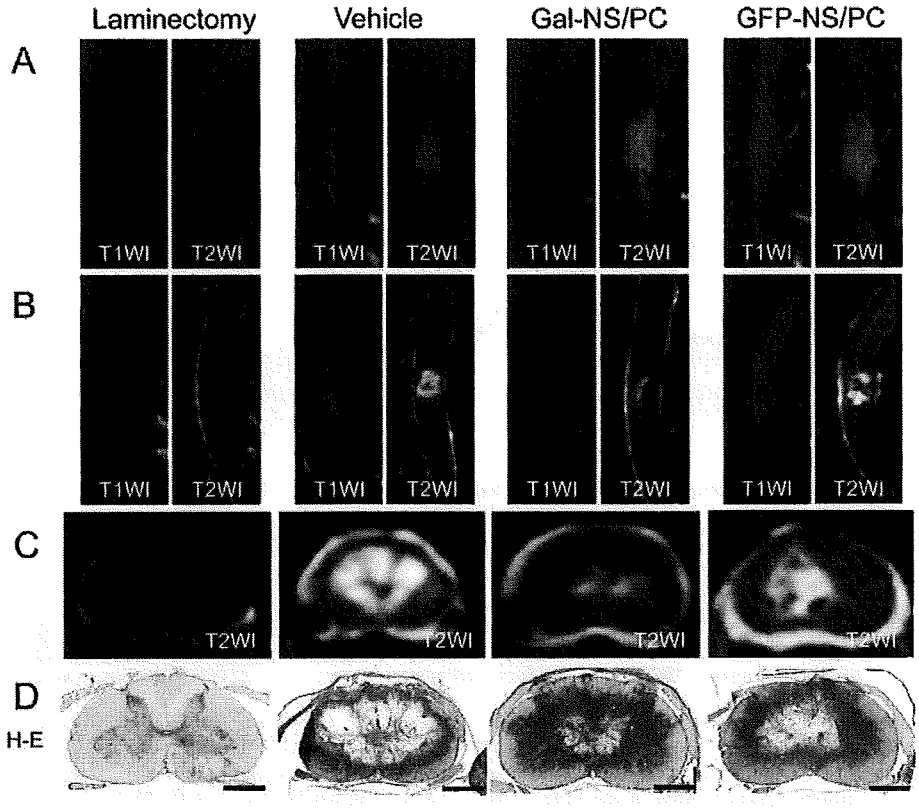

Fig. 2. Comparisons of the injured spinal cord by MRI and histology. MR images of the spinal cord from animals of the laminectomy, vehicle control, Gal-NS/PC, and GFP-NS/PC groups 3 days after SCI (**A**, sagittal) and 12 weeks after transplantation (**B**, sagittal; **C**, axial). The lesion epicenter appeared as a low-signal-intensity area in the T1WI and as a high-signal-intensity area in the T2WI. **D**: H-E staining. Representative H-E-stained specimens from the laminectomy, vehicle control, Gal-NS/PC, and GFP-NS/PC groups. Scale bars = 1 mm.

GFP, neurofilament 200 kD (NF200; NF200 antibody, 1:1,000 mouse IgG, Chemicon, Temecula, CA), and antimyelin basic protein (MBP; 1:200) was performed.

Grafted NS/PCs that were colabeled with both GFP and cell-type-specific markers were detected with a confocal microscope equipped with an argon-krypton laser (LSM510; Carl Zeiss Co., Oberkochen, Germany) and a fluorescence microscope (Axioskop 2 Plus; Carl Zeiss, Munich, Germany).

Statistical Analysis

A one-way ANOVA followed by the Turkey-Kramer test for multiple comparisons was applied to the behavioral analyses and histological quantifications. All data are shown as the mean ± SEM, with $P < 0.05$ regarded as statistically significant.

RESULTS

Characterization of hNS/PCs In Vitro

To examine the expression of h-galectin-1 in each type of NS/PC (Gal-NS/PCs, GFP-NS/PCs, and naive NS/PCs), we performed Western blotting. The amount of h-galectin-1 in the cell lysate of Gal-NS/PCs was significantly greater than in the lysate of the GFP-NS/PCs or naive NS/PCs (Fig. 1C). Although the amount of h-galectin-1 in the conditioned medium from all the NS/PCs was too small to detect by typical Western blotting, we could detect it in the conditioned medium from all the NS/PCs using the avidin-biotin complex (ABC) method. The amount of h-galectin-1 in the conditioned medium of Gal-NS/PCs was significantly greater than in that of GFP-NS/PCs or naive NS/PCs (data not shown).

We next examined the effect of lentiviral h-galectin-1 transduction on the proliferation and differentiation of NS/PCs. The proliferation rate of each type of NS/PC was quantified by ATP assay. There was no significant difference in the doubling time among the three types of NS/PCs (approximately 150 hr; Fig. 1D).

Gal-NS/PCs, GFP-NS/PCs, and naive NS/PCs differentiated into Tuj1-positive neurons and GFAP-positive astrocytes (Fig. 1E) but not into CNPase-positive oligodendrocytes, 1 week after their differentiation was induced in vitro. Quantitative analysis of their phenotypes revealed no significant differences in the percentages of neurons and astrocytes among the three types of NS/PCs (Fig. 1F).

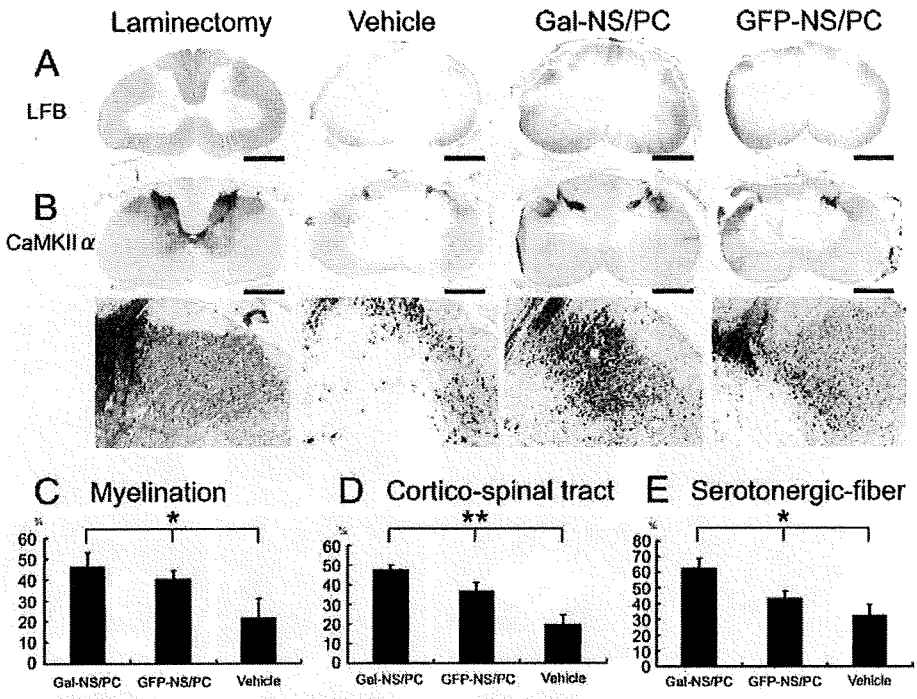

Fig. 3. Myelination, corticospinal fibers, and serotonergic fibers after SCI. Representative axial sections of lesion sites stained with LFB (**A**) and for CaMKIIα (**B**) in the laminectomy, vehicle control, Gal-NS/PC, and GFP-NS/PC groups. Quantification of the LFB^+ myelinated (**C**), $CaMKII\alpha^+$ corticospinal fiber (**D**), and $5HT^+$ serotonergic fiber (**E**) areas at the lesion site. The percentage of each area relative to the corresponding area in the laminectomy group was calculated using MCID. $*P < 0.05$, $**P < 0.01$. Scale bars = 1 mm.

To determine the effect of lentiviral h-galectin-1 transduction on the neurite growth of each type of NS/PC, the neurite lengths of the neurons derived from Gal-NS/PCs and GFP-NS/PCs were quantified 1 week after their differentiation. The Tuj1/GFP double-positive neurons derived from the Gal-NS/PCs possessed significantly longer neurites than the GFP-NS/PC-derived neurons (Fig. 1G).

Transplantation of Galectin-1-NS/PCs Into the Injured Spinal Cord Reduced the Size of the Lesion Site

In all groups except the laminectomy group, an area of isosignal intensity on the T1-weighted images (T1WI) and a diffuse area of high signal intensity on the T2-weighted images (T2WI) were observed at the lesion epicenter 3 days after the injury, and there was no significant difference in the area of intramedullary T2-high signal among the three groups (Fig. 2A). At 12 weeks after the transplantation, the lesion site appeared as a clearly demarcated low-signal-intensity area on the T1WI and a high-signal-intensity area on the T2WI in all the injury groups, but the lesion appeared smaller in the Gal-NS/PC group than in the GFP-NS/PC and vehicle control groups (Fig. 2B,C). Histological analyses revealed that the intramedullary signal changes observed on the MRI corresponded to the formation of a cavity at the lesion epicenter. The cavity area was smaller in the Gal-NS/PC group than in the GFP-NS/PC and vehicle control groups, which was consistent with the MRI findings (Fig. 2C,D).

Transplantation of Galectin-1 NS/PCs Decreased Demyelination and Increased Corticospinal and Serotonergic Fibers After SCI

Compared with the case in the laminectomy group, the contusive SCI resulted in severe loss of myelin sheath and corticospinal fibers at the lesion site (approximately 20% of the value of the laminectomy group). In both the Gal-NS/PC and the GFP-NS/PC groups, more myelin sheath and corticospinal fibers were observed at the lesion epicenter than in the vehicle control group, and the areas in the Gal-NS/PC group were greater than in the GFP-NS/PC group (Fig. 3A,B). The myelin sheath and corticospinal fibers of the three groups were measured and expressed as a percentage of the corresponding area in the laminectomy group. There were significant differences in the areas of both LFB-positive myelin sheath and α-CaMKII-positive corticospinal fibers among the three groups (Fig. 3C,D).

5HT-positive serotonergic fibers were also lost after SCI at the lesion epicenter. The area of 5HT-positive fibers after injury was approximately 30% of the corresponding area of the laminectomy group. The transplan-

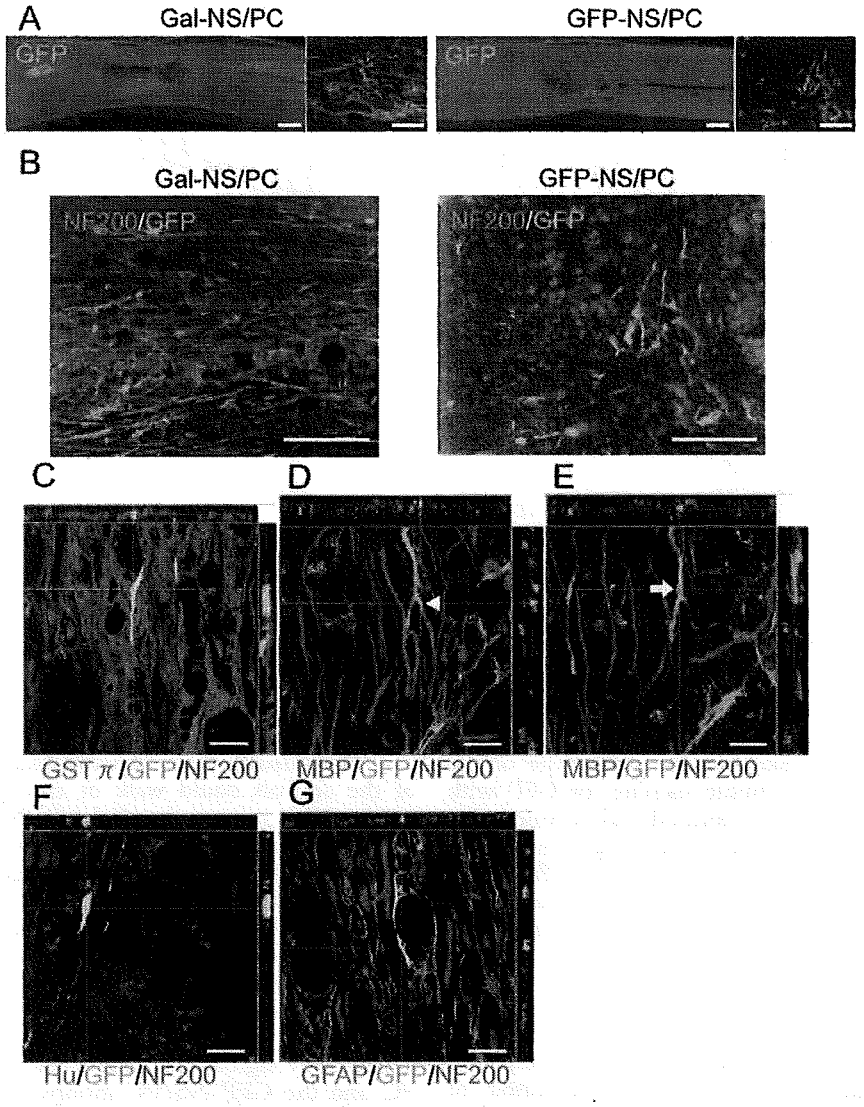

Fig. 4. Grafted human NS/PCs survived and differentiated into oligodendrocytes, neurons, and astrocytes within the injured marmoset spinal cord. **A**: Anti-GFP immunostaining of sagittal sections from the Gal-NS/PC and GFP-NS/PC groups. **B**: Double staining for NF200 and GFP revealed that the differentiated Gal-NS/PC-derived cells possessed longer processes compared with the GFP-NS/PC-derived cells, and these processes were often closely apposed to $NF200^+$ nerve fibers. Confocal images of triple labeling with anti-GFP, anti-GSTpi, and anti-NF200 (**C**) and with anti-GFP, anti-MBP, and anti-NF200 (**D,E**). Grafted Gal-NS/PC-derived oligodendrocytes were closely associated with host neurofilaments (E, arrow) and host myelin sheath (D, arrowhead). Confocal images of triple labeling with anti-GFP, anti-NF200, and anti-Hu (**F**) or anti-GFP, anti-NF200, and anti-GFAP (**G**) revealed that some Gal-NS/PCs also differentiated into neurons or astrocytes. Scale bars = 1 mm at left in A; 50 μm at right in A; 50 μm in B; 20 μm in C–G.

tation of Gal-NS/PCs or GFP-NS/PCs increased the 5HT-positive serotonergic fibers at the epicenter, and the area of serotonergic fibers at the lesion epicenter in the Gal-NS/PC group was greater than in the GFP-NS/PC group (Fig. 3E).

Grafted NS/PCs Survived and Differentiated Into Neurons, Astrocytes, and Oligodendrocytes

To examine the fate of the NS/PCs grafted into the injured spinal cord, fluorescent immunostaining for GFP was performed. In both the Gal-NS/PC and the GFP-NS/PC groups, GFP-positive cells were detected mainly around the cavity (Fig. 4A), but some were distributed as far as 10 mm rostral and caudal to the epicenter, 13 weeks after transplantation. No evidence of tumor formation was observed in either the Gal-NS/PC or the GFP-NS/PC group at 13 weeks after transplantation. Double immunostaining for GFP and NF200 showed that the Gal-NS/PCs possessed longer processes than the GFP-NS/PCs, and these processes were often

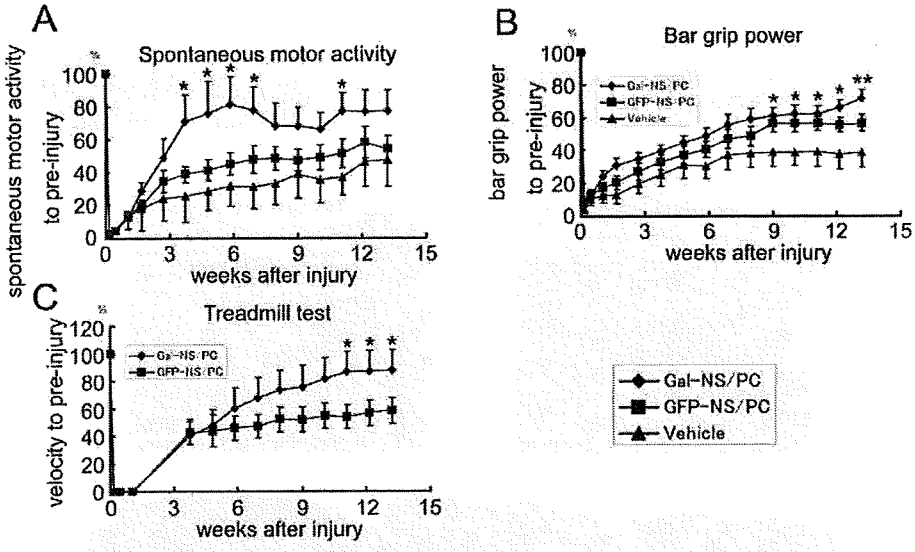

Fig. 5. Behavioral analyses. **A**: Spontaneous motor activity. **B**: Bar grip power. **C**: Treadmill test. The vehicle control group was excluded from the treadmill test, because some animals in the vehicle control group could not walk on the treadmill. $*P < 0.05$, $**P < 0.01$.

aligned with and in close apposition to NF200-positive nerve fibers, which was not observed in the GFP-NS/PC group (Fig. 4B). To examine the phenotype of the transplanted Gal-NS/PCs, double staining for GFP with GSTpi, Hu, or GFAP was performed and revealed that some NS/PCs had differentiated into oligodendrocytes (Fig. 4C), neurons (Fig. 4F), or astrocytes (Fig. 4G). The Gal-NS/PC-derived cells were closely associated with $NF200^+$ fibers (Fig 4E) and MBP^+ myelin sheath (Fig. 4D).

Behavioral Analyses

Spontaneous motor activity. Immediately after injury, the spontaneous motor activity decreased to approximately 0% of the preinjury level, and then increased in all groups. The Gal-NS/PC group showed a rapid increase, reaching a plateau of about 80% by 4 weeks after SCI. The GFP-NS/PC and vehicle control groups showed a more gradual increase. There were significant differences among the three groups by multiple comparisons 4–7 weeks after injury. By 13 weeks after the injury, the motor activities had returned to approximately 80%, 50%, and 40% in the Gal-NS/PC, GFP-NS/PC, and vehicle control groups, respectively (Fig. 5A).

Bar grip power. The maximal grip strength after injury relative to that before injury (percentage grip strength) decreased to approximately 5% in all the groups 1 day after the injury. The grip strength gradually recovered thereafter, reaching a plateau of approximately 70%, 60%, and 40% in the Gal-NS/PC, GFP-NS/PC, and vehicle control groups, respectively. Significant differences in the percentage grip strength were observed among the three groups 9 weeks after injury and thereafter (Fig. 5B).

Treadmill test. Immediately after injury, none of the animals could walk or run by themselves. By 4 weeks after injury, the Gal-NS/PC and GFP-NS/PC groups could walk at approximately 40% of their maximum velocity preinjury (percentage velocity). Because some animals in the vehicle control group could not walk on the treadmill, the vehicle control group was excluded from this analysis. The velocity rapidly recovered to approximately 90% in the Gal-NS/PC group, whereas, in the GFP-NS/PC group, it initially recovered quickly but reached a plateau of approximately 50%. There were significant differences between the Gal-NS/PC and the GFP-NS/PC groups at 10 weeks after SCI and thereafter (Fig. 5C).

DISCUSSION

Stem cell transplantation shows promise as a potential therapy for treating SCI. In particular, ES cells and induced pluripotent stem cells (iPS cells; Takahashi and Yamanaka, 2006) represent possible donor sources for transplantation therapy because of their high pluripotency and potential for proliferation. However, a major problem associated with ES and iPS cell-based therapies is tumor formation (Arnhold et al., 2004; Yoshizaki et al., 2004; Chung et al., 2006; Wernig et al., 2008; Miura et al., 2009), because the grafted cells contain undifferentiated cells even after differentiation is induced. In contrast, no tumor formation has been reported in rodent or primate SCI models subjected to NS/PC-based cell therapy (Ogawa et al., 2002; Ishibashi et al., 2004; Cummings et al., 2005; Iwanami et al., 2005a),

although one recent case report showed brain tumor formation following intracerebellar and intrathecal transplantation of human fetal NS/PCs into an ataxia telangiectasia patient within immune deficiency (Amariglio et al., 2009).

Previous studies (Ogawa et al., 2002; Ishibashi et al., 2004; Cummings et al., 2005; Iwanami et al., 2005a) have shown significant functional recovery after NS/PC transplantation in SCI models, but the improvement has not been sufficient to extrapolate to clinical trials. Therefore, we have been seeking to enhance these effects, by combining hNS/PC transplantation with gene therapy. We showed that galectin-1 applied as an induced gene using a lentiviral vector had neural regenerative effects and improved the recovery of sensorimotor function in an animal model of stroke (Ishibashi et al., 2007).

Lentivirus is broadly used as an ex vivo gene transfer tool for viral gene therapy applications, from basic research to clinical studies (Campbell and Hope, 2005; Young et al., 2006). In fact, several studies have used lentivirus to insert transgenes into NS/PCs (Consiglio et al., 2004; Okada et al., 2005). In the present study, NS/PCs were infected with a recombinant lentivirus carrying h-galectin-1 and also Venus, to track the graft-derived cells in vivo. One advantage of lentivirus is that, unlike retrovirus, which infects dividing cells, nondividing cells can be infected, including most NS/PCs, which divide very slowly (Englund et al., 2000). Another advantage is that, unlike adenovirus, lentivirus can integrate a transgene into the host genome, which prevents dilution of the labeling by cell division. In this study, 80% or more of the NS/PCs were infected with the recombinant lentivirus, and the ratio of the infected cells remained the same after several passages.

One disadvantage of lentivirus is that where and how many copies of reporter genes are inserted into the host genome are uncertain and could affect the cell's phenotype, depending on the site of insertion. Therefore, we compared the proliferation rate and differentiation potential of the Gal-NS/PCs, GFP-NS/PCs, and naive NS/PCs using an ATP doubling time assay and differentiation assay, respectively, and found no significant differences. In addition, there was no significant difference among the Gal-NS/PCs, GFP-NS/PCs, and naive NS/PCs in the differentiation rate of Tuj-1-positive neurons or GFAP-positive astrocytes, consistent with previously reported results (Li et al., 2005; Fig. 1E,F). Thus, there was no change in the differentiation and proliferation rates of the NS/PCs before vs. after lentiviral infection. Furthermore, the proportion of infected cells after several passages was almost constant, and there was no tumor formation for at least 13 weeks after transplantation. Taken together, our findings indicate that the lentivirus infection did not affect the nature of the NS/PCs in any major way.

Galectin-1 has reported functions in a variety of processes, including cell proliferation, differentiation, apoptosis, cell adhesion, metastasis of tumor cells, and inflammation. Galectin-1 is expressed by various stem cells, including embryonic (Ramalho-Santos et al., 2002), neural (Sakaguchi et al., 2006), hematopoietic (Vas et al., 2005), mesenchymal (Silva et al., 2003), and kertinocyte (Tumbar et al., 2004) stem cells. In particular, galectin-1 induced the proliferation of adult NS/PCs in the subventricular zone (SVZ; Sakaguchi et al., 2006) and in hippocampus (Kajitani et al., 2009). Furthermore, in the SVZ, this action was shown to be mediated by galectin-1's carbohydrate-binding activity (Sakaguchi et al., 2006). On the other hand, oxidized galectin-1, which lacks lectin activity, promotes peripheral nerve regeneration (Horie et al., 1999).

In this study, we showed that the neurite growth length of Gal-NS/PC-derived neurons reached 1.5 times that of GFP-NS/PC-derived neurons in vitro, probably because extracellular galectin-1 expressed by the Gal-NS/PCs affected the axonal growth through autocrine and/or paracrine mechanisms similar to those of peripheral nerves (Horie et al., 1999). Therefore, we expected that galectin-1 transfected into NS/PCs would enhance neurite growth in the injured spinal cord.

Our histological analyses revealed that NS/PC transplantation reduced the size of the demyelinated area in the injured spinal cord. Only a residual myelinated area was observed at the outer border of the lesion epicenter in the vehicle control group, whereas, in both the Gal-NS/PC and the GFP-NS/PC groups, more myelin sheath was observed, especially at the anterior and posterior funiculus. In both the Gal-NS/PC and the GFP-NS/PC groups, a much greater area of corticospinal fibers was also observed at the lesion epicenter compared with the vehicle control group. Intriguingly, there was a significantly greater area of corticospinal fibers in the Gal-NS/PC group than in the GFP-NS/PC group. Furthermore, there was a significant difference in the area of the serotonergic fibers, which are closely associated with motor function, among the Gal-NS/PC, GFP-NS/PC, and vehicle control groups.

Both Gal-NS/PCs and GFP-NS/PCs survived and differentiated into neurons, astrocytes, and oligodendrocytes around the cavity. Surviving Gal-NS/PCs were observed up to 10 mm from the transplanted site. The cells that differentiated from the Gal-NS/PCs possessed longer processes than those from GFP-NS/PCs, and these processes were closely apposed to NF200-positive nerve fibers, with some processes enclosing the NF200-positive fibers entirely. It is possible that the processes produced myelin sheath or contributed to the guidance of host-cell axonal growth. These possibilities were supported by the finding that the NF200-positive fibers expressed β1-integrin (data not shown), which is one of the binding proteins for galectin-1 (Moiseeva et al., 2003).

Our results collectively suggest that both Gal-NS/PCs and GFP-NS/PCs supported the regenerative processes of the host spinal cord after injury and that the effect of the Gal-NS/PCs was much greater than that of the GFP-NS/PCs, because of the h-galectin-1 secreted from the transplanted cells. In previous studies, the fol-

lowing mechanisms for the effects of transplanted NS/PCs have been proposed (Horner and Gage, 2000; Okano, 2002b; Schwab, 2002; Okano et al., 2003; Iwanami et al., 2005a). First, immature astrocytes derived from the grafted NS/PCs play an important role in axonal guidance (Hofstetter et al., 2002; Silver and Miller, 2004). Second, the neurons differentiated from the NS/PCs form new synapses with the host neurons, helping to reform the neuronal circuits that had been disrupted by the injury (Ogawa et al., 2002; Okano et al., 2003). Third, trophic effects also improve the survival of the host cells within the injured spinal cord, leading to functional recovery (Namiki et al., 2000; Koda et al., 2002). We believe, based on our finding that Gal-NS/PC-grafted animals showed significantly better functional recovery than GFP-NS/PC-grafted animals at the early phase after transplantation, that the h-galectin-1 produced by the NS/PCs enhanced the third mechanism, although the possibility that Gal-NS/PCs enhanced the effects of the first and second mechanisms cannot be excluded.

It is important to evaluate the cell source for the clinical application to SCI. Accordingly, it will be necessary to compare the merits and demerits of various potential cell sources for the therapeutics of SCI, which include not only NS/PCs but also ES cells (Keirstead et al., 2005), MSCs (Hofstetter et al., 2002), OECs (Li et al., 1997), and nestin-expressing multipotent hair follicle stem cells (Amoh et al., 2005, 2008), from the aspects of safety, effectiveness, and availability.

We previously developed several methods for assessing the motor function of marmosets after SCI, including the measurement of spontaneous motor activity and the bar grip test (Iwanami et al., 2005a,b). Here we observed significant differences between the Gal-NS/PC or the GFP-NS/PC group and the vehicle group in their spontaneous motor activity and bar grip power. However, there was no significant difference between the Gal-NS/PC and the GFP-NS/PC groups when evaluated by these two post hoc tests, because it can be difficult to detect significant differences between mild-injury groups by these evaluations. Therefore, as a new evaluation for motor function to detect the difference between mild-injury groups, we developed a treadmill test. Insofar as the ability to walk or run combines muscle power with coordination of the extremities, this test allows us to discriminate between animals that can run and those that can only walk, a difference that is not recognized by our other evaluation methods. Using this test, we observed significant differences between the Gal-NS/PC and the GFP-NS/PC groups 9–11 weeks after injury.

Intriguingly, significantly greater functional recovery was seen in the Gal-NS/PC group in the spontaneous motor activity and treadmill tests during the early phase, about 5 weeks after injury. This functional improvement might have been due to the greater amounts of LFB-positive myelin sheath, CaMKIIα-positive corticospinal tract, and 5HT-positive serotonergic fibers in the Gal-NS/PC group.

The bar grip power test is used to assess segmental symptoms, mainly involving the motoneurons at the anterior horn. In this study, there was no significant difference in the number of choline acetyltransferase-positive motoneurons at the lesion epicenter, although the Gal-NS/PC group appeared to have more of these cells compared with the GFP-NS/PC and vehicle groups (data not shown). Consistently with this observation, there were no significant differences in the bar grip power during the early phase but significant differences at the late phase, probably because of the compensatory function of the spinal cord to reorganize denervated muscles.

In conclusion, here we have shown that the transplantation of Gal-NS/PCs or GFP-NS/PCs into a primate SCI model improved the recovery of motor functions and that the Gal-NS/PCs had a greater effect than the GFP-NS/PCs. Human NS/PCs expressing h-galectin-1 may therefore have a higher therapeutic potential than control NS/PCs for treating SCI.

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Review Article

Physiological significance of astrogliosis after CNS injury

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In the injured central nervous system (CNS), reactive astrocytes form a glial scar and they are considered a physical barrier to prevent axonal regeneration by producing axonal growth inhibitors, such as chondroitin sulfate proteoglycans. However, the physiological role of reactive astrocytes remains to be elucidated. In this review, we showed that reactive astrocytes play a crucial role in wound healing and functional recovery. At the subacute phase of spinal cord injury (SCI), reactive astrocytes eventually migrated to the lesion epicenter and gradually compacted the infiltrated inflammatory cells and contracted the lesion area, and functional recovery was observed only in this repair phase. Selective deletion of the signal transducer and activator of transcription-3 (STAT3) in reactive astrocytes resulted in their limited migration associated with zinc signaling, markedly widespread damaged area and severe motor deficits. These results suggest that STAT3 is a key regulator of reactive astrocytes migration in the healing process after SCI, providing a beneficial aspect of reactive astrocytes after CNS injury.

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Key words spinal cord injury, reactive astrocytes, STAT3

Introduction

Since the regenerative capability of mammalian CNS is poor, SCI causes severe motor/sensory dysfunction and deficits can often be permanent. It is now generally accepted that SCI is a two-step process involving the primary mechanical injury and a following cascade of auto-destructive injury. Mechanical trauma

rapidly lead to blood brain barrier disruption, neuronal cell death, axonal damage and demyelination, followed by a cascade of secondary injury that expands the additional inflammatory reaction at the lesion area[1,2]. This concept of "secondary" injury encompassing both necrotic and programmed cell death was postulated more than half a century ago, and has long been considered a major therapeutic target aimed at sparing tissue and function for anti-inflammatory and anti-apoptotic agents[3,4], although primary mechanical injury is considered irreversible.

However, the actual clinical paralyses of SCI patients as well as experimental SCI of rodents almost always exhibit severest state just after SCI, and gradual improvement to some extent with time course (except for complete paralysis), and there are very few patients with permanent deterioration[5,6]. If the secondary injury has a critical influence on the paralysis outcome, there would be a greater number of patients with deterioration after injury. Although the clinical time course of paralysis is suggesting that there is some sort of self-repair system after CNS injury even in rodents and primates, the mechanisms of gradual improvement in subacute phase is poorly understood and referred to as withdrawal of "spinal shock". Understanding of the self-repair mechanism inherent in mammals is surely to lead to novel therapeutic strategy for the treatment of CNS injury.

Astrogliosis and functional recovery

To interpret the process of paralysis improvement in the subacute phase, we examined serial histological sections of contused spinal cords and followed motor function in wild-type mice after produced contusion injury at thoracic 12 levels[7]. In this incomplete paralysis model, gradual functional recovery was observed until the subacute phase of injury (~ 2 weeks after injury), followed by limited recovery afterward.

Firstly, we tried to confirm the secondary injury process in the acute phase and found that the area of neural cell loss gradually enlarged in a rostral-caudal direction within a few days after SCI. Some portions of neurons were positive for cleaved caspase-3 indicated that the secondary injury process lasted for several days in this model during which we observed limited functional recovery. Meanwhile, astrocytes surrounding the lesion underwent a typical change of hypertrophy, process extension and increased expression of intermediate filaments such as GFAP and Nestin by 7 d after SCI, characteristic of "reactive astrocytes".

Notably, these responsive astrocytes eventually migrated centripetally to the lesion epicenter and gradually compacted the CD11b-positive inflammatory cells, contracting the lesion area up until subacute phase after SCI as shown in Fig.1A. During this process, we observed repair of injured tissue and gradual functional improvement, and reactive astrocytes formed a physical barrier against inflammatory cells, commonly referred to as glial scar (Fig.1B). After the migration of reactive astrocytes and completion of glial scar, functional improvement reached a plateau around 2 weeks after injury.

This glial scar contains extracellular matrix molecules that chemically inhibit axonal regeneration as well as physically, and has only been considered to definitely play a crucial part in CNS regeneration failure in the chronic phase of SCI[8]. However, the process observed at subacute phase strongly suggested that the emergence and migration of reactive astrocytes have a prominent role in the repair of injured tissue and the restoration of motor function before completion of the glial scar.

The migration mechanism and Stat3 signaling

The regulatory mechanisms behind the reactive response of astrocytes remain elusive. We investigated the role of Stat3 signaling since Stat3 is a principal mediator in a variety of biological processes including wound healing and the movement of various types of cells[9,10]. In addition, several reports suggested Stat3 mediates certain aspects of astrogliosis downstream of the action of cytokines such as interleukin (IL)-6, leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) after CNS injury[11-14].

In the injured spinal cord, phosphorylated Stat3 prominently increased at 12 h after injury, which remained detectable with western blotting for 2 weeks. We observed phosphorylation and nuclear translocation of Stat3 mainly in reactive astrocytes surrounding the lesion in immunohistochemistry, but not in distant areas for several days after injury.

To elucidate the role of Stat3 in reactive astrocytes, we performed experiments by using mice with a selective deletion of STAT3 under the control of Nestin gene promoter/enhancer[15] ($STAT3^{N-/-}$), which are activated in reactive astrocytes after SCI. $STAT3^{N-/-}$ mice showed no apparent abnormalities in motor function and development, although they showed signs of hyperphagia and leptin resistance[15]. At 2 weeks after injury, widespread tissue damage, demyelination and severe motor deficit were observed in this conditional STAT3 knockout mouse compared to wild-type mice (Fig.2A,C). Interestingly, although the comparable tissue damage and reactive gliosis was observed around the lesion at acute phase of injury in both type of mice, the configuration of these cells remained relatively unchanged for the chronic phase of injury owing to their limited migration. As a

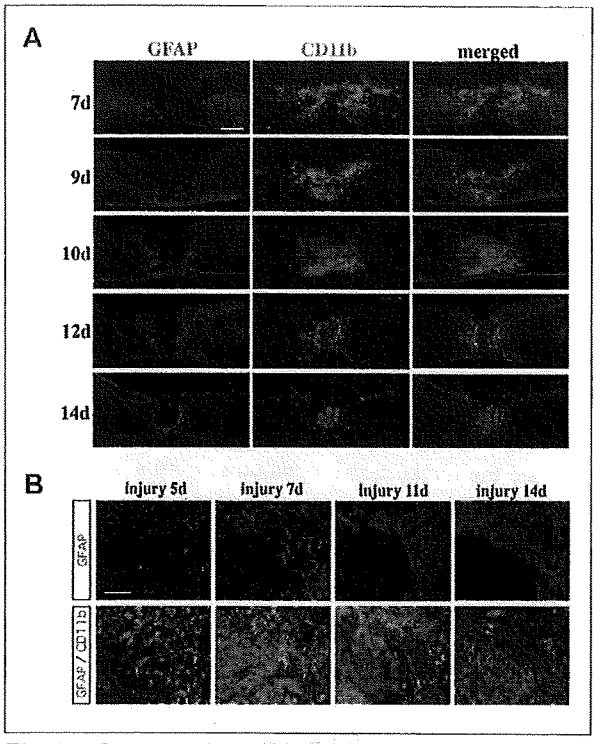

Fig.1 Compaction of inflammatory cells by reactive astrocytes and glial scar formation (Figure adapted from Okada et al.[7]).

A) GFAP-positive reactive astrocytes gradually confine the area of CD11b-positive inflammatory cell infiltration. Scale bar, 500 μm.

B) GFAP-positive reactive astrocytes gradually form glial scar and seclude inflammatory cells. Scale bar, 100 μm.

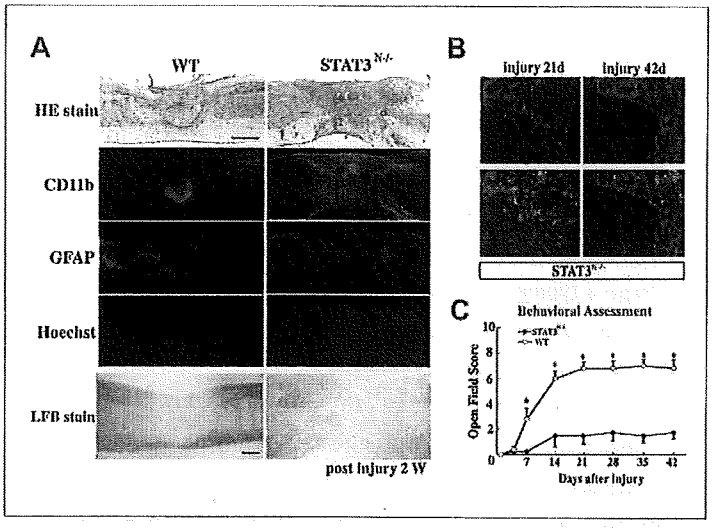

Fig.2 Compaction of inflammatory cells by reactive astrocytes and functional recovery were limited in STAT3$^{N-/-}$ mice (Figure adapted from Okada et al.[7]).

A) Compared to WT mice, the infiltration of CD11b-positive cells, GFAP negative area and demyelination were greater in STAT3$^{N-/-}$ mice at 2 weeks after injury. Scale bar, 500 μm and 300 μm.

B) Glial scar formation and seclusion of inflammatory cells were insufficient in STAT3$^{N-/-}$ mice even at 3 weeks after injury.

C) Time course of functional recovery of lower limbs (open filed score). While gradual recovery was observed in the subacute phase in WT mice, little improvement was observed in STAT3$^{N-/-}$ mice. Data are mean $\pm$ s.e.m. $*p<0.01$, and $\star p<0.05$.

result, the impaired contraction of inflammatory cells by reactive astrocytes brought about widespread damage and limited recovery in only STAT3$^{N-/-}$ mice (Fig.2B,C).

To further investigate the relationship between STAT3 signaling and function of reactive astrocytes, analysis of SCI in SOCS3$^{N-/-}$ mice[16] was conducted. SOCS3 is the negative feedback molecule of STAT3 and the "bipolar" relationship between STAT3 and SOCS3 has been noted in several selective deletion experiments[15,16]. In the injured spinal cord in SOCS3$^{N-/-}$ mice, rapid migration of reactive astrocytes to seclude inflammatory cells, enhanced contraction of lesion area and dramatic improvement in functional recovery were observed. These results suggest that STAT3 signaling associated with the migration of reactive astrocytes is key regulator in the healing process after SCI.

Regarding the downstream of Stat3, several reports indicated a possible molecular link between Stat3-zinc signaling and cell movement[17]. The zinc transporter LIV1 was found to be the transcriptional downstream target of Stat3 and essential for the nuclear localization of Snail, a transcriptional repressor of the Cdh1 gene which encodes E-cadherin. The absence of Stat3 therefore causes dysregulation of cell adhesion and impairs cell movement. In this model, selective deletion of Stat3 in reactive astrocytes brought about their limited migration and impaired healing process after SCI. In addition, another study reported that zinc deficiency impaired compaction of inflammatory cells by reactive astrocytes after CNS injury similar to STAT3$^{N-/-}$ mice[18,19]. On the other hand, astrocyte-targeted IL-6-expressing transgenic mice showed prompt migration of reactive astrocytes and compaction of inflammatory cells after CNS injury similar to SOCS3$^{N-/-}$ mice[20]. It stands to reason that enhanced phosphorylation of Stat3 in reactive astrocytes brought about the similar phenotype to SOCS3$^{N-/-}$ mice after SCI in this transgenic mice. We also confirmed the robust expression of LIV1 mRNA in reactive astrocytes of wild-type mice but limited expression in STAT3$^{N-/-}$ mice in this model[7]. Thus, Stat3-Zinc signaling could

be a radically new therapeutic target for the treatment of CNS injury.

The pleiotropic role for astrogliosis

Astrogliosis is intrinsically loosely defined term. After CSN injury, the astrocytes around the lesion response to injury and undergo a typical change of hypertrophy, process extension. These reactive astrocytes are gradually integrated and form a physical barrier, commonly referred to as glial scar[21] as shown in Fig.1B. This process after CNS injury is roughly described as "reactive gliosis" or "astrogliosis". As mentioned above, astrogliosis is considered to be detrimental for regeneration of CNS since they secrete chondroitin sulphate proteoglycans (CSPGs), which inhibit axonal outgrowth[8]. Owing to this inhibitory molecule, severed axons within long myelinated tracts cannot regenerate past the lesion. In fact, treatment with chondroitinase after SCI resulted in degradation of CSPGs at the lesion site, and allowed axonal regeneration and recovery of locomotor and proprioceptive functions[22]. In mice lacking both GFAP and vimentin, reduced astroglial reactivity resulted in improved sprouting of axons and functional restoration after SCI[23].

However, the basic phenomena of reactive gliosis appear conserved throughout vertebrate evolution. Thus, reactive gliosis has advantages for functional restoration or survival. Actually, glial scar provide several important beneficial functions for stabilizing fragile CNS tissue and repair of the blood-brain barrier after injury. Their primary role is to seclude the injury site from healthy tissue, preventing a cascading wave of uncontrolled tissue damage[8]. The selective ablation of dividing astrocytes using ganciclovir and GFAP-TK transgenic mice resulted in severe leukocyte infiltration, tissue disruption, demyelination and neuronal death[24]. Here, we showed that Stat3 signaling in reactive astrocytes have a considerable role in the repair of injured tissue and the recovery of motor function.

Although these results seem to conflict with one another, consideration of the timeframe in which these events were observed suggests a possible phase-dependent role of reactive astrocytes. In mice lacking both GFAP and vimentin, functional recovery was observed later than 2 weeks after injury[23], whereas substantial recovery was completed within 2 weeks after injury in Nes-Stat3-/- and Nes-Socs3-/- mice, suggesting that reactive astrocytes in the subacute phase repair tissue and restore function, whereas in the chronic phase of injury they impair axonal regeneration as a physical and chemical barrier. These reports also indicate that different potential effects of reactive gliosis are likely to be context dependent and regulated by different intracellular signaling pathways.

Concluding remarks

In this review, we have shown that Stat3 is a key regulator of reactive astrocytes migration and beneficial aspects of reactive astrocytes after CNS injury. Stimulation of reactive astrocytes migration might thus represent a potential target for intervention in the treatment of CNS trauma. However, the precise mechanism of reactive response in astrocytes as well as the functional recovery by reactive astrocytes remains elusive. For functional restoration, reorganization of interactions between descending inputs and the lumbosacral locomotor circuits is required. In addition, developed glial scar actually inhibit the regeneration and reorganization of spinal circuits in the chronic phase of injury. Future elucidation of both intrinsic and extrinsic astrocytes response mechanisms might contribute to achieve a better understanding of the pathophysiology of CNS injury.

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PROSPECT

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Neural Crest-Derived Stem Cells Display a Wide Variety of Characteristics

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ABSTRACT

A recent burst of findings has shown that neural crest-derived stem cells (NCSCs) can be found in diverse mammalian tissues. In addition to their identification in tissues that are known to be derived from the neural crest, recent studies have revealed NCSCs in tissues that are not specifically derived from the neural crest, such as bone marrow. NCSCs can express a wide range of characteristics, and which properties are expressed mainly depends on their tissue sources and the ontogenic stage of the animal. The identification of NCSCs in various tissues opens an entirely new avenue of approach to developing autologous cell replacement therapies for use in regenerative medicine. In this review, we discuss the origin, migration, and lineage potential of NCSCs from various mammalian tissue sources. J. Cell. Biochem. 107: 1046–1052, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: NEURAL CREST-DERIVED STEM CELLS; BONE MARROW; DORSAL ROOT GANGLIA; SKIN; EMBRYO; ADULT

The neural crest is a transient embryonic tissue that originates at the neural folds during vertebrate development. The neural crest-derived cells delaminate from the dorsal neural tube and migrate to various locations, where they differentiate into a vast array of cell types, from neural to mesenchymal [Le Douarin and Kalcheim, 1999]. In addition, some neural crest-derived cells are maintained in an undifferentiated state as neural crest-derived stem cells (NCSCs) throughout the life of the animal. Although how NCSCs are defined has varied among reports to date, it is clear that NCSCs have a self-renewal ability and the potential to differentiate into several different neural-crest lineages, including neurons, glial cells, myofibroblasts, melanocytes, adipocytes, chondrocytes, osteocytes, and connective tissues [Crane and Trainor, 2006; Delfino-Machin et al., 2007]. Recent studies have demonstrated the presence of NCSCs in a number of different tissues. This review will consider the present status of NCSC research, focusing on the origin, migration, and characteristics of mammalian NCSCs, and highlighting the differences in the phenotypes of NCSCs derived from different tissues.

ISOLATION AND CHARACTERIZATION OF EMBRYONIC NCSCs

Stemple and Anderson [1992] first described mammalian NCSCs, which they isolated separately from the rat embryonic neural tube. These NCSCs were isolated by flow cytometry set to select cells expressing low-affinity nerve growth factor (NGF) receptor ($p75^{LNTR}$). The frequency of colony formation was significantly higher in the $p75^{+}$ fraction than the $p75^{-}$ one. The $p75^{+}$ colony-forming cells had self-renewal activity and gave rise to neurons, glial cells, and myofibroblasts. These colony-forming cells are now well accepted as being the NCSCs.

Cells with similar properties to the NCSCs have been isolated from rat embryonic sciatic nerve in the post-migratory phase of neural crest development [Morrison et al., 1999]. Because glial cells in the sciatic nerve also express p75, the authors selected the cell fraction that was both positive for p75 and negative for P0 (peripheral myelin protein). The isolated NCSCs showed a significant enrichment of colony-forming cells, with self-renewal and multipotent

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differentiation potential. An in vivo study in mouse revealed that NCSCs in the peripheral nerve generate Schwann cells and endoneurial fibroblasts during embryonic development [Joseph et al., 2004]. These reports demonstrate that multipotent NCSCs persist at least until late gestation, after the onset of neural crest migration, and suggest that they persist in other tissues as well, during embryogenesis.

Rat NCSCs were also isolated from the gut at E14.5 by selecting for $p75^{+}$ and $\alpha4$ integrin^{+} fractions [Bixby et al., 2002]. The authors compared the characteristics of the NCSCs from the gut and sciatic nerve, and demonstrated that gut NCSCs are sensitive to neurogenic factors, while sciatic nerve NCSCs are sensitive to gliogenic factors in vitro. Upon transplantation of both types of NCSCs into the developing peripheral nerve in vivo, gut NCSCs gave rise primarily to neurons, while sciatic nerve NCSCs gave rise to glial cells. These results suggest that the phenotype of NCSCs mainly depends on their post-migratory tissue source. Furthermore, NCSCs express characteristics in a combinatorial manner that reflects regional environmental differences as well as cell-intrinsic differences [Bixby et al., 2002]. However, the mechanisms that control the lineage determination and/or the timing of differentiation among the tissue sources remain unclear. Therefore, of interest will be the clarification of the molecular mechanisms operating on epigenetic modifications of a differentiation-related gene that is involved in the regulation of the spatial and temporal specifications of NCSCs.

INSTRUCTIVE SIGNALS FOR NCSCs

After migration, the NCSCs undergo specific differentiation steps that are influenced by environmental signals. BMP2 acts as a growth factor and instructs embryonic NCSCs to become autonomic neurons of the peripheral nervous system [Shah et al., 1996]. Wnt signaling instructs embryonic NCSCs to adopt a sensory neuronal phenotype [Lee et al., 2004]. Interestingly, the combined action of BMP2 and Wnt1 does not induce the NCSCs to differentiate into a particular cell fate, but rather maintains the undifferentiated state [Kleber et al., 2005]. Other factors involved in embryonic NCSC differentiation are glial growth factors, which induce the generation of peripheral glia, and transforming growth factor-β (TGF-β), which promotes the generation of smooth muscle cells [Le Douarin and Dupin, 2003]. Thus, the developmental processes of differentiation and proliferation in NCSCs require precise coordination and control.

MIGRATORY PATHWAYS OF NCSCs

Once the neural crest-derived cells detach from the dorsal neural tube, they migrate along specific pathways. The neural crest-derived cells of both the branchial region and the trunk of vertebrate embryos migrate in segmentally restricted streams. The cephalic neural crest cells migrate into the branchial arches, where they differentiate into specific bones, cartilages, and cranial ganglia [Kontges and Lumsden, 1996]. The trunk neural crest cells travel two pathways, dorsolaterally, between the somites and the overlying ectoderm, and ventromedially, through the somatic mesoderm or between the neural tube and the somites (Fig. 1) [Le Douarin and

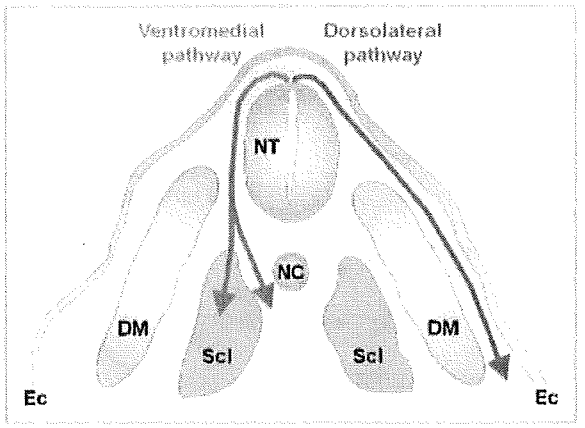

Fig. 1. Schematic diagram of the trunk region of the embryo. Trunk neural crest cells travel by two pathways: the dorsolateral pathway, which runs between the dermomyotome and the ectoderm, and the ventromedial pathway, which runs between the neural tube and the sclerotome or through the sclerotome. Ec, ectoderm; NT, neural tube; DM, dermomyotome; Scl, sclerotome; NC, notochord; Ao, aorta.

Kalcheim, 1999]. Neural crest cells that travel medially contribute to the sensory and sympathetic ganglia, and generate Schwann cells and chromaffin cells. Neural crest that migrates dorsolaterally generates melanocytes.

Recent work has identified the signaling mechanism for this migration [Kuriyama and Mayor, 2008]. Briefly, neural crest cells are known to undergo epithelial–mesenchymal transition when they delaminate from the dorsal neural tube, a process controlled by *cadherins, connexins, snail, twist,* and *matrix metalloproteases (MMPs)* genes. Once the neural crest cells delaminate from the dorsal neural tube, their well-regulated migration is controlled by some specific signals such as Eph/ephrins, semaphorins/neuropilins, and Slit/Robo, which play important roles as repulsive signals modulating the migration of neural crest cells into target areas of embryo [Kuriyama and Mayor, 2008].

The migratory routes and destinations chosen by transplanted NCSCs that were isolated from adult rodent heart were examined in the chick embryo. These NCSCs migrated to the dorsal root ganglia (DRG) and ventral spinal nerve by the medial route, and to the outflow tract and conotruncus of the developing heart by the lateral route [Tomita et al., 2005]. Similarly, skin-derived NCSCs, also called skin-derived precursors (SKPs), that were harvested from rodent trunk skin and transplanted into the dorsal neural tube of the chick embryo, migrated back to the skin by the lateral route and to the DRG and spinal nerve by the medial route [Fernandes et al., 2004]. These studies showed that transplanted NCSCs could migrate like other neural crest cells and contribute to a variety of neural crest-derived tissues when reintroduced into the embryonic environment.

Recently, our group reported that NCSCs also migrate into the aorta-gonad-mesonephros (AGM) region in embryonic mice [Nagoshi et al., 2008]. The AGM region is a transient embryonic tissue in which the first adult-type hematopoietic stem cells (HSCs) are generated. Late in embryogenesis, the HSCs migrate within the

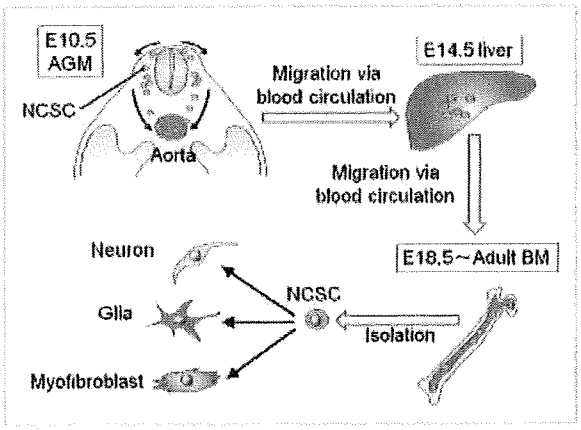

Fig. 2. Simplified cartoon of the NCSC migratory stream from the dorsal neural tube to the BM. During embryogenesis, some NCSCs migrate into the AGM region, enter the blood circulation, pass through the fetal liver, and reach the BM just before birth. The migrated NCSCs persist in the BM throughout life, and maintain the potential to differentiate into various types of neural-crest lineage cells.

bloodstream, from which they pass through fetal liver; the HSCs then enter the bone marrow (BM) just before the mice are born [Dzierzak and Speck, 2008]. We found that, like the HSCs, NCSCs migrate from the AGM region through the bloodstream, and the fetal liver to the BM (Fig. 2) [Nagoshi et al., 2008]. The timing of the NCSC migration coincides with that of the HSCs, implying that some undefined relationship exists between the NCSCs and hematopoiesis.

NCSCs IN ADULT TISSUES

NCSCs have been found in diverse adult mammalian tissues. Because the methods for harvesting and analyzing the NCSCs differ among reports, as do the NCSCs' reported characteristics, in the following text, these findings are systematically compared and summarized (Table I).

BONE MARROW

We demonstrated the existence of NCSCs in the BM of adult rodents [Nagoshi et al., 2008] using the Cre-lox system-mediated lineage analysis and sphere culture technique. Transgenic mice expressing Cre recombinase under control of the P0 promoter (P0-Cre) [Yamauchi et al., 1999] or Wnt1 promoter/enhancer (Wnt1-Cre) [Danielian et al., 1998] were mated with EGFP reporter mice (CAG-CAT$^{loxP/loxP}$-EGFP) [Kawamoto et al., 2000] to obtain P0-Cre/CAG-EGFP or Wnt1-Cre/CAG-EGFP double-transgenic mice [Nagoshi et al., 2008]. P0 promoter expresses genes after differentiation of neural crest cells from the dorsal neural tube [Yamauchi et al., 1999], and Wnt1 promoter/enhancer expresses genes in the dorsal neural tube and roof-plate from the onset of neural crest migration [Danielian et al., 1998]. In these transgenic mice, the transient activation of the P0 promoter and Wnt1 promoter/enhancer induces Cre-mediated recombination in premigratory neural crest cells, indelibly tagging neural crest-derived cells with EGFP expression. Prospectively isolated EGFP$^+$ cells from the BM of P0 and Wnt1-Cre/CAG-EGFP adult mice proliferated in vitro to form clonal spheres, showed self-renewal activity, and differentiated into neurons, glial cells, and myofibroblasts [Nagoshi et al., 2008]. The presence of NCSCs in the BM is also supported by a recent report using the same P0-Cre/CAG-EGFP reporter mice to demonstrate that a portion of mesenchymal stem cells (MSCs) in the BM of the lower extremities are of neural-crest lineage [Takashima et al., 2007]. In addition, we recently showed that neural crest-derived cells contribute to MSCs, which can give rise to adipocytes, chondrocytes, and osteocytes [Morikawa et al., 2009]. Considering that a part of MSCs are derived from neural crest, NCSCs might play a role as an HSC niche by controlling HSC maintenance, proliferation, differentiation, and recruitment in the BM [Uccelli et al., 2008].

Several groups have demonstrated the presence of stem or precursor cells in the BM that generate neurons. For example, bone marrow stromal cells (BMSCs) harvested from rat and human express Nestin and differentiate into neurons and glial cells in vitro [Sanchez-Ramos et al., 2000]. Another report demonstrated that rat and human BMSCs cultured with FBS expand as undifferentiated cells, and upon differentiation, they become neurons [Woodbury et al., 2000]. The differentiation of BMSCs into functional neurons is enhanced by Noggin [Kohyama et al., 2001]. However, the

TABLE I. Identification of NCSCs From Various Tissues

Refs.	Age	Place	Isolation	Marker	Animal	Genotype
Stemple and Anderson [1992]	E10.5	Neural tube	Retrospective	p75+	Rat	W/T
Morrison et al. [1999]	E14.5	Sciatic nerve	Prospective	p75+P0−	Rat	W/T
Bixby et al. [2002]	E14.5	Gut	Prospective	p75+α4+	Rat	W/T
Kruger et al. [2002]	Adult	Gut	Prospective	p75+	Rat	W/T
Hagedorn et al. [1999]	E14	DRG	Retrospective	p75+	Rat	W/T
Hjerling-Leffler et al. [2005]	E11.5	DRG	Retrospective		Mouse	W/T
Li et al. [2007]	Adult	DRG	Retrospective		Rat	W/T
Nagoshi et al. [2008]	Adult	DRG, whisker pad, bone marrow	Prospective	EGFP	Mouse	P0 and Wnt1-Cre/CAG-EGFP
Toma et al. [2001, 2005]	Juvenile and adult	Skin (face and back)	Retrospective		Mouse	W/T
	Adult	Skin (scalp)	Retrospective		Human	
Sieber-Blum et al. [2004]	Adult	Skin (bulge)	Retrospective		Mouse	Wnt1-Cre/ROSA-LacZ
Wong et al. [2006]	Adult	Skin (back)	Retrospective		Mouse	W/T
	Adult	Skin (back)	Prospective	EYFP	Mouse	Dhh and Dct-Cre/ROSA-EYFP
Toma et al. [2001, 2005]	Children	Foreskin	Retrospective		Human	
Tomita et al. [2005]	Juvenile and adult	Heart	Prospective	SP cells	Mouse	W/T, P0-Cre/CAG-EGFP
Yoshida et al. [2006]	Adult	Cornea	Retrospective		Mouse	W/T, P0, and Wnt1-Cre/CAG-EGFP
Pardal et al. [2007]	Adult	Carotid body	Retrospective		Rat	W/T
	Adult	Carotid body	Prospective	EGFP	Mouse	GFAP promoter-EGFP

developmental origin and differentiation potential of the BMSCs remain unclear, largely because it is difficult to understand how neurons, which are ectodermal in origin, are generated by the BM-derived cells. Our results demonstrating the presence of NCSCs in the BM suggest that this unusual differentiation potential is owing to NCSCs inhabiting the BM. It will be interesting to clarify the relationship between NCSCs and the BM-derived stem cells that are reported to generate neural cells.

DRG

Neural crest cells were first discovered in chick embryos as the precursors of the spinal sensory ganglia, the DRGs [His, 1868]. In recent years, detailed analyses of mammalian NCSCs in the DRG have been carried out. In one report, single cells dissociated from rat embryonic DRGs were labeled with p75 by live-cell staining, and the identified $p75^+$ NCSCs were shown to give rise to neurons, glial cells, and smooth muscle-like cells in response to instructive extracellular cues, but their self-renewal activity was not assessed [Hagedorn et al., 1999]. A recent study showed that neural crest boundary cap cells, found in embryos, can generate neurons and satellite cells [Maro et al., 2004]. Boundary cap cells are neural crest derivatives that form clusters at the entry and exit points of peripheral nerve roots; they migrate to and colonize the DRG during embryogenesis. Boundary cap clusters contain multipotent NCSCs that self-renew, show multipotency, and differentiate into mature sensory neurons and Schwann cells under appropriate conditions [Hjerling-Leffler et al., 2005; Aquino et al., 2006]. These findings raised the possibility that NCSCs might persist in the DRGs throughout life, and this has now been demonstrated [Li et al., 2007]. Interestingly, the NCSCs probably originate from satellite cells [Li et al., 2007]. Given that some of the satellite cells are thought to derive from boundary cap cells [Zirlinger et al., 2002; Maro et al., 2004], these data indicate that the NCSCs, which form a subpopulation of the boundary cap cells and migrate into the DRG during embryogenesis, are maintained in an undifferentiated state throughout the life of the animal.

Our group also confirmed the existence of NCSCs in the DRG of adult mice [Nagoshi et al., 2008]. In this recent study, we compared the characteristics of NCSCs in various tissues of adult mice by examining the expression levels of the NCSC markers *sox10* [Paratore et al., 2001] and *p75* [Stemple and Anderson, 1992] and of markers for neural stem/progenitor cells, *nestin* [Lendahl et al., 1990] and *musashi1* [Sakakibara et al., 1996; Okano et al., 2002]. We found that these markers were expressed at higher levels in NCSCs from the DRG than in NCSCs from the whisker pad or BM. These results may reflect the self-renewal activity and multipotency of NCSCs, because the DRG-derived NCSCs displayed a greater ability to form secondary spheres and displayed a higher proportion of cells that maintained a multilineage differentiation potential [Nagoshi et al., 2008]. Although the methods for identifying NCSCs and the culture conditions were different in the reports cited above, the combined findings support the idea that the DRG contains a high proportion of NCSCs.

SKIN

One of the more surprising findings about NCSCs is their existence in the skin. Isolated cells from juvenile and adult rodent skin proliferate to form spheres and differentiate into several types of cells: neurons, glial cells, smooth muscle cells, and adipocytes [Toma et al., 2001]. Importantly, a single cell can also form a sphere that is self-renewed for at least 5 months of passaging and still shows the multilineage differentiation into cells of both neural and mesodermal origins. These cells are called SKPs [Toma et al., 2001]. Although the developmental origin of the SKPs was unclear when the report was published, the same group has since demonstrated that the SKPs in facial skin are derived from the neural crest [Fernandes et al., 2004].

Fernandes et al. [2004] used Wnt-Cre/ROSA-LacZ double-transgenic mice, a line used for neural-crest lineage tracing, and showed that whisker follicle dermal papillae are entirely neural crest-derived. In addition, SKP-derived spheres from the facial skin of the Wnt1-Cre/ROSA-LacZ mice were positive for β-galactosidase. The SKPs expressed the transcription factor genes *slug*, *snail*, *twist*, *Pax3*, and *Sox9*, which are also expressed in embryonic NCSCs [Fernandes et al., 2004].

Another group has demonstrated the existence of different NCSCs, "epidermal neural crest cells" (eNCSCs), in the adult mouse whisker follicle, using a different approach [Sieber-Blum et al., 2004]. This group used Wnt1-Cre reporter mice to show marker expression in the bulge region of the follicle. In explants of whisker follicle bulges, migrating eNCSCs were observed. An in vitro analysis of the emigrated eNCSCs revealed that they have self-renewal capacity and the potential to differentiate into neurons, Schwann cells, smooth muscle cells, and melanocytes, a finding that highlights the pluripotency of individual clones [Sieber-Blum et al., 2004]. The gene expression profile of eNCSCs was examined by longSAGE (long serial analysis of gene expression) [Hu et al., 2006]. The authors identified 19 genes expressed in common between eNCSCs and embryonic NCSCs. Although eNCSCs and the epidermal stem cells that generate keratinocytes share the bulge as their stem-cell niche, they are clearly distinguishable by their gene expression profiles. Interestingly, these authors also examined the expression of cell markers for SKPs in the eNCSCs, but the eNCSCs did not express any of them, showing that eNCSCs are very different from SKPs [Fernandes et al., 2004; Hu et al., 2006].

In addition to the whisker follicle of the facial skin, a recent study showed that a subpopulation of sphere-initiating cells from the murine trunk skin is also of neural crest origin [Wong et al., 2006]. Spheres derived from trunk skin contain cells that express the NCSC markers p75 and Sox10, display self-renewal capacity over more than 20 passages, and differentiate into neurons, glial cells, smooth muscle cells, chondrocytes, melanocytes, and adipocytes.

Wong et al. also used Desert Hedgehog (Dhh)-Cre/ROSA-LacZ mice, which express Cre recombinase in the peripheral glial lineage, and found that *LacZ*-positive cells in the bulge region of trunk skin were positive for p75 and Sox10, suggesting the existence of NCSCs which could give rise to glial lineage. Moreover, in Dct-Cre/ROSA-*LacZ* mice, which express Cre recombinase in melanocytes, *LacZ*-positive cells in the bulge region and hair follicle bulb were positive for p75 and Sox10, suggesting that NCSCs, which could give rise to melanocyte lineage, also exist in the bulge region and bulb [Wong et al., 2006].

When these authors prospectively isolated enhanced yellow fluorescent protein (EYFP)-positive cells from the trunk skin of Dhh-

Cre/ROSA-EYFP and Dct-Cre/ROSA-EYFP mice, the cells proliferated to form spheres, and the spheres contained cells positive for p75 and Sox10. The authors concluded that NCSCs or neural crest-derived progenitor cells that are restricted to the glial and melanocyte lineages also exist in the trunk skin of adult mice [Wong et al., 2006].

Stem cells from human skin have been identified as well. SKPs from the human scalp express Nestin by immunohistochemstry and differentiate into neurons [Toma et al., 2001]. The same group has also shown that SKPs exist in juvenile human foreskin that show self-renewal activity by several passages, differentiate into neurons, glial cells, smooth muscle cells, and adipocytes, and express the neural crest-specific markers *Pax3*, *Snail*, and *Slug* by RT-PCR [Toma et al., 2005]. Similar results were observed in the adult human dermis, which contains sphere-initiating cells [Joannides et al., 2004]. These spheres expressed Nestin and Musashi1, and differentiated into neurons, fibronectin, and smooth muscle cells. However, they did not express neural crest markers or differentiated glial cells [Joannides et al., 2004]. Since the sphere-initiating cells from human skin were retrospectively identified in all of these reports (Table I), it remains uncertain that these cells are derived from neural crest. Future studies will help identify novel neural crest markers to allow the prospective isolation of NCSCs and their selective enrichment from other sources.

OTHER TISSUES

NCSCs have been identified in adult rat gut as well as that of the embryo. A comparison of fetal and adult gut NCSCs showed that the adult NCSCs self-renew less efficiently and differentiate into a narrower range of neuronal subtypes [Bixby et al., 2002; Kruger et al., 2002]. Considering that the characteristics of these NCSCs are different both spatially and temporally [Bixby et al., 2002; Kruger et al., 2002; Nagoshi et al., 2008], it is not possible to categorize NCSCs as a homogenous population. It will be important to classify these populations according to their differentiation potential and self-renewal activity, and to elucidate the molecular mechanisms for the maintenance and lineage determination of NCSCs in both spatial and temporal aspects.

Another type of NCSC has been identified in the heart of adult mice [Tomita et al., 2005]. Cardiac side population (SP) cells contain a subpopulation of NCSCs, which can generate spheres and differentiate into neurons, glial cells, smooth muscle cells, and cardiomyocytes. By using P0-Cre/CAG-EGFP adult heart tissue for immunohistochemistry, Nestin-positive cells were identified among the EGFP-positive ones that proliferated to form spheres in vitro. These findings suggest that NCSCs that can differentiate into various cell types remain in the heart of adult mice.

NCSCs have also been identified in the adult mouse cornea [Yoshida et al., 2006]. Cornea-derived spheres express Nestin and Musashi1, self-renew over several passages, and differentiate into neural- and mesenchymal-lineage cells. The NCSCs in the cornea are also enriched in the SP cells, like the cardiac NCSCs [Tomita et al., 2005; Yoshida et al., 2006]. Cornea-derived cells from P0-Cre/CAG-EGFP and Wnt1-Cre/CAG-EGFP adult mice proliferate to form $EGFP^+$ spheres, indicating the existence of NCSCs in the adult cornea.

A recent study demonstrated the existence of NCSCs in the carotid body, an oxygen-sensing organ of the sympathoadrenal lineage that grows under conditions of hypoxemia [Pardal et al., 2007]. $GFAP^+$ cells in the rat carotid body incorporate BrdU in vivo, and proliferate in vitro to form spheres that differentiate into tyrosine hydroxylase (TH)-positive neurons and smooth muscle cells, suggesting that the $GFAP^+$ cells are the stem/progenitor cells that resemble NCSCs in some aspects. Although the $GFAP^+$ stem cells are reversibly converted to $Nestin^+$ progenitors in re-normoxia, the equilibrium is displaced toward the $Nestin^+$ progenitors, which give rise to TH^+ neurons under hypoxic conditions [Pardal et al., 2007].

Although NCSCs from various adult tissues have been reported, it would be rash to conclude that all tissue-derived stem cells are NCSCs. For example, multipotent precursors that generate neural- and pancreatic-lineages have been identified in the adult mouse pancreas [Seaberg et al., 2004], and they did not express the neural crest markers *Pax3*, *Twist*, *Sox10*, or *Wnt1* by RT-PCR. The authors concluded that the precursors are not neural crest derivatives. However, these cells did express *slug*, *snail*, and *p75*, and therefore the possibility that they are derived from neural crest cannot be excluded, especially because the expression patterns of neural crest markers in NCSCs are quite different, depending on the tissue source [Nagoshi et al., 2008].

COMPARISON OF METHODS FOR IDENTIFYING NCSCs

So far, a perfect single marker has not been identified for the isolation of NCSCs, although several research groups have established their original methods for the identification of NCSCs (Table I). Sophisticated purification methods for NCSCs are expected to enhance the progress in this field. When comparing the respective protocols for identification, prospective rather than retrospective isolation seems much better for the purification with native condition. Retrospective isolation raises the possibility that there might be contamination by various non-NCSCs and that the characteristics of NCSCs will change during the cell culture procedure. In the rodent study, p75 is one of the good markers for the prospective isolation of NCSCs, and it has been widely used for purification by several groups [Morrison et al., 1999; Bixby et al., 2002; Kruger et al., 2002]. Considering that genetic lineage labeling techniques such as P0-Cre and/or Wnt1-Cre/CAG-EGFP are available for mice, until now, one of the best ways for NCSC purification has been the isolation of $p75^+$ $EGFP^+$ cells by flow cytometry. Although NCSC markers including p75 have been identified in rodents for prospective isolation, no such valuable markers have been established for human NCSCs. Needless to say, genetic lineage labeling techniques are not available for human. The possible identification of novel specific surface antigens for human NCSCs needs to be pursued further.

APPLICATION OF NCSCs TO REGENERATIVE MEDICINE

The NCSC is one of the most intriguing cells in the field of regenerative medicine, because it is easily harvested from accessible

peripheral tissues, which could make autologous transplantation possible. Autologous transplantation would avoid immunological complications as well as the ethical concerns associated with the use of embryonic stem cells. Of the various NCSCs, research on skin-derived NCSCs is the most advanced because of their accessibility. One of the critical questions for the application of NCSCs to regenerative medicine is whether cells that are differentiated from NCSCs are functional. Some evidence supports this. Cultured rodent and human SKPs generate Schwann cells when treated with neuregulins, and myelinate host axons after transplantation to an injured peripheral nerve [McKenzie et al., 2006]. These Schwann cells also myelinate axons in the CNS when transplanted into the brain. Furthermore, the SKP-derived Schwann cells were transplanted into the injured spinal cord of the rat, and improved locomotor function [Biernaskie et al., 2007]. This was the first report that NCSC-derived cells could contribute to the recovery of function following central nervous system injury, but these SKP-derived Schwann cells were harvested from neonatal murine trunk skin, not adult [Biernaskie et al., 2007]. The development of methods for the efficient collection of adult NCSCs that require only small tissue samples will be needed to accomplish the goal of using NCSCs clinically in autologous cell transplantation.

CONCLUSION

Numerous reports show that NCSCs survive in a wide range of tissues for the entire life of the animal, and other tissues may still harbor unidentified NCSCs. It is not altogether clear why the NCSCs persist in adult mammals. However, from the reports that some NCSCs maintain the potential to help tissue recover from damage, it is generally thought that the stem cells from various adult tissues retain the capacity for tissue repair; it is also likely that these cells have undiscovered biological roles that may be extremely helpful in the treatment of human disease. Even the currently known properties of adult NCSCs make them attractive for clinical application in regenerative therapies such as cell replacement therapy. NCSCs from different tissues have distinct characteristics, and further study of these NCSCs will hopefully lead to the culture and transplantation of NCSCs that are the most appropriate for treating specific lesions.

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