Tuj-1) (1:200; Sigma, St. Louis, MO), anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) (1:200; Sigma), anti-Hu (1:1000; a gift from Dr. Robert Darnell, The Rockefeller University), and anti-APC (CC-1) (1:200; Oncogene, Cambridge, MA). Hoechst 33342 was used to counterstain the nuclei (Molecular Probes, Eugene, OR) and then samples were incubated with Alexa Fluor-conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR). Images were obtained by fluorescence microscopy (Axiocam; Carl Zeiss, Munich, Germany) or confocal microscopy (LSM700; Carl Zeiss). For diaminobenzidine staining, the sections were incubated at 4°C with anti-GFP (1:500; MBL), followed by biotinylated secondary antibodies (1:500; Jackson Immunoresearch). Biotinylated antibodies were visualized using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), followed by diaminobenzidine (Sigma). Quantitative analysis of the NS/PCs after their differentiation in vitro was performed as described previously (47).

RESULTS

In Vitro Imaging and Differentiation Assay of Lentivirally Transduced NS/PCs

To identify and monitor the transplanted cells, NS/PCs were labeled by lentiviral infection with ffLuc expressed under the EF1α promoter (Fig. 1A). Owing to the stable and strong emission of ffLuc, which is a fusion protein of Venus and luciferase [(39), Hara et al., in preparation] we could observe the fluorescent signals of the Venus by fluorescence microscopy (Fig. 1B) and the bioluminescent signals of luciferase with BLI (Fig. 1C). FACS Calibur analysis revealed that >70% of these cells were positive for Venus (data not shown). The minimum number of cells that could be measured by their photon counts with BLI was approximately 100 cells in vitro. There was a linear correlation between the number of labeled cells and the photon count (Fig. 1D). The NS/PCs expressing ffLuc showed about 10 times stronger bioluminescence intensity in vitro than the reported intensity of NS/PCs expressing conventional luciferase with IRES-Venus (47). To confirm that the lentiviral transduction did not alter the properties of the NS/PCs, we performed differentiation assays on transduced NS/PCs. The transfected NS/PCs could be maintained and passaged using the conventional neurosphere method, and an in vitro differentiation assay revealed that the trasduced NS/PCs differentiated into Tuj1-positive (21.1 ± 2.1%), GFAP-positive (50.0 ± 2.8%), and CNPase-positive (17.9 ± 8.1%) cells (Fig. 1E–H), which are markers for neurons, astrocytes, and oligodendrocytes, respectively. These results showed the lentivirus-transduced NS/PCs had a similar multipotency to that reported for nontransduced NS/PCs (47).

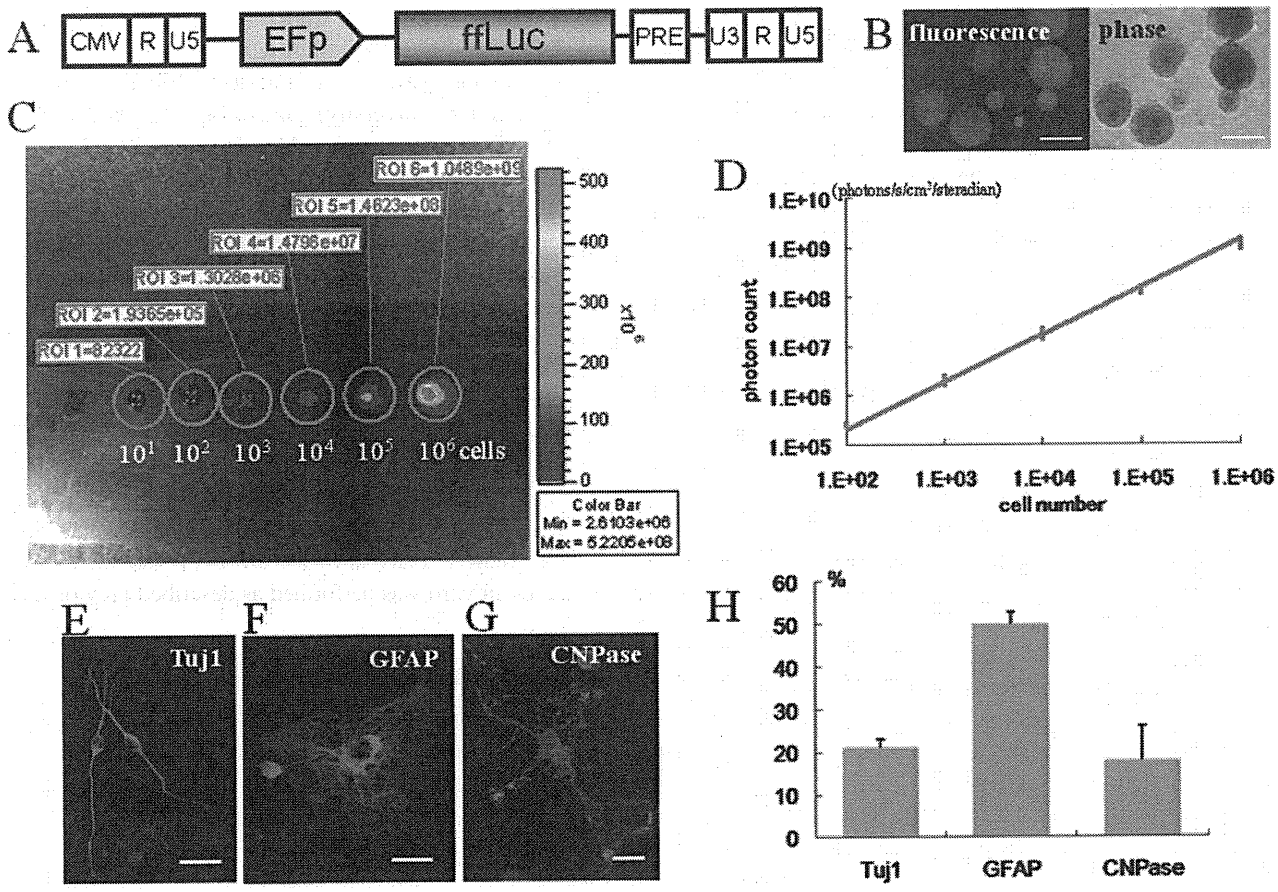

Figure 1. In vitro imaging and differentiation of lentivirally transduced NS/PCs. (A) Structure of the HIV-1-based lentivirus vector that expresses ffLuc under the elongation factor (EF)-1α promoter. (B) Fluorescent (left) and phase-contrast (right) images of neurospheres derived from ffLuc-expressing NS/PCs. (C) The ffLuc-expressing NS/PCs showed strong luciferase intensity. (D) There was a linear correlation between the number of labeled NS/PCs and their photon count in vitro. Data are the mean ± SEM; $n = 10$. (E–G) The ffLuc-expressing NS/PCs differentiated into Tuj1-positive (E), GFAP-positive (F), and CNPase-positive (G) cells. (H) Quantitative analysis of the ffLuc-expressing NS/PC phenotype after differentiation. Data are the mean ± SEM; $n = 3$. Scale bars: 200 μm (B), 20 μm (E–G).

Detection Limits for In Vivo Imaging of NS/PCs Transplanted Into Intact Spinal Cord

When transplanted into the intact spinal cord of adult C57BL/6 mice, the transfected NS/PCs showed one tenth of the photon count that was observed in vitro (Fig. 2A). There was a linear correlation between the numbers of grafted cells and the photon count in vivo (Fig. 2B), and the minimum number of grafted cells that could be measured with BLI was approximately 1,000 cells (Fig. 2C). As few as 100 grafted cells could be detected as bioluminescence in the normal spinal cord after opening the skin at the transplantation site (Fig. 2D).

In Vivo Imaging of NS/PCs Grafted Into the Injured Spinal Cord

To examine the survival and distribution of the transplanted cells in vivo, the photon counts of the grafted cells in each mouse were measured weekly by BLI for 6 weeks after transplantation. In the IL group, the photon counts of the grafted cells were detected at the lesion site for all 6 weeks, in spite of their decrease to approximately 30% of the initial level at 7 days and to 9.4 ± 0.03% at 6 weeks after the transplantation (Fig. 3A, B). In the IT group, the grafted cells were distributed along the entire subarachnoid space immediately after transplantation, and some were detected at the lesion site 7 day later. The bioluminescence of the grafted cells at the lesion site gradually disappeared thereafter. The photon counts of the grafted cells also decreased, to about 5% of the initial level at 1 week and 0.3 ± 0.0%, which is almost background measurement value, at 6 weeks after transplantation (Fig. 3C, D). In the IV group, no bioluminescence was detected at either the site of injury or in the uninjured spinal cord at any of the time points examined (Fig. 3E, F). In contrast, strong biolumines-

cence was observed in the bilateral chest immediately after the injection of the NS/PCs in all 10 animals of the IV group (Fig. 3G), and 3 of the 10 animals died immediately after the injection. In these mice, we confirmed the strong bioluminescence of the grafted cells in the lung (Fig. 3H). Although the luminescence in the bilateral chest became undetectable the next day in the surviving mice, two of the other seven mice died within 4 days of the transplantation. Five of the original 10 mice in the IV group survived for the full 6 weeks following the cell injection.

Immunohistochemistry

To examine the survival and distribution of the grafted cells histologically, immunohistochemistry with an anti-GFP antibody, which could detect ffLuc protein, was performed 6 weeks after transplantation to identify the ffLuc-positive grafted cells. Among the three groups, the IL group showed the best survival of ffLuc-positive cells at the lesion site (Fig. 4A, B). In the IT group, only a few grafted cells were detected at the lesion site (Fig. 4C, D). We could not detect any grafted cells at the lesion site in the IV group (Fig. 4E, F). In IL group, these grafted cells differentiated into neurons, astrocytes, and oligodendrocytes within the injured spinal cord (Fig. 4G–I), as previously reported (47). In the IT group, there were some ffLuc-positive cell clusters that lay on the surface of the spinal cord (Fig. 5A, C, D) and the cauda equina (Fig. 5B, E, F). These cells, which also differentiated into neurons, astrocytes, and oligodendrocytes (Fig. 5G–I), attached on the surface of pia matter of the uninjured spinal cord, but not invaded into the spinal cord tissue (Fig. 5C–F). In the IV group, ffLuc-positive cells were observed in the lung and spleen, and GFP-positive fragments were found in the glomeruli of the kidney (Fig. 6), although no ffLuc-positive cells were detected at the lesion site. There was no tumor formation, at least 6 weeks after transplantation, in any of the three groups.

DISCUSSION

In the present study we used a new reporter gene, ffLuc, and BLI for the longitudinal tracking and quantification of grafted cells in vivo. Our findings demonstrated that the IL injection of NS/PCs resulted in the best survival of the grafted cells among the three appli-

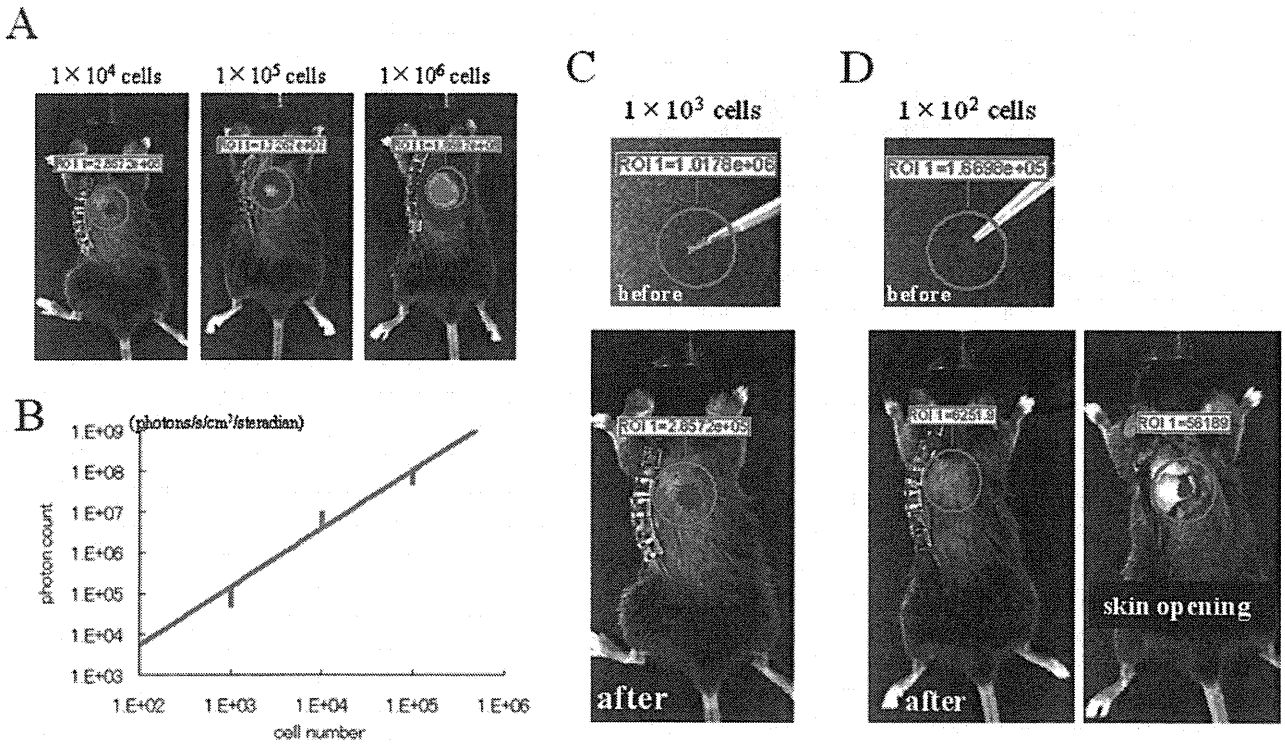

Figure 2. In vivo imaging of the grafted NS/PCs in the intact spinal cord. (A) Luminescence of the grafted NS/PCs was detected in vivo. In vivo, their photon counts were approximately one tenth of those observed in vitro. (B) There was a linear correlation between the number of grafted NS/PCs and the photon count in vivo. Data are the mean ± SEM; $n = 3$. (C) The luminescence of 1,000 cells transplanted into the normal spinal cord was detectable by photon count. (D) The luminescence of 100 cells grafted into the normal spinal cord was undetectable without opening the skin.

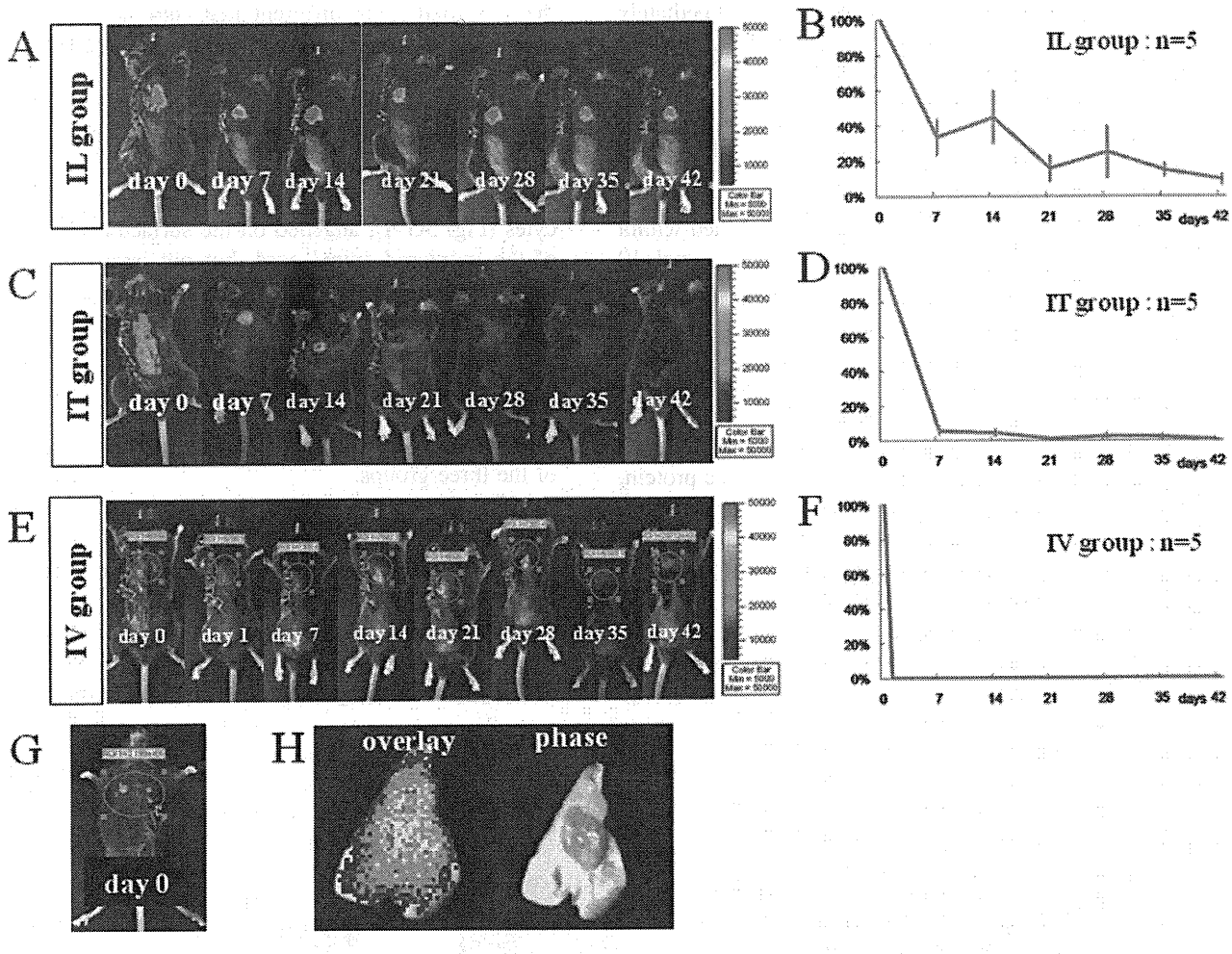

Figure 3. (A, C, E) Representative in vivo images of NS/PCs transplanted by the three application methods: (A) IL group, (C) IT group, and (E) IV group. (B, D, F) Quantitative analysis of the photon counts of the grafted NS/PCs in the IL group (B), IT group (D), and IV group (F). Data are the mean ± SEM; $n = 5$. (G) Strong luminescence was observed in the bilateral chest immediately after transplantation in all 10 animals of the IV group. (H) A resected lung from the IV group showed strong luminescence from the transplanted cells.

cations, and no complications were seen in the recipient mouse. Our analysis also revealed the distribution of the cells transplanted by the three methods.

To analyze the kinetics of cells after their transplantation in vivo, several previous studies have used immunohistochemistry, gamma scintigraphic imaging (1,11,20, 24), and magnetic resonance imaging (MRI) (3,26,29, 53). However, none of these methods permit the quantitative and continuous evaluation of the transplanted cells. Immunohistochemical analysis cannot track the fate of grafted cells in the same animal, because the animals need to be sacrificed at serial posttransplant time points to examine the grafted cells. Scintigraphic imaging using a radioactive tracer to label grafted cells enables the examination of their survival and distribution in vivo, but they can only be observed for a short time, owing to the tracer's short half-life and the dilution effect by cell division. MRI can detect the distribution of the transplanted cells at their initial deposition with high sensitivity, but the MRI signals from dead cells cannot be distinguished from those of live signals, which diminishes the usefulness of this method over time.

To overcome these problems, we used a BLI system, which enabled us to trace only living grafted cells in the same animal at multiple time points and to quantify the bioluminescence intensity of the grafted cells as a photon count (17,22,25,35,56,60,66). Although there are previous reports in which grafted cells were analyzed by BLI in injured spinal cord (33,47), the intensity of the bioluminescence in those studies was not strong enough to detect small numbers of cells. In the present study, we used a novel fusion protein, ffLuc. NS/PCs express-

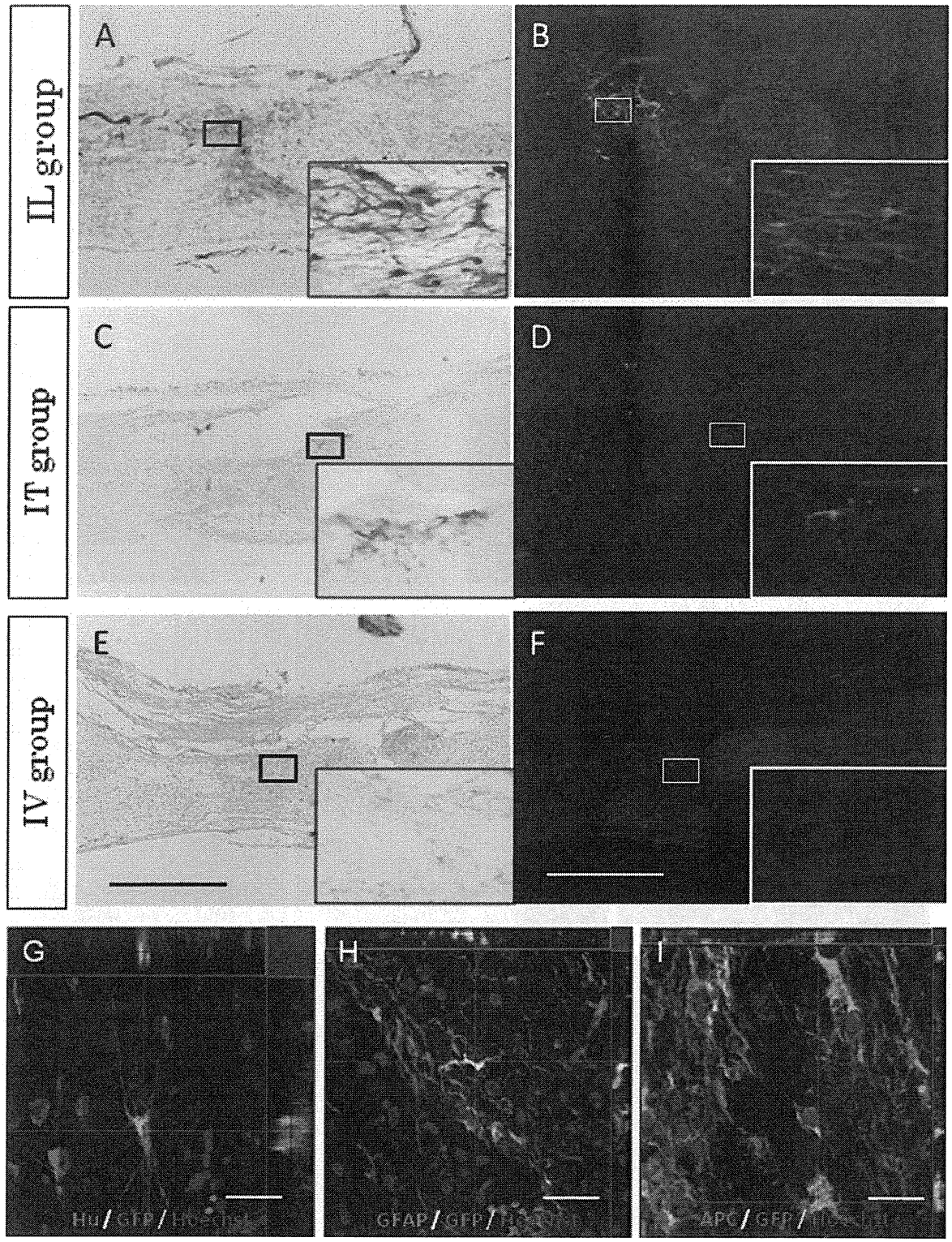

Figure 4. Immunohistochemical analysis of the injured spinal cord 6 weeks after transplantation. The grafted cells were identified as ffLuc-positive cells by diaminobenzidine (A, C, E) and immunofluorescence (B, D, F) staining. (A, B) In the IL group, the grafted cells survived well at the lesion site. (C, D) In the IT group, a few grafted cells survived, and some were present at the surface of the lesion site. (E, F) In the IV group, no grafted cells could be detected at the lesion site. (G, H, I) In the IL group, the grafted cells differentiated into Hu-positive (G), GFAP-positive (H) and APC-positive (I) cells within the injured spinal cord. Scale bars: 500 μm at magnification 25×, inset at magnification 400× (A–F); 20 μm (G–I).

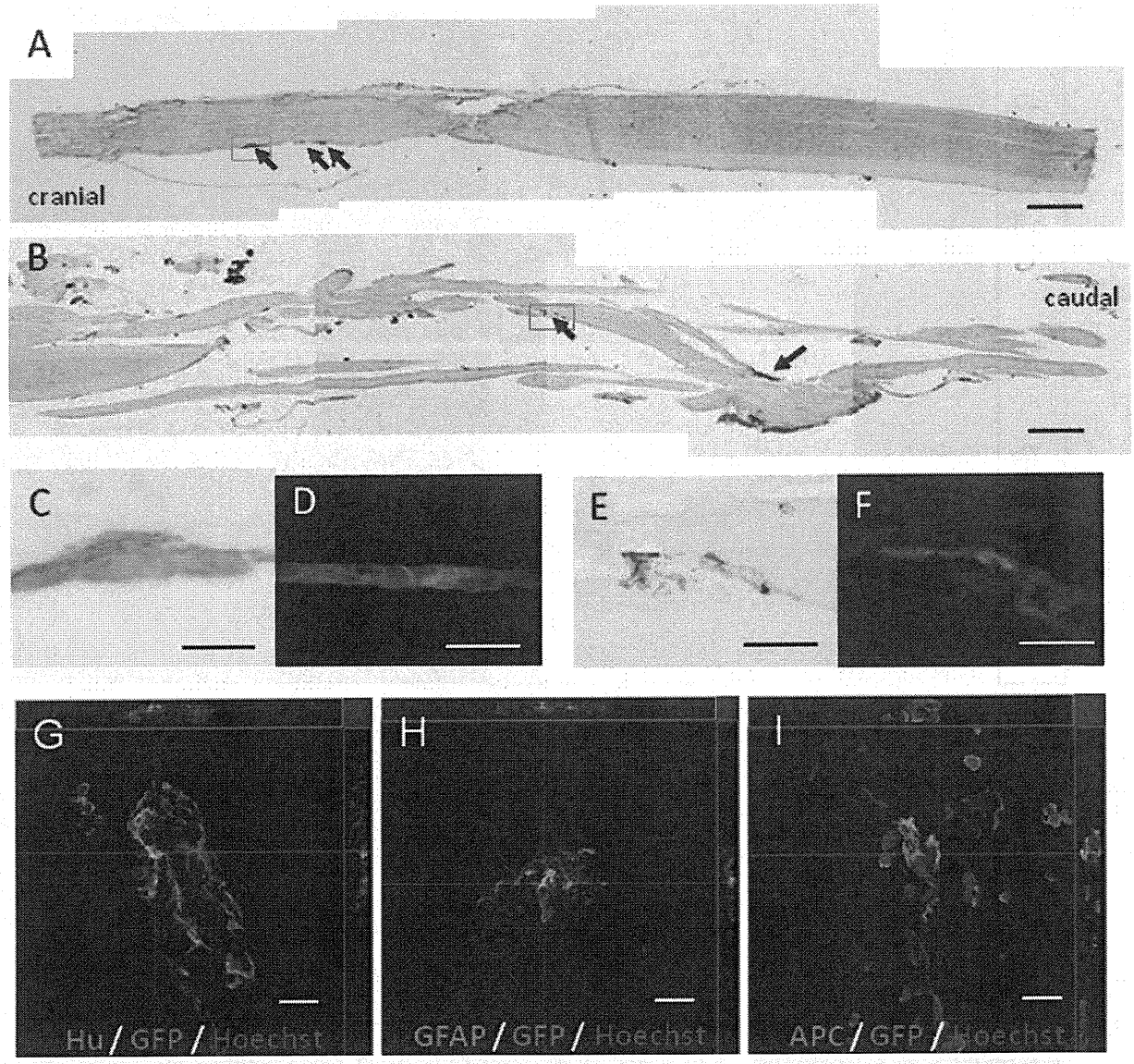

Figure 5. Immunohistochemical analysis of the IT group. (A, B) The grafted cells (arrow) were distributed on the surface of the uninjured spinal cord (A) and the cauda equina at 6 weeks after transplantation (B). (C, D) High magnification of the box in (A); the grafted cells were attached to the spinal cord surface, as shown by diaminobenzidine (C) and immunofluorescence (D) staining. (E, F) High magnification of the box in (B); the grafted cells were attached to the cauda equina, as shown by diaminobenzidine (E) and immunofluorescence (F) staining. (G, H, I) In the IT group, few grafted cells survived and differentiated into Hu-positive (G), GFAP-positive (H), and APC-positive (I) cells on the surface of the uninjured spinal cord and the cauda equina. Scale bars: 500 μm (A, B), 100 μm (C–F), 20 μm (G–I).

ing ffLuc emitted bioluminescent signals almost 10 times stronger than those of NS/PCs expressing conventional luciferase with IRES-Venus (47), thus enabling the detection of the bioluminescent signals of transplanted NS/PCs, even though they were present in small number or deep inside the mouse body. Because the ffLuc that is transfected by lentivirus is expressed in the NS/PCs permanently, we can trace the grafted NS/PCs accurately for a long period after transplantation.

From the viewpoint of clinical trials for cell transplantation to treat SCI, there is concern about causing additional damage to the injured spinal cord during the cell transplantation procedure. However, several researchers who have performed IL cell transplantation for SCI demonstrated that motor function is not degraded by the IL application (16); rather, the transplantation of cells by IL injection promotes functional recovery (19, 30,33,42,44,47).

The IT application is performed in two ways: one is by injection into the fourth ventricle (4,45,69,70) and the other is by lumbar puncture (5,21,36). Because the IT application via lumbar puncture is considered less invasive than transplantation by IL injection, this procedure has been used for cell transplantation into SCI patients in some hospitals (12,59,64). Previous studies also demonstrated that grafted NS/PCs transplanted through an IT application survive within the injured spinal cord (4,36,69). In the present study, however, cells transplanted by IT initially dispersed throughout the entire subarachnoid space, resulting in low survival at the lesion site. These findings suggested that the IT application is an uncertain method for delivering cells to the site of injury. Moreover, a small number of the grafted cells survived on the surface of the uninjured spinal cord and cauda equina. Although there was no evidence of tumor formation during the observation period of this study, it was previously reported that neural cells deposited at uninjured spinal cord sites had formed enlarging clusters of cells within a couple of weeks after transplantation (4). Furthermore, in a recent clinical procedure using an IT application, human NS/PCs caused a donor-derived brain tumor in a patient with ataxia telangiectasia, which was associated with comprised immunity (2). Therefore, it is highly likely that intrathecally applied cells adhering to the surface of the spinal cord could cause tumor formation in SCI therapy, too.

IV injection has been considered the most minimally invasive application procedure for several types of cells (31,61,63), and is already used for clinical therapies such as hematopoietic stem cell transplantation. However, in the present study, the intravenously injected NS/PCs were distributed throughout the body, and no grafted cells were detected within the injured spinal cord, although some grafted cells were trapped in the lung, spleen, and kidney. The ffLuc-positive fragments found in the kidney were broken grafted cells. Previous reports demonstrated that intravenously injected nonneural cells could be observed at a lesion site shortly after the transplantation, and the mechanisms for functional recovery in this situation are thought to be trophic support of the grafted cells and/or activation of the intrinsic neuronal cells, rather than the displacement of lost neurons and glia at the lesion site, especially for mesenchymal stem cell or bone-marrow-derived stem cell transplantation (52,62). However, the important mechanisms for functional recovery from SCI are not only trophic support, but also neural circuit reconstruction, axonal induction, and remyelination (8,50,65). The cells grafted into the host spinal cord play a role in maintaining the improved performance. Cummings et al. showed that se-

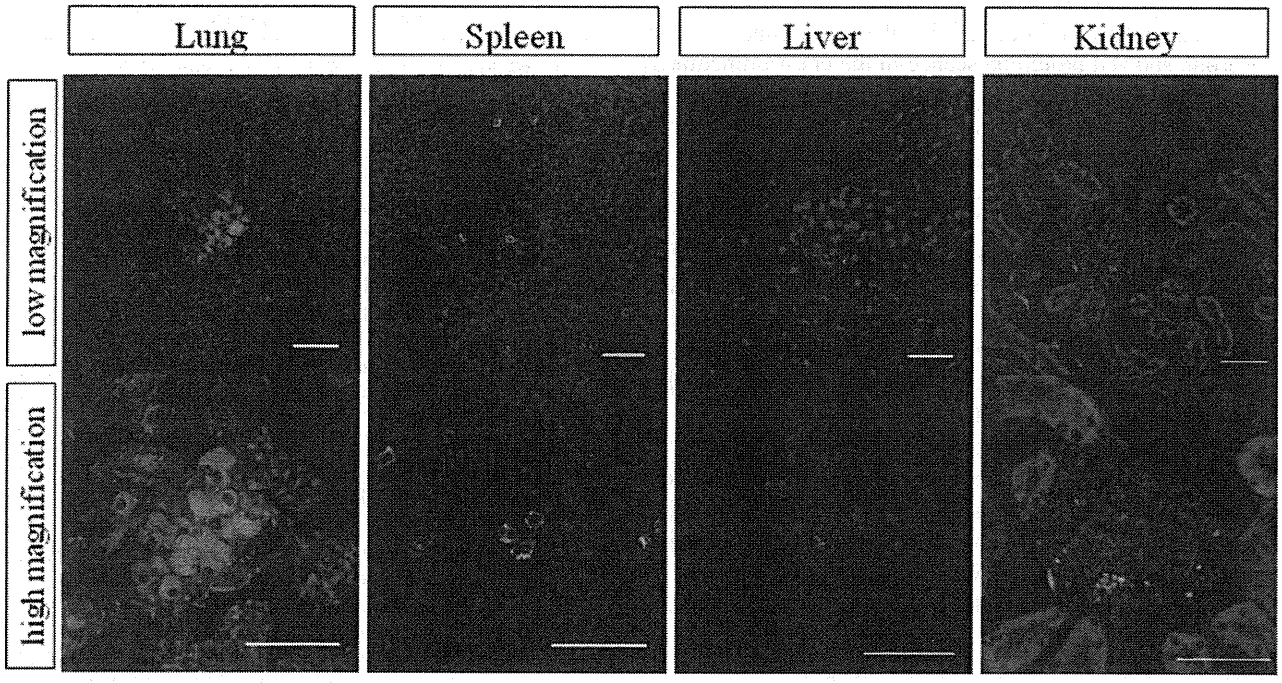

Figure 6. Immunohistochemical analysis of the several organs in the IV group. ffLuc-positive cells were found in the lung and spleen at 6 weeks after transplantation. Many ffLuc-positive fragments were also observed in the kidney, suggesting that degraded grafted cells had been trapped in the glomeruli. Scale bar: 50 μm.

lective ablation of the grafted cells in the host spinal cord causes the deterioration of motor function (19), indicating that the long-term survival of grafted cells in the host spinal cord is very important for functional recovery from SCI.

In the present study, we recognized the functional recovery in the IL group according to the behavioral analysis by BMS score (9) as reported previously (27,44,47), but not in the IT and IV groups (data not shown). However, there remained several possibilities that some mice technically might have received cauda equina impediment by IT injection and that some mice transplanted by femoral vein injection might also have suffered from postoperative scar formation producing hip joint contracture. Thus, further studies would be necessary to obtain a conclusive result in terms of functional recovery. The timing of cell transplantation is critical for the survival of grafted cells because microenvironments of injured spinal cord such as neurotrophic factors, proinflammatory cytokines, chemokines, and free radicals are different among the acute, subacute, and chronic phases of SCI (23,41,49,58). Previous studies indicated that the intralesionally grafted cells at the subacute phase showed better survival than at the acute phase (51). In the present study, we dared to choose the acute phase transplantation because the repair of blood–brain barrier and glial scar formation, which occur at the subacute and chronic phase, could prevent the grafted cells from infiltrating into the injured spinal cord after IT or IV injection at the subacute or chronic phase (57,68).

In the present study, all 10 of the animals in the IV group showed bioluminescence in the chest immediately after transplantation, three died immediately after the injection, and two more died within 4 days, due to pulmonary embolism. To reduce the risk of embolism, the grafted cells should be dissociated completely into single cells, or large cell clusters should be eliminated by filtration. However, complete dissociation of the grafted cells could decrease cell viability compared with the viability of small clusters. Furthermore, because both the IV and IT applications resulted in grafted cells homing to nonlesion sites, longer term observation is needed to evaluate tumor formation.

In conclusion, the transplantation of NS/PCs by IL injection resulted in the best survival of grafted cells among the three methods investigated, and no complications were seen afterwards. Transplantation by IT injection resulted in low survival of the grafted cells at the lesion site, and some cells were detected on the surface of the uninjured spinal cord, along the subarachnoid space. After the IV application, no grafted cells were detected at the SCI site, whereas luminescence was observed in the bilateral chest of all these mice, suggesting that the grafted cells caused pulmonary embolism immediately after the IV injection. Taken together, our findings indicate that the best method for the transplantation of NS/PCs into the SCI site is by IL injection, in terms of both grafted cell survival and safety of the recipient.

ACKNOWLEDGMENTS: We thank T. Harada for expert animal care and S. Miyao for technical support. This work was supported by grants from the Key Technology Project for the Realization of Regenerative Medicine from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Japan to M.N. and H.O.; the General Insurance Association of Japan to M.N. and Y.T.; and a Grant-in-Aid for the Global COE program from MEXT to Keio University.

REFERENCES

1. Aicher, A.; Brenner, W.; Zuhayra, M.; Badorff, C.; Massoudi, S.; Assmus, B.; Eckey, T.; Henze, E.; Zeiher, A. M.; Dimmeler, S. Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling. Circulation 107(16):2134–2139; 2003.
2. Amariglio, N.; Hirshberg, A.; Scheithauer, B. W.; Cohen, Y.; Loewenthal, R.; Trakhtenbrot, L.; Paz, N.; Koren-Michowitz, M.; Waldman, D.; Leider-Trejo, L.; Toren, A.; Constantini, S.; Rechavi, G. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med. 6(2):e1000029; 2009.
3. Arbab, A. S.; Yocum, G. T.; Kalish, H.; Jordan, E. K.; Anderson, S. A.; Khakoo, A. Y.; Read, E. J.; Frank, J. A. Efficient magnetic cell labeling with protamine sulfate complexed to ferumoxides for cellular MRI. Blood 104(4):1217–1223; 2004.
4. Bai, H.; Suzuki, Y.; Noda, T.; Wu, S.; Kataoka, K.; Kitada, M.; Ohta, M.; Chou, H.; Ide, C. Dissemination and proliferation of neural stem cells on the spinal cord by injection into the fourth ventricle of the rat: A method for cell transplantation. J. Neurosci. Methods 124(2):181–187; 2003.
5. Bakshi, A.; Barshinger, A. L.; Swanger, S. A.; Madhavani, V.; Shumsky, J. S.; Neuhuber, B.; Fischer, I. Lumbar puncture delivery of bone marrow stromal cells in spinal cord contusion: A novel method for minimally invasive cell transplantation. J. Neurotrauma 23(1):55–65; 2006.
6. Bakshi, A.; Hunter, C.; Swanger, S.; Lepore, A.; Fischer, I. Minimally invasive delivery of stem cells for spinal cord injury: Advantages of the lumbar puncture technique. J. Neurosurg. Spine 1(3):330–337; 2004.
7. Ban, D. X.; Kong, X. H.; Feng, S. Q.; Ning, G. Z.; Chen, J. T.; Guo, S. F. Intraspinal cord graft of autologous activated Schwann cells efficiently promotes axonal regeneration and functional recovery after rat's spinal cord injury. Brain Res. 1256:149–161; 2009.
8. Barnabe-Heider, F.; Frisen, J. Stem cells for spinal cord repair. Cell Stem Cell 3(1):16–24; 2008.
9. Basso, D. M.; Fisher, L. C.; Anderson, A. J.; Jakeman, L. B.; McTigue, D. M.; Popovich, P. G. Basso mouse scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J. Neurotrauma 23(5):635–659; 2006.
10. Bottai, D.; Madaschi, L.; Di Giulio, A. M.; Gorio, A. Viability-dependent promoting action of adult neural precursors in spinal cord injury. Mol. Med. 14(9–10):634–644; 2008.
11. Brenner, W.; Aicher, A.; Eckey, T.; Massoudi, S.; Zuhayra, M.; Koehl, U.; Heeschen, C.; Kampen, W. U.;

Zeiher, A. M.; Dimmeler, S.; Henze, E. 111In-labeled CD34+ hematopoietic progenitor cells in a rat myocardial infarction model. J. Nucl. Med. 45(3):512–518; 2004.
12. Callera, F.; de Melo, C. M. Magnetic resonance tracking of magnetically labeled autologous bone marrow CD34+ cells transplanted into the spinal cord via lumbar puncture technique in patients with chronic spinal cord injury: CD34+ cells' migration into the injured site. Stem Cells Dev. 16(3):461–466; 2007.
13. Cao, Q. L.; Zhang, Y. P.; Howard, R. M.; Walters, W. M.; Tsoulfas, P.; Whittemore, S. R. Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Exp. Neurol. 167(1): 48–58; 2001.
14. Cho, S. R.; Kim, Y. R.; Kang, H. S.; Yim, S. H.; Park, C. I.; Min, Y. H.; Lee, B. H.; Shin, J. C.; Lim, J. B. Functional recovery after the transplantation of neurally differentiated mesenchymal stem cells derived from bone barrow in a rat model of spinal cord injury. Cell Transplant. 18(12):1359–1368; 2009.
15. Chow, S. Y.; Moul, J.; Tobias, C. A.; Himes, B. T.; Liu, Y.; Obrocka, M.; Hodge, L.; Tessler, A.; Fischer, I. Characterization and intraspinal grafting of EGF/bFGF-dependent neurospheres derived from embryonic rat spinal cord. Brain Res. 874(2):87–106; 2000.
16. Cloutier, F.; Siegenthaler, M. M.; Nistor, G.; Keirstead, H. S. Transplantation of human embryonic stem cell-derived oligodendrocyte progenitors into rat spinal cord injuries does not cause harm. Regen. Med. 1(4):469–479; 2006.
17. Contag, C. H.; Bachmann, M. H. Advances in in vivo bioluminescence imaging of gene expression. Annu. Rev. Biomed. Eng. 4:235–260; 2002.
18. Coutts, M.; Keirstead, H. S. Stem cells for the treatment of spinal cord injury. Exp. Neurol. 209(2):368–377; 2008.
19. Cummings, B. J.; Uchida, N.; Tamaki, S. J.; Salazar, D. L.; Hooshmand, M.; Summers, R.; Gage, F. H.; Anderson, A. J. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc. Natl. Acad. Sci. USA 102(39):14069–14074; 2005.
20. de Haro, J.; Zurita, M.; Ayllon, L.; Vaquero, J. Detection of 111In-oxine-labeled bone marrow stromal cells after intravenous or intralesional administration in chronic paraplegic rats. Neurosci. Lett. 377(1):7–11; 2005.
21. Eaton, M. J.; Wolfe, S. Q.; Martinez, M.; Hernandez, M.; Furst, C.; Huang, J.; Frydel, B. R.; Gomez-Marin, O. Subarachnoid transplant of a human neuronal cell line attenuates chronic allodynia and hyperalgesia after excitotoxic spinal cord injury in the rat. J. Pain 8(1):33–50; 2007.
22. Edinger, M.; Cao, Y. A.; Hornig, Y. S.; Jenkins, D. E.; Verneris, M. R.; Bachmann, M. H.; Negrin, R. S.; Contag, C. H. Advancing animal models of neoplasia through in vivo bioluminescence imaging. Eur. J. Cancer 38(16): 2128–2136; 2002.
23. Gal, P.; Kravcukova, P.; Mokry, M.; Kluchova, D. Chemokines as possible targets in modulation of the secondary damage after acute spinal cord injury: A review. Cell. Mol. Neurobiol. 29(6–7):1025–1035; 2009.
24. Gao, J.; Dennis, J. E.; Muzic, R. F.; Lundberg, M.; Caplan, A. I. The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs 169(1):12–20; 2001.
25. Hara, M.; Murakami, T.; Kobayashi, E. In vivo bioimaging using photogenic rats: Fate of injected bone marrow-derived mesenchymal stromal cells. J. Autoimmun. 30(3): 163–171; 2008.
26. Hoehn, M.; Kustermann, E.; Blunk, J.; Wiedermann, D.; Trapp, T.; Wecker, S.; Focking, M.; Arnold, H.; Hescheler, J.; Fleischmann, B. K.; Schwindt, W.; Buhrle, C. Monitoring of implanted stem cell migration in vivo: A highly resolved in vivo magnetic resonance imaging investigation of experimental stroke in rat. Proc. Natl. Acad. Sci. USA 99(25):16267–16272; 2002.
27. Hofstetter, C. P.; Holmstrom, N. A.; Lilja, J. A.; Schweinhardt, P.; Hao, J.; Spenger, C.; Wiesenfeld-Hallin, Z.; Kurpad, S. N.; Frisen, J.; Olson, L. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nat. Neurosci. 8(3):346–353; 2005.
28. Iwanami, A.; Kaneko, S.; Nakamura, M.; Kanemura, Y.; Mori, H.; Kobayashi, S.; Yamasaki, M.; Momoshima, S.; Ishii, H.; Ando, K.; Tanioka, Y.; Tamaoki, N.; Nomura, T.; Toyama, Y.; Okano, H. Transplantation of human neural stem cells for spinal cord injury in primates. J. Neurosci. Res. 80(2):182–190; 2005.
29. Jendelova, P.; Herynek, V.; Urdzikova, L.; Glogarova, K.; Kroupova, J.; Andersson, B.; Bryja, V.; Burian, M.; Hajek, M.; Sykova, E. Magnetic resonance tracking of transplanted bone marrow and embryonic stem cells labeled by iron oxide nanoparticles in rat brain and spinal cord. J. Neurosci. Res. 76(2):232–243; 2004.
30. Keirstead, H. S.; Nistor, G.; Bernal, G.; Totoiu, M.; Cloutier, F.; Sharp, K.; Steward, O. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J. Neurosci. 25(19):4694–4705; 2005.
31. Khalatbary, A. R.; Tiraihi, T. Localization of bone marrow stromal cells in injured spinal cord treated by intravenous route depends on the hemorrhagic lesions in traumatized spinal tissues. Neurol. Res. 29(1):21–26; 2007.
32 Kim, B. G.; Hwang, D. H.; Lee, S. I.; Kim, E. J.; Kim, S. U. Stem cell-based cell therapy for spinal cord injury. Cell Transplant. 16(4):355–364; 2007.
33. Kumagai, G.; Okada, Y.; Yamane, J.; Nagoshi, N.; Kitamura, K.; Mukaino, M.; Tsuji, O.; Fujiyoshi, K.; Katoh, H.; Okada, S.; Shibata, S.; Matsuzaki, Y.; Toh, S.; Toyama, Y.; Nakamura, M.; Okano, H. Roles of ES cell-derived gliogenic neural stem/progenitor cells in functional recovery after spinal cord injury. PLoS ONE 4(11): e7706; 2009.
34. Lee, J.; Kuroda, S.; Shichinohe, H.; Ikeda, J.; Seki, T.; Hida, K.; Tada, M.; Sawada, K.; Iwasaki, Y. Migration and differentiation of nuclear fluorescence-labeled bone marrow stromal cells after transplantation into cerebral infarct and spinal cord injury in mice. Neuropathology 23(3):169–180; 2003.
35. Lee, Z.; Dennis, J. E.; Gerson, S. L. Imaging stem cell implant for cellular-based therapies. Exp. Biol. Med. (Maywood) 233(8):930–940; 2008.
36. Lepore, A. C.; Bakshi, A.; Swanger, S. A.; Rao, M. S.; Fischer, I. Neural precursor cells can be delivered into the injured cervical spinal cord by intrathecal injection at the lumbar cord. Brain Res. 1045(1–2):206–216; 2005.
37. McDonald, J. W.; Liu, X. Z.; Qu, Y.; Liu, S.; Mickey, S. K.; Turetsky, D.; Gottlieb, D. I.; Choi, D. W. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat. Med. 5(12):1410–1412; 1999.

38. Mikami, Y.; Okano, H.; Sakaguchi, M.; Nakamura, M.; Shimazaki, T.; Okano, H. J.; Kawakami, Y.; Toyama, Y.; Toda, M. Implantation of dendritic cells in injured adult spinal cord results in activation of endogenous neural stem/progenitor cells leading to de novo neurogenesis and functional recovery. J. Neurosci. Res. 76(4):453–465; 2004.
39. Morikawa, S.; Mabuchi, Y.; Kubota, Y.; Nagai, Y.; Niibe, K.; Hiratsu, E.; Suzuki, S.; Miyauchi-Hara, C.; Nagoshi, N.; Sunabori, T.; Shimmura, S.; Miyawaki, A.; Nakagawa, T.; Suda, T.; Okano, H.; Matsuzaki, Y. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. J. Exp. Med. 206(11):2483–2496; 2009.
40. Nagai, T.; Ibata, K.; Park, E. S.; Kubota, M.; Mikoshiba, K.; Miyawaki, A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat. Biotechnol. 20(1):87–90; 2002.
41. Nakamura, M.; Bregman, B. S. Differences in neurotrophic factor gene expression profiles between neonate and adult rat spinal cord after injury. Exp. Neurol. 169(2): 407–415; 2001.
42. Nakamura, M.; Okano, H.; Toyama, Y.; Dai, H. N.; Finn, T. P.; Bregman, B. S. Transplantation of embryonic spinal cord-derived neurospheres support growth of supraspinal projections and functional recovery after spinal cord injury in the neonatal rat. J. Neurosci. Res. 81(4):457–468; 2005.
43. Neuhuber, B.; Barshinger, A. L.; Paul, C.; Shumsky, J. S.; Mitsui, T.; Fischer, I. Stem cell delivery by lumbar puncture as a therapeutic alternative to direct injection into injured spinal cord. J. Neurosurg. Spine 9(4):390–399; 2008.
44. Ogawa, Y.; Sawamoto, K.; Miyata, T.; Miyao, S.; Watanabe, M.; Nakamura, M.; Bregman, B. S.; Koike, M.; Uchiyama, Y.; Toyama, Y.; Okano, H. Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. J. Neurosci. Res. 69(6):925–933; 2002.
45. Ohta, M.; Suzuki, Y.; Noda, T.; Ejiri, Y.; Dezawa, M.; Kataoka, K.; Chou, H.; Ishikawa, N.; Matsumoto, N.; Iwashita, Y.; Mizuta, E.; Kuno, S.; Ide, C. Bone marrow stromal cells infused into the cerebrospinal fluid promote functional recovery of the injured rat spinal cord with reduced cavity formation. Exp. Neurol. 187(2):266–278; 2004.
46. Ohta, Y.; Takenaga, M.; Tokura, Y.; Hamaguchi, A.; Matsumoto, T.; Kano, K.; Mugishima, H.; Okano, H.; Igarashi, R. Mature adipocyte-derived cells, dedifferentiated fat cells (DFAT), promoted functional recovery from spinal cord injury-induced motor dysfunction in rats. Cell Transplant. 17(8):877–886; 2008.
47. Okada, S.; Ishii, K.; Yamane, J.; Iwanami, A.; Ikegami, T.; Katoh, H.; Iwamoto, Y.; Nakamura, M.; Miyoshi, H.; Okano, H. J.; Contag, C. H.; Toyama, Y.; Okano, H. In vivo imaging of engrafted neural stem cells: Its application in evaluating the optimal timing of transplantation for spinal cord injury. FASEB J. 19(13):1839–1841; 2005.
48. Okano, H. Stem cell biology of the central nervous system. J. Neurosci. Res. 69(6):698–707; 2002.
49. Okano, H.; Okada, S.; Nakamura, M.; Toyama, Y. Neural stem cells and regeneration of injured spinal cord. Kidney Int. 68:1927–1931; 2005.
50. Okano, H.; Sakaguchi, M.; Ohki, K.; Suzuki, N.; Sawamoto, K. Regeneration of the central nervous system using endogenous repair mechanisms. J. Neurochem. 102(5): 1459–1465; 2007.
51. Parr, A. M.; Kulbatski, I.; Tator, C. H. Transplantation of adult rat spinal cord stem/progenitor cells for spinal cord injury. J. Neurotrauma 24(5):835–845; 2007.
52. Parr, A. M.; Tator, C. H.; Keating, A. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. Bone Marrow Transplant. 40(7):609–619; 2007.
53. Partlow, K. C.; Chen, J.; Brant, J. A.; Neubauer, A. M.; Meyerrose, T. E.; Creer, M. H.; Nolta, J. A.; Caruthers, S. D.; Lanza, G. M.; Wickline, S. A. 19F magnetic resonance imaging for stem/progenitor cell tracking with multiple unique perfluorocarbon nanobeacons. FASEB J. 21(8):1647–1654; 2007.
54. Paul, C.; Samdani, A. F.; Betz, R. R.; Fischer, I.; Neuhuber, B. Grafting of human bone marrow stromal cells into spinal cord injury: A comparison of delivery methods. Spine 34(4):328–334; 2009.
55. Reynolds, B. A.; Tetzlaff, W.; Weiss, S. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci. 12(11):4565–4574; 1992.
56. Rice, B. W.; Cable, M. D.; Nelson, M. B. In vivo imaging of light-emitting probes. J. Biomed. Opt. 6(4):432–440; 2001.
57. Rolls, A.; Shechter, R.; Schwartz, M. The bright side of the glial scar in CNS repair. Nat. Rev. Neurosci. 10(3): 235–241; 2009.
58. Rowland, J. W.; Hawryluk, G. W.; Kwon, B.; Fehlings, M. G. Current status of acute spinal cord injury pathophysiology and emerging therapies: Promise on the horizon. Neurosurg. Focus 25(5):E2; 2008.
59. Saito, F.; Nakatani, T.; Iwase, M.; Maeda, Y.; Hirakawa, A.; Murao, Y.; Suzuki, Y.; Onodera, R.; Fukushima, M.; Ide, C. Spinal cord injury treatment with intrathecal autologous bone marrow stromal cell transplantation: The first clinical trial case report. J. Trauma 64(1):53–59; 2008.
60. Sher, F.; van Dam, G.; Boddeke, E.; Copray, S. Bioluminescence imaging of Olig2-neural stem cells reveals improved engraftment in a demyelination mouse model. Stem Cells 27(7):1582–1591; 2009.
61. Sykova, E.; Jendelova, P. Magnetic resonance tracking of implanted adult and embryonic stem cells in injured brain and spinal cord. Ann. NY Acad. Sci. 1049:146–160; 2005.
62. Sykova, E.; Jendelova, P.; Urdzikova, L.; Lesny, P.; Hejcl, A. Bone marrow stem cells and polymer hydrogels—two strategies for spinal cord injury repair. Cell. Mol. Neurobiol. 26(7–8):1113–1129; 2006.
63. Takeuchi, H.; Natsume, A.; Wakabayashi, T.; Aoshima, C.; Shimato, S.; Ito, M.; Ishii, J.; Maeda, Y.; Hara, M.; Kim, S. U.; Yoshida, J. Intravenously transplanted human neural stem cells migrate to the injured spinal cord in adult mice in an SDF-1- and HGF-dependent manner. Neurosci. Lett. 426(2):69–74; 2007.
64. Tator, C. H. Review of treatment trials in human spinal cord injury: Issues, difficulties, and recommendations. Neurosurgery 59(5):957–987; 2006.
65. Thuret, S.; Moon, L. D.; Gage, F. H. Therapeutic interventions after spinal cord injury. Nat. Rev. Neurosci. 7(8): 628–643; 2006.
66. Togel, F.; Yang, Y.; Zhang, P.; Hu, Z.; Westenfelder, C. Bioluminescence imaging to monitor the in vivo distribu-

tion of administered mesenchymal stem cells in acute kidney injury. Am. J. Physiol. Renal Physiol. 295(1):F315–321; 2008.
67. Vaquero, J.; Zurita, M.; Oya, S.; Santos, M. Cell therapy using bone marrow stromal cells in chronic paraplegic rats: Systemic or local administration? Neurosci. Lett. 398(1–2):129–134; 2006.
68. Whetstone, W. D.; Hsu, J. Y.; Eisenberg, M.; Werb, Z.; Noble-Haeusslein, L. J. Blood–spinal cord barrier after spinal cord injury: Relation to revascularization and wound healing. J. Neurosci. Res. 74(2):227–239; 2003.
69. Wu, S.; Suzuki, Y.; Noda, T.; Bai, H.; Kitada, M.; Kataoka, K.; Nishimura, Y.; Ide, C. Immunohistochemical and electron microscopic study of invasion and differentiation in spinal cord lesion of neural stem cells grafted through cerebrospinal fluid in rat. J. Neurosci. Res. 69(6): 940–945; 2002.
70. Yoshihara, T.; Ohta, M.; Itokazu, Y.; Matsumoto, N.; Dezawa, M.; Suzuki, Y.; Taguchi, A.; Watanabe, Y.; Adachi, Y.; Ikehara, S.; Sugimoto, H.; Ide, C. Neuroprotective effect of bone marrow-derived mononuclear cells promoting functional recovery from spinal cord injury. J. Neurotrauma 24(6):1026–1036; 2007.

Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury

Osahiko Tsuji[a,b,1], Kyoko Miura[a,c,1], Yohei Okada[a,d], Kanehiro Fujiyoshi[a,b], Masahiko Mukaino[a,e], Narihito Nagoshi[a,b,f], Kazuya Kitamura[a,b], Gentaro Kumagai[a,g], Makoto Nishino[a], Shuta Tomisato[a], Hisanobu Higashi[a], Toshihiro Nagai[h], Hiroyuki Katoh[a,b,f], Kazuhisa Kohda[a], Yumi Matsuzaki[a], Michisuke Yuzaki[a], Eiji Ikeda[i,j], Yoshiaki Toyama[b], Masaya Nakamura[b,2], Shinya Yamanaka[c], and Hideyuki Okano[a,2]

Departments of [a]Physiology and [b]Orthopedic Surgery, School of Medicine, Keio University, Shinjuku, Tokyo 160-8582, Japan; [c]Center for Induced Pluripotent Stem Cell Research and Application, Kyoto University, Kyoto 606-8507, Japan; [d]Kanrinmaru-Project and Departments of [e]Rehabilitation Medicine, [h]Electron Microscope Laboratory, and [i]Pathology, School of Medicine, Keio University, Tokyo 160-8582, Japan; [f]Department of Orthopedic Surgery, National Hospital Organization, Murayama Medical Center, Tokyo 208-0011, Japan; [g]Department of Orthopedic Surgery, Graduate School of Medicine, Hirosaki University, Aomori 036-8560, Japan; and [j]Department of Pathology, Graduate School of Medicine, Yamaguchi University, Yamaguchi 755-8505, Japan

Edited by Fred Gage, Salk Institute, San Diego, CA, and approved June 3, 2010 (received for review September 3, 2009)

Various types of induced pluripotent stem (iPS) cells have been established by different methods, and each type exhibits different biological properties. Before iPS cell-based clinical applications can be initiated, detailed evaluations of the cells, including their differentiation potentials and tumorigenic activities in different contexts, should be investigated to establish their safety and effectiveness for cell transplantation therapies. Here we show the directed neural differentiation of murine iPS cells and examine their therapeutic potential in a mouse spinal cord injury (SCI) model. "Safe" iPS-derived neurospheres, which had been pre-evaluated as nontumorigenic by their transplantation into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse brain, produced electrophysiologically functional neurons, astrocytes, and oligodendrocytes in vitro. Furthermore, when the safe iPS-derived neurospheres were transplanted into the spinal cord 9 d after contusive injury, they differentiated into all three neural lineages without forming teratomas or other tumors. They also participated in remyelination and induced the axonal regrowth of host 5HT$^+$ serotonergic fibers, promoting locomotor function recovery. However, the transplantation of iPS-derived neurospheres pre-evaluated as "unsafe" showed robust teratoma formation and sudden locomotor functional loss after functional recovery in the SCI model. These findings suggest that pre-evaluated safe iPS clone-derived neural stem/progenitor cells may be a promising cell source for transplantation therapy for SCI.

neural stem/progenitor cell | cell transplantation | regenerative medicine | remyelination | axonal regrowth

Given their ability to generate all types of neural cells, neural stem/progenitor cells (NS/PCs) are a promising source for cell replacement therapy for various intractable CNS disorders (reviewed in refs. 1–6). Notably, ES cells have great developmental plasticity and can be induced to become NS/PCs with specific differentiation potentials (7–11), making them a major candidate for cell replacement therapies for CNS disorders (12–16). The clinical use of ES cells is complicated, however, by ethical and immunological concerns, both of which might be overcome by using pluripotent stem cells derived directly from a patient's own somatic cells (17).

We recently reported the establishment of induced pluripotent stem (iPS) cells from mouse fibroblasts by the retroviral introduction of four factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) with selection for *Fbxo15* expression (18) and *Nanog* expression (19, 20). Compared with *Fbxo15*-selected iPS cells, *Nanog*-selected iPS cells more closely resembled ES cells' gene-expression pattern and could contribute to germline-competent adult chimeras (19–21). More recently, we and others (22, 23) generated iPS cells without using *c-Myc* retroviruses, albeit with lower efficiency. The successful establishment of these iPS cell lines, along with initial reports showing efficacy in the therapeutic use of iPS cells in rodent models of sickle cell anemia (24) and Parkinson disease (25), led us to examine the use of iPS cells as a treatment for spinal cord injury (SCI).

A number of important issues need to be addressed before a clinical trial using iPS cells as a cell-therapy source for SCI is initiated. First, a detailed evaluation of iPS cells' potential to generate neural cells compared with ES cells is required. Second, iPS cells are likely to carry a higher risk of tumorigenicity than ES cells, due to the inappropriate reprogramming of these somatic cells, the activation of exogenous transcription factors, or other reasons (25–27). Thus, it is essential to confirm the safety of grafted iPS-derived NS/PCs. Finally, the effectiveness of iPS-derived NS/PC transplantation as a treatment for SCI must be evaluated.

In the previous study, we pre-evaluated iPS clones for safety by transplanting iPS-derived neurospheres into the NOD/SCID mouse brain (27). Here, we show that the transplantation of neurospheres derived from safe iPS cell clones into the injured spinal cord promoted functional recovery without any tumor formation. In contrast, the transplantation of neurospheres derived from unsafe iPS cells, showing robust teratoma formation in the NOD/SCID mouse brain, also resulted in initial functional recovery, but was later followed by teratoma formation and deterioration of locomotor function. These data suggest that the evaluation of in vitro differentiation and in vivo tumorigenicity are important for identifying safe iPS clones for cell therapy, and that the NS/PCs derived from iPS clones deemed safe by such pre-evaluation are a promising source for cell therapy for SCI.

Results

Pre-Evaluated Safe MEF-iPS Cells Exhibit ES-Like Neural Differentiation Potentials in Vitro. We previously reported the neural differentiation of 36 independent murine iPS cell clones (27). The results of this study led us to classify several iPS clones as safe or unsafe

Author contributions: O.T., K.M., M. Nakamura, S.Y., and H.O. designed research; O.T., K.M., Y.O., K.F., M.M., N.N., K. Kitamura, G.K., M. Nishino, S.T., H.H., T.N., H.K., E.I., and H.O. performed research; O.T. and K.M. contributed new reagents/analytic tools; O.T., K.M., Y.O., K.F., M.M., N.N., K. Kitamura, G.K., H.K., K. Kohda, Y.M., M.Y., E.I., Y.T., M. Nakamura, S.Y., and H.O. analyzed data; and O.T., K.M., Y.O., K.F., H.K., E.I., M. Nakamura, and H.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]O.T. and K.M. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: hidokano@sc.itc.keio.ac.jp or masa@sc.itc.keio.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.0910106107/-/DCSupplemental.