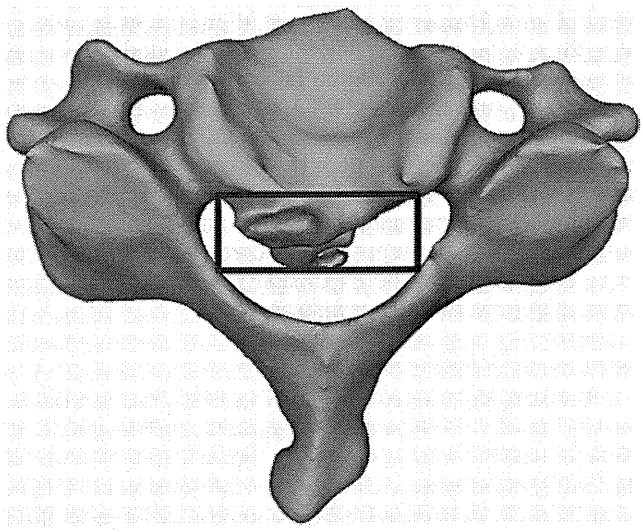

FIG. 2. The volume of OPLL was defined as the area bounded by the rectangle.

migration was measured by marker-based registration, providing gold-standard data. Accuracy was defined as the closeness to the true value; the RMSE was assessed.

Reproducibility of Volume Measurement

To assess reproducibility of measurement of OPLL volume, 3 observers measured the volume of 10 OPLL segments twice with 1 week elapsing between each measurement. Intraobserver and interobserver ICC, RMSE, and CV were assessed.

Results

Clinical Data

The mean patient age was 63 years (range 43–71 years), and the mean duration of follow-up monitoring was 3.1 years (range 2.1–3.8 years). The mean score on the Japanese Orthopaedic Association scale was 8.5 before surgery and 15.6 after surgery; the maximum possible score is 17. All patients experienced functional improvement after surgery. Four of the 5 patients had a mixed type of OPLL, and 1 had a localized type of OPLL (Table 1).

Progression of OPLL

Ossification of the posterior longitudinal ligament progressed 0.5 mm or greater in all patients. The average maximum progression length was 4.7 mm, and the progression rate was 1.5 mm/year (Table 1). The maximum progression occurred in the youngest patient (Case 1). Ossification of the posterior longitudinal ligament tends to grow actively around intervertebral areas and less in vertebral areas (Figs. 3 and 4).

Volume Increase of OPLL

The mean volume increase was 1622 mm^3 per patient, and the mean expansion rate was 37% during the follow-up period. The mean volume increase rate was 484 mm^3/year (Table 1).

Accuracy of Voxel-Based Registration and Reproducibility of Volume Measurement

The RMSE was 0.19° in flexion-extension, 0.13° in axial rotation, 0.21° in lateral bending, 0.13 mm in lateral translation, 0.15 mm in superoinferior translation, and 0.31 mm in anteroposterior translation (Table 2).

For intraobserver reproducibility of the volume measurement, the mean ICC, RMSE, and CV were 0.987, 16.0 mm^3, and 1.7%, respectively (Table 3). For interobserver reproducibility, the mean values were 0.968, 33.1 mm^3, and 3.4%, respectively (Table 4).

Illustrative Cases

Case 1

This 43-year-old man presented with a mixed type of OPLL. Before surgery, a plain lateral radiograph showed a continuous type of OPLL at the C2–3 level; however, the extent of OPLL at the C4–5 level was unclear (Fig. 3). Before surgery, the CT model clearly revealed a segmental type of OPLL at C-4, C-5, C-6, and C-7 (Fig. 4). Four years after laminoplasty, growth was apparent on radiographs; however, quantitative changes were unclear, especially at the levels of C-6 and C-7. The postoperative CT model revealed that the C3–4, C4–5, and C5–6 intervertebral spaces were bridged by growing OPLL. The ossified posterior longitudinal ligament behind the C-2

TABLE 1: Clinical data for 5 patients with OPLL*

Parameter	Case 1	Case 2	Case 3	Case 4	Case 5	Mean
age (yrs)	43	64	71	65	71	63
duration of follow-up monitoring (yrs)	3.6	2.6	3.5	2.1	3.8	3.1
JOA score (preop/postop)	7.5/15.5	9.5/17	8.5/15	6.5/14.5	10.5/16	8.5/15.6
OPLL type	mixed	localized	mixed	mixed	mixed	
progression length (mm)	8.9	6.4	3.6	2.5	2.1	4.7
progression rate (mm/yr)	2.4	2.5	1.0	1.2	0.6	1.5
vol increase (mm^3)	3851	369	2139	728	1022	1622
vol increase rate (mm^3/yr)	1056	143	608	346	268	484
expansion rate (%)	89	40	22	15	18	37

* JOA = Japanese Orthopaedic Association.

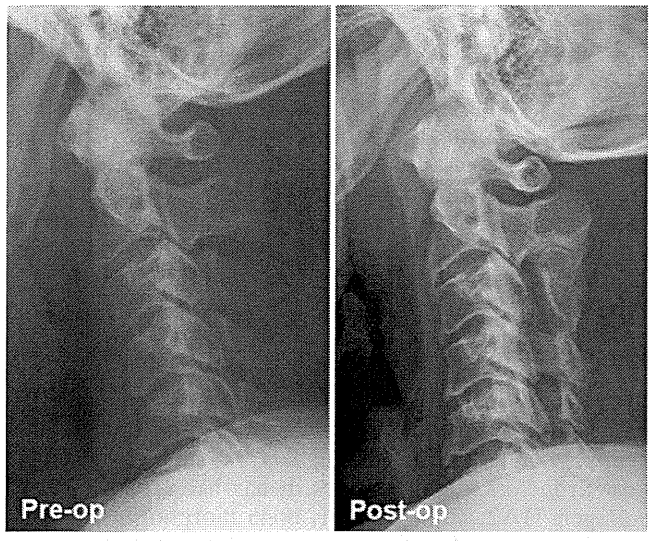

FIG. 3. Case 1. Plain lateral radiographs showing the mixed type of OPLL with the continuous type at C2–3 and the segmental type below C-2. However, it was difficult to recognize the segmental type of OPLL below the C-4 level on the preoperative radiograph.

vertebra had grown 8.9 mm cranially, and the volume increase was 769 mm^3. The ossified posterior longitudinal ligament of the C-4 vertebra had expanded; the volume increase was 977 mm^3. However, there was little progression in the C2–3 intervertebral space, which had already fused and had little mobility before surgery. The segmental type of OPLL at the C-6 level had grown only 1.2 mm, with a volume increase of 129 mm^3. Meanwhile, the segmental type of OPLL of the C-7 vertebra had grown 7.2 mm, with a volume increase of 708 mm^3. There was a big difference in growth between ossifications.

Case 2

This 64-year-old man with a localized type OPLL

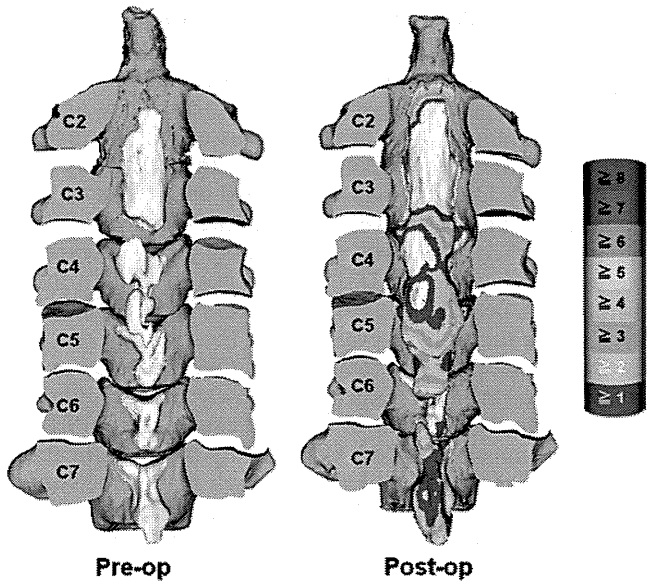

FIG. 4. Case 1. Three-dimensional models. The *bar* at the right shows the relationship between progression length and color.

TABLE 2: Accuracy of voxel-based registration of the cervical spine

Position	Rx (°)	Ry (°)	Rz (°)	Tx (mm)	Ty (mm)	Tz (mm)
1	0.26	0.08	−0.03	0.13	0.00	0.36
2	0.22	0.14	−0.11	−0.13	0.16	−0.30
3	0.06	0.08	0.29	0.10	0.03	0.41
4	−0.10	0.04	-0.28	0.02	0.07	−0.07
5	0.00	0.16	-0.09	0.13	−0.10	0.08
6	−0.21	−0.10	0.32	−0.17	0.34	0.14
7	−0.29	−0.25	−0.18	0.01	−0.05	0.58
8	0.17	0.08	−0.24	−0.19	0.12	−0.13
RMSE	0.19	0.13	0.21	0.13	0.15	0.31

* Rx = flexion-extension; Ry = axial rotation; Rz = lateral bending; Tx = lateral translation; Ty = superoinferior translation; Tz = anteroposterior translation.

underwent surgery. It was difficult to recognize OPLL on preoperative radiography (Fig. 5). However, CT scanning clearly revealed the OPLL attached to the C-4 vertebra (Fig. 6). Two years after laminoplasty, the OPLL had grown 6.4 mm in the cranial direction, with a volume increase of 369 mm^3.

Discussion

Advantages of CT Evaluation

Although several studies have reported on progression of OPLL, 2D evaluation with plain lateral radiographs has some limitations. Three-dimensional evaluation with CT scanning is better suited to measuring OPLL growth. For example, in the lower cervical spine, OPLL is likely to be masked by the shoulder girdle shadows. In young patients, OPLL may be less distinct because the ossification is less densely calcified. Although growth can occur in any direction, only craniocaudal and ventrodorsal progression are depicted on plain radiographs. Growth in the oblique direction is projected only orthographically onto a radiograph. Compared with radiography, CT scanning has several advantages. Computed tomography scanning is the most sensitive diagnostic method for detecting small ossifications or calcifications of the ligament, which are likely to be missed on radiographs.[15] However, conventional CT scanning also has the limitation of slice thickness. Measurement of OPLL on conventional radiographs is unreliable because the slice is likely to be thick, owing to the limited number of films used. It is also difficult to slice OPLL on the same level and angle to compare past and present

TABLE 3: Intraobserver reproducibility of volume measurement

Observer	ICC (95% CI)	RMSE (mm^3)	CV (%)
1	0.992 (0.970–0.998)	10.7	1.0
2	0.980 (0.928–0.995)	17.3	2.1
3	0.990 (0.962–0.998)	20.0	1.9
mean	0.987 (0.953–0.997)	16.0	1.7

TABLE 4: Interobserver reproducibility of volume measurement

Measurement	ICC (95% CI)	RMSE (mm^3)	CV (%)
1st	0.956 (0.842–0.989)	39.2	4.1
2nd	0.980 (0.919–0.995)	27.1	2.7
mean	0.968 (0.880–0.992)	33.1	3.4

ossification. However, these limitations can be overcome using helical scanning with multidetector CT and digital viewers. Additionally, accurate superimposition by voxel-based registration facilitates comparison of OPLL before and after surgery. The Orthopedic Viewer that we used was developed specifically for our study; however, the program was written using Visualization Toolkit (http://www.vtk.org/), an open-source free software system. Therefore, the measurement method that we used can be universally applied using similar viewers with equivalent function.

In our study, 3D evaluation, including volume increase, depicted minor changes in OPLL with high sensitivity. Hori et al.[5,6] reported that progression of greater than 2 mm on a plain radiograph occurred in 56.5%–75% of patients 2 years after laminoplasty. However, our method revealed that progression of greater than 0.5 mm occurred in all patients and that the mean rate of volume increase was 484 mm^3/year. Despite the growth of OPLL, myelopathy did not worsen in any patient in our study, partly because decompression was enough to forestall it. If enough space for the spinal cord can be obtained through laminoplasty, the growth of ossification could help decrease the dynamic factor and might not always be a clinical problem. However, the growth of OPLL after surgery could be a cause for revision surgery in some cases.[4,11,21] Fujiyoshi et al.[3] reported that additional fusion for mobile segments in laminoplasty could slow the progression of OPLL. Ono et al.[16] reported that etidronate disodium had the potential to slow progression. Because OPLL is a kind of ectopic bone formation, some inhibitors of bone formation can prevent OPLL growth. It is essential to have an accurate and reliable measurement method to determine the efficacy of such drugs or of surgical procedures to decrease growth. The method that we used can be a useful tool for the future study of the natural progression process or the efficacy of drugs or surgery in treating OPLL.

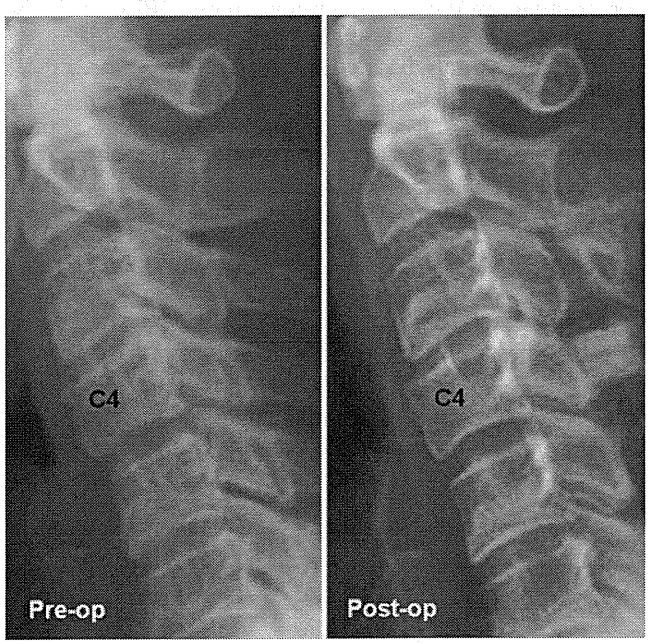

FIG. 5. Case 2. Plain lateral radiographs showing the localized type of OPLL at C-4. It was difficult to recognize ossification on the preoperative radiograph **(left)**. The postoperative radiograph **(right)** shows ossification; however, the length of progression was unclear.

Factors in Ossification Growth

Factors in ossification growth are roughly classified as either general or local. General factors include age, sex, and some hormonal factors, such as genetics, growth factors, and cytokines. It has been reported that collagen 11A2, collagen 6A1, bone morphogenetic protein–2, and transforming growth factor–β are related to progression of OPLL.[18,19] Local factors include mechanical stress, types of OPLL, and types of surgical procedures. Some researchers have reported that the segmental type of OPLL does not progress much in general.[2,5] Chiba et al.[2] reported that progression occurred in 53.3% of the continuous type of OPLL, 27.3% of the segmental type, 67.3% of the mixed type, and 40% of the localized type. They noted that the incidence of progression was significantly higher in patients younger than 59 years than in those older than 60 years. Hori et al.[5] reported that longitudinal progression occurred in 85% of the continuous type of OPLL, 29% of the segmental type, 100% of the mixed type, and 100% of the localized type. They also reported that thickening of ossifications occurred in 38% of the continuous type of OPLL, 0% of the segmental type, 41% of the mixed type, and 0% of the localized type.[6] They speculated that the pathological entity might be different for the continuous or mixed type of OPLL versus the segmental type. However, some reports of studies with longer follow-up periods noted that the segmental type of OPLL went on to become the continuous or mixed type of OPLL.[14,20] Murakami et al.[14] reported that the segmental type of OPLL had become the continuous type by 10 years of follow-up. They argued that the segmental type of OPLL was an initial stage of the continuous or the mixed type and that the entity was not different. Our findings correspond with those of Murakami et al.; however, we found that the extent of growth between OPLL types was considerably different even in the same patient (Case 1). It is not easy to explain these differences, because the growth process is not influenced by a single factor. However, one of the possible reasons for this is that the segmental type of OPLL located at the middle of a vertebral body grows slowly because it is not subjected to dynamic factors to the extent that OPLL in other locations is affected. Once the tip of an ossification reaches the intervertebral area, growth may be activated by increased dynamic factors. Hirabayashi et al.[4] reported that pseudarthrosis-like thickening occurred at mobile intervertebral spaces and that these changes stopped only after elimination of mobility. Our results correspond with theirs. In our study, ossifications grew actively in inter-

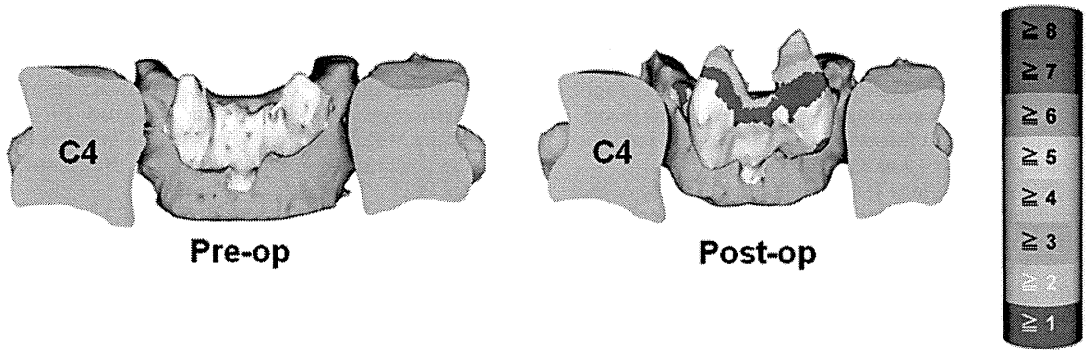

FIG. 6. Case 2. Three-dimensional models. The *bar* at the right shows the relationship between progression length and color.

vertebral areas as if they were bridging gaps to stabilize the mobile segments (Fig. 4). This finding suggests that dynamic factors are strongly involved in growth.[13] We speculate that the growth of OPLL decreases with the stabilizing effect of ossifications themselves and of laminoplasty.

In discussing the reasons for the different growth rates in different types of OPLL, the problem is the difficulty of detecting OPLL at its onset. A small ossification in the initial stage may sometimes be accidentally found, but most ossifications are found at a mature stage because a certain level of ossification must usually be attained before patients experience symptoms. Many studies have reported that ossifications do not grow at a constant speed but instead have growth spurts.[5,20] Therefore, it is theoretically possible that the mixed type of OPLL may be a form of the activated phase of the OPLL growth process. To answer these questions, long-term observation of OPLL starting from the initial stage is necessary. Our accurate 3D evaluation method can be an appropriate tool for further observation.

Conclusions

A novel 3D method for measuring OPLL growth, involving scanning by multidetector CT, showed that progression of greater than 0.5 mm occurred in all patients. After laminoplasty, the mean maximum progression rate was 1.5 mm/year, and the mean volume increase rate was 484 mm^3/year. This method can accurately detect changes in OPLL and thus can be a useful, reliable tool for examining OPLL growth.

Disclosure

This work was supported by a grant-in-aid from the Investigation Committee on Ossification of the Spinal Ligaments, Japanese Ministry of Public Health, Labor, and Welfare and a grant-in-aid for Scientific Research C (KAKENHI:22591632) from the Japan Society for the Promotion of Science. No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

Author contributions to the study and manuscript preparation include the following. Conception and design: Fujimori, Iwasaki, Ishii. Acquisition of data: Fujimori, Iwasaki. Analysis and interpretation of data: Fujimori, Nagamoto. Drafting the article: Fujimori. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Fujimori. Statistical analysis: Fujimori. Administrative/technical/material support: Iwasaki, Yoshikawa, Sugamoto. Study supervision: Iwasaki, Yoshikawa, Sugamoto.

Acknowledgments

The authors thank Aya Sasaki. Katharine O'Moore-Klopf, E.L.S. (East Setauket, NY) provided professional English-language editing of this article.

References

1. Chiba K, Kato Y, Tsuzuki N, Nagata K, Toyama Y, Iwasaki M, et al: Computer-assisted measurement of the size of ossification in patients with ossification of the posterior longitudinal ligament in the cervical spine. **J Orthop Sci 10:**451–456, 2005
2. Chiba K, Yamamoto I, Hirabayashi H, Iwasaki M, Goto H, Yonenobu K, et al: Multicenter study investigating the postoperative progression of ossification of the posterior longitudinal ligament in the cervical spine: a new computer-assisted measurement. **J Neurosurg Spine 3:**17–23, 2005
3. Fujiyoshi T, Yamazaki M, Okawa A, Kawabe J, Hayashi K, Endo T, et al: Static versus dynamic factors for the development of myelopathy in patients with cervical ossification of the posterior longitudinal ligament. **J Clin Neurosci 17:**320–324, 2010
4. Hirabayashi K, Miyakawa J, Satomi K, Maruyama T, Wakano K: Operative results and postoperative progression of ossification among patients with ossification of cervical posterior longitudinal ligament. **Spine (Phila Pa 1976) 6:**354–364, 1981
5. Hori T, Kawaguchi Y, Kimura T: How does the ossification area of the posterior longitudinal ligament progress after cervical laminoplasty? **Spine (Phila Pa 1976) 31:**2807–2812, 2006
6. Hori T, Kawaguchi Y, Kimura T: How does the ossification area of the posterior longitudinal ligament thicken following cervical laminoplasty? **Spine (Phila Pa 1976) 32:**E551–E556, 2007
7. Hsieh PC, Wang MY: Introduction. Ossification of the posterior longitudinal ligament. **Neurosurg Focus 30(3):**Introduction, 2011
8. Inoue S, Goto S, Nagase J, Tanaka Y: Investigation of window width and window level of computed tomography for OPLL, in: **Annual Report of Taskforce of Research for Ossification of the Spinal Ligaments.** Tokyo: Japanese Ministry Of Public Health and Welfare, Vol 58, 1984, pp 237–239
9. Ishii T, Mukai Y, Hosono N, Sakaura H, Nakajima Y, Sato Y, et al: Kinematics of the upper cervical spine in rotation: in vivo three-dimensional analysis. **Spine (Phila Pa 1976) 29:** E139–E144, 2004
10. Iwasaki M, Kawaguchi Y, Kimura T, Yonenobu K: Long-term

results of expansive laminoplasty for ossification of the posterior longitudinal ligament of the cervical spine: more than 10 years follow up. **J Neurosurg 96 (2 Suppl):**180–189, 2002
11. Kawaguchi Y, Kanamori M, Ishihara H, Nakamura H, Sugimori K, Tsuji H, et al: Progression of ossification of the posterior longitudinal ligament following en bloc cervical laminoplasty. **J Bone Joint Surg Am 83-A:**1798–1802, 2001
12. Kokubun S, Sato T, Nishihira T: Computed tomography of the ossification of the spinal ligaments, in: **Seikeigeka Mook.** Tokyo: Kanehara Shuppan, Vol 50, pp 59–71, 1987
13. Matsunaga S, Sakou T, Taketomi E, Nakanisi K: Effects of strain distribution in the intervertebral discs on the progression of ossification of the posterior longitudinal ligaments. **Spine (Phila Pa 1976) 21:**184–189, 1996
14. Murakami M, Seichi A, Chikuda H, Takeshita K, Nakamura K, Kimura A: Long-term follow-up of the progression of ossification of the posterior longitudinal ligament. Case report. **J Neurosurg Spine 12:**577–579, 2010
15. Nagata K, Sato K: Diagnostic imaging of cervical ossification of the posterior longitudinal ligament, in Yonenobu K, Nakamura K, Toyama Y (eds): **OPLL: Ossification of the Posterior Longitudinal Ligament, ed 2.** Tokyo: Springer, 2006, pp 127–143
16. Ono K, Yonenobu K, Sako T, Kawai S, Nagata K: [Prevention of progression of ossification of the posterior longitudinal ligament (OPLL) by the administration of etidronate disodium (EHDP) after posterior decompression.] **Nippon Sekitsui Geka Gakkai Zasshi 9:**432–442, 1998 (Jpn)
17. Saetia K, Cho D, Lee S, Kim DH, Kim SD: Ossification of the posterior longitudinal ligament: a review. **Neurosurg Focus 30(3):**E1, 2011
18. Stapleton CJ, Pham MH, Attenello FJ, Hsieh PC: Ossification of the posterior longitudinal ligament: genetics and pathophysiology. **Neurosurg Focus 30(3):**E6, 2011
19. Stetler WR, La Marca F, Park P: The genetics of ossification of the posterior longitudinal ligament. **Neurosurg Focus 30(3):** E7, 2011
20. Terayama K, Ohtsuka K, Tsuama N, Ohtani K, Yamauchi Y, Yamaura I, et al: Investigation of ossification of the spinal ligaments with more than 5 years follow-up, in: **Annual Report of Taskforce of Research for Ossification of the Spinal Ligaments.** Tokyo: Japanese Ministry Of Public Health and Welfare, Vol 58, 1984, pp 85–96
21. Tokuhashi Y, Ajiro Y, Umezawa N: A patient with two resurgeries for delayed myelopathy due to progression of ossification of the posterior longitudinal ligaments after cervical laminoplasty. **Spine (Phila Pa 1976) 34:**E101–E105, 2009

Manuscript submitted June 2, 2011.
Accepted November 7, 2011.
Please include this information when citing this paper: published online December 16, 2011; DOI: 10.3171/2011.11.SPINE11502.

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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.

Rec./Acc. 12/8/2008, pp35-39

This work was supported in parts by Grant-in-aid for Young Scientists, 19689031 from Ministry of Education, Culture, Sports, Science and Technology, and research foundations from the general insurance association of Japan, Takeda Science Foundation, and Japan Orthopaedics and Traumatology foundation.

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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

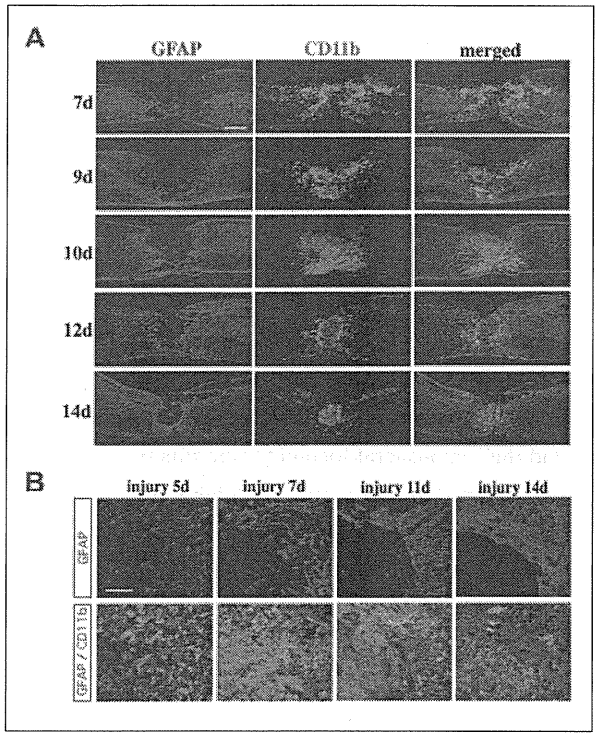

Fig.1 Compaction of inflammatory cells by reactive astrocytes and glial scar formation (Figure adapted from Okada et al.[7]).

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.

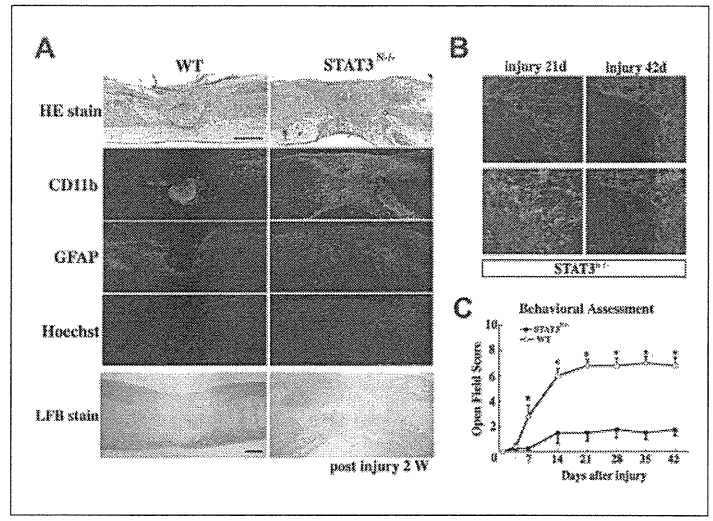

Fig.2 Compaction of inflammatory cells by reactive astrocytes and functional recovery were limited in STAT3$^{N-/-}$ mice (Figure adapted from Okada et al.[7]).

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.

References

1) Beattie MS: Inflammation and apoptosis: linked therapeutic targets in spinal cord injury. Trends Mol Med, 10: 580-583, 2004.
2) Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, and Beattie MS: Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. Nat Med, 3: 73-76, 1997.
3) Demjen D, Klussmann S, Kleber S, Zuliani C, Stieltjes B, Metzger C, Hirt UA, Walczak H, Falk W, Essig M, Edler L, Krammer PH, Martin-Villalba A: Neutralization of CD95 ligand promotes regeneration and functional recovery after spinal cord injury. Nat Med, 10: 389-395, 2004.
4) Okada S, Nakamura M, Mikami Y, Shimazaki T, Mihara M, Ohsugi Y, Iwamoto Y, Yoshizaki K, Kishimoto T, Toyama Y, Okano H: Blockade of interleukin-6 receptor suppresses reactive astrogliosis and ameliorates functional recovery in experimental spinal cord injury. J Neurosci Res, 15: 265-276, 2004.
5) Frankel HL, Hancock DO, Hyslop G, Melzak J, Michaelis LS, Ungar GH, Vernon JD, Walsh JJ: The value of postural reduction in the initial management of closed injuries of the spine with paraplegia and tetraplegia. I Paraplegia, 7: 179-192, 1969.
6) Ueta T, Shiba K: Cervical Cord Injuries; Comprehensive Treatment in the Acute Stage and Neurological Prognosis. The Jounal of the Japan Spine Research Society, 12: 389-417, 2001.

7) Okada S, Nakamura M, Katoh H, Miyao T, Shimazaki T, Ishii K, Yamane J, Yoshimura A, Iwamoto Y, Toyama Y, Okano H. Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. Nat Med, 12:829-34, 2006.
8) Silver J, Miller JH: Regeneration beyond the glial scar. Nat Rev Neurosci, 5: 146-156, 2004.
9) Sano S, Itami S, Takeda K, Tarutani M, Yamaguchi Y, Miura H, Yoshikawa K, Akira S, Takeda J: Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. EMBO J, 18: 4657-68, 1999.
10) Yamashita S, Miyagi C, Carmany-Rampey A, Shimizu T, Fujii R, Schier AF, Hirano T. Stat3 Controls Cell Movements during Zebrafish Gastrulation. Dev Cell, 2: 363-375, 2002.
11) Sriram K, Benkovic SA, Hebert MA, Miller DB, O'Callaghan JP: Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astrogliosis in vivo? J Biol Chem, 279: 19936-19947, 2004.
12) Kerr BJ, Patterson PH: Potent pro-inflammatory actions of leukemia inhibitory factor in the spinal cord of the adult mouse. Exp Neurol, 188: 391-407, 2004.
13) Xia XG, Hofmann HD, Deller T, Kirsch M: Induction of STAT3 signaling inactivated astrocytes and sprouting septal neurons following entorhinal cortex lesion in adult rats. Mol Cell Neurosci, 21: 379-392, 2002.
14) Klein MA, Möller JC, Jones LL, Bluethmann H, Kreutzberg GW, Raivich G: Impaired neuroglial activation in interleukin-6 deficient mice. Glia, 19, 227-233, 1997.
15) Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, Shulman GI, Fu XY: Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. Proc Natl Acad Sci USA, 101: 4661-4666, 2004.
16) Mori H, Hanada R, Hanada T, Aki D, Mashima R, Nishinakamura H, Torisu T, Chien KR, Yasukawa H, Yoshimura A: Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. Nat Med, 10: 739-743, 2004.
17) Yamashita S, Miyagi C, Fukada T, Kagara N, Che YS, Hirano T: Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. Nature, 429: 298-302, 2004.
18) Penkowa M, Giralt M, Thomsen PS, Carrasco J, Hidalgo J: Zinc or copper deficiency-induced impaired inflammatory response to brain trauma may be caused by the concomitant metallothionein changes. J Neurotrauma, 18: 447-463, 2001.
19) Penkowa M, Carrasco J, Giralt M, Moos T, Hidalgo J: CNS wound healing is severely depressed in metallothionein I- and II-deficient mice. J Neurosci, 19: 2535-2545, 1999.
20) Penkowa M, Giralt M, Lago N, Camats J, Carrasco J, Hernández J, Molinero A, Campbell IL, Hidalgo J: Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. Exp Neurol, 181: 130-148, 2003.
21) Frisén J, Johansson CB, Török C, Risling M, Lendahl U: Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. J Cell Biol, 131: 453-464, 1995.
22) Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB: Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature, 416: 636-640, 2002.
23) Menet V, Prieto M, Privat A, Giménez y Ribotta M: Axonal plasticity and functional recovery after spinal cord injury in mice deficient in both glial fibrillary acidic protein and vimentin genes. Proc Natl Acad Sci USA, 100: 8999-9004, 2003.
24) Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV: Reactive astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci, 24: 2143-2155, 2004.

PROSPECT
Journal of Cellular Biochemistry 107:1046–1052 (2009)

Journal of Cellular Biochemistry

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.

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

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology; Grant sponsor: General Insurance Association of Japan.
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Received 4 September 2008; Accepted 21 April 2009 • DOI 10.1002/jcb.22213 • © 2009 Wiley-Liss, Inc.
Published online 28 May 2009 in Wiley InterScience (www.interscience.wiley.com).

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 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.

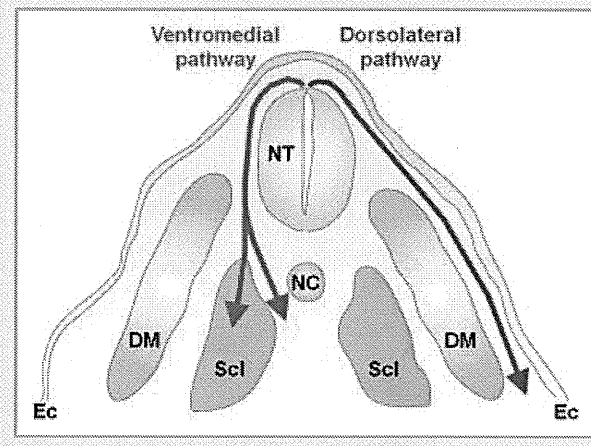

Fig. 1. Schematic diagram of the trunk region of the embryo. Trunk neural crest cells travel by two pathways: the dorsolateral pathway, which runs between the dermomyotome and the ectoderm, and the ventromedial pathway, which runs between the neural tube and the sclerotome or through the sclerotome.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

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

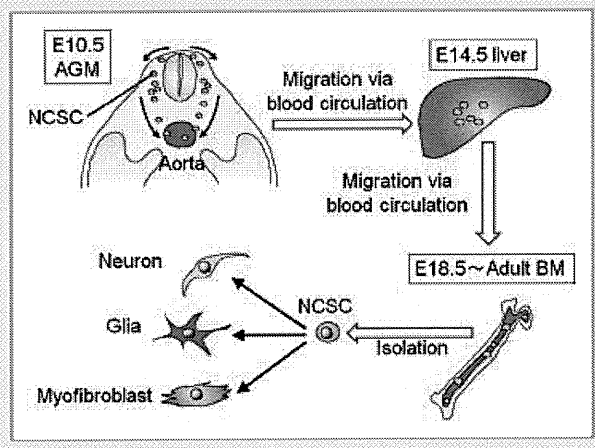

Fig. 2. Simplified cartoon of the NCSC migratory stream from the dorsal neural tube to the BM. During embryogenesis, some NCSCs migrate into the AGM region, enter the blood circulation, pass through the fetal liver, and reach the BM just before birth. The migrated NCSCs persist in the BM throughout life,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

ACKNOWLEDGMENTS

The author is grateful to Yo Mabuchi for drawing the illustrations. 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) to H.O.; the General Insurance Association of Japan to M.N. and Y.T.; and a Grant-in-Aid for the Global century COE program from MEXT to Keio University.

REFERENCES

Aquino JB, Hjerling-Leffler J, Koltzenburg M, Edlund T, Villar MJ, Ernfors P. 2006. In vitro and in vivo differentiation of boundary cap neural crest stem cells into mature Schwann cells. Exp Neurol 198:438–449.

Biernaskie J, Sparling JS, Liu J, Shannon CP, Plemel JR, Xie Y, Miller FD, Tetzlaff W. 2007. Skin-derived precursors generate myelinating Schwann cells that promote remyelination and functional recovery after contusion spinal cord injury. J Neurosci 27:9545–9559.

Bixby S, Kruger GM, Mosher JT, Joseph NM, Morrison SJ. 2002. Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity. Neuron 35: 643–656.

Crane JF, Trainor PA. 2006. Neural crest stem and progenitor cells. Annu Rev Cell Dev Biol 22:267–286.

Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. 1998. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol 8:1323–1326.

Delfino-Machin M, Chipperfield TR, Rodrigues FS, Kelsh RN. 2007. The proliferating field of neural crest stem cells. Dev Dyn 236:3242–3254.

Dzierzak E, Speck NA. 2008. Of lineage and legacy: The development of mammalian hematopoietic stem cells. Nat Immunol 9:129–136.

Fernandes KJ, McKenzie IA, Mill P, Smith KM, Akhavan M, Barnabe-Heider F, Biernaskie J, Junek A, Kobayashi NR, Toma JG, Kaplan DR, Labosky PA, Rafuse V, Hui CC, Miller FD. 2004. A dermal niche for multipotent adult skin-derived precursor cells. Nat Cell Biol 6:1082–1093.

Hagedorn L, Suter U, Sommer L. 1999. P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF-beta family factors. Development 126:3781–3794.

His W. 1868. Untersuchungen uber die erste Analge des Wirbeltierleibes. Die erste Entwicklung des Hunchens im Ei Leipzig 16:237.

Hjerling-Leffler J, Marmigere F, Heglind M, Cederberg A, Koltzenburg M, Enerback S, Ernfors P. 2005. The boundary cap: A source of neural crest stem cells that generate multiple sensory neuron subtypes. Development 132: 2623–2632.

Hu YF, Zhang ZJ, Sieber-Blum M. 2006. An epidermal neural crest stem cell (EPI-NCSC) molecular signature. Stem Cells 24:2692–2702.

Joannides A, Gaughwin P, Schwiening C, Majed H, Sterling J, Compston A, Chandran S. 2004. Efficient generation of neural precursors from adult human skin: Astrocytes promote neurogenesis from skin-derived stem cells. Lancet 364:172–178.

Joseph NM, Mukouyama YS, Mosher JT, Jaegle M, Crone SA, Dormand EL, Lee KF, Meijer D, Anderson DJ, Morrison SJ. 2004. Neural crest stem cells undergo multilineage differentiation in developing peripheral nerves to generate endoneurial fibroblasts in addition to Schwann cells. Development 131:5599–5612.

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.

Kleber M, Lee HY, Wurdak H, Buchstaller J, Riccomagno MM, Ittner LM, Suter U, Epstein DJ, Sommer L. 2005. Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. J Cell Biol 169:309–320.

Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A. 2001. Brain from bone: Efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation 68:235–244.

Kontges G, Lumsden A. 1996. Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. Development 122:3229–3242.

Kruger GM, Mosher JT, Bixby S, Joseph N, Iwashita T, Morrison SJ. 2002. Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. Neuron 35:657–669.

Kuriyama S, Mayor R. 2008. Molecular analysis of neural crest migration. Philos Trans R Soc Lond B Biol Sci 363:1349–1362.

Le Douarin NM, Dupin E. 2003. Multipotentiality of the neural crest. Curr Opin Genet Dev 13:529–536.

Le Douarin NM, Kalcheim C. 1999. The neural crest. UK: Cambridge University Press.

Lee HY, Kleber M, Hari L, Brault V, Suter U, Taketo MM, Kemler R, Sommer L. 2004. Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. Science 303:1020–1023.

Lendahl U, Zimmerman LB, McKay RD. 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595.

Li HY, Say EH, Zhou XF. 2007. Isolation and characterization of neural crest progenitors from adult dorsal root ganglia. Stem Cells 25:2053–2065.

Maro GS, Vermeren M, Voiculescu O, Melton L, Cohen J, Charnay P, Topilko P. 2004. Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci 7:930–938.

McKenzie IA, Biernaskie J, Toma JG, Midha R, Miller FD. 2006. Skin-derived precursors generate myelinating Schwann cells for the injured and dysmyelinated nervous system. J Neurosci 26:6651–6660.

Morikawa S, Mabuchi Y, Niibe K, Suzuki S, Nagoshi N, Sunabori T, Shimmura S, Nagai Y, Nakagawa T, Okano H, Matsuzaki Y. 2009. Development of mesenchymal stem cells partially originate from the neural crest. Biochem Biophys Res Commun 379:1114–1119.

Morrison SJ, White PM, Zock C, Anderson DJ. 1999. Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. Cell 96:737–749.

Nagoshi N, Shibata S, Kubota Y, Nakamura M, Nagai Y, Satoh E, Morikawa S, Okada Y, Mabuchi Y, Katoh H, Okada S, Fukuda K, Suda T, Matsuzaki Y, Toyama Y, Okano H. 2008. Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad. Cell Stem Cell 2:392–403.

Okano H, Imai T, Okabe M. 2002. Musashi: A translational regulator of cell fate. J Cell Sci 115:1355–1359.

Paratore C, Goerich DE, Suter U, Wegner M, Sommer L. 2001. Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development 128:3949–3961.

Pardal R, Ortega-Saenz P, Duran R, Lopez-Barneo J. 2007. Glia-like stem cells sustain physiologic neurogenesis in the adult mammalian carotid body. Cell 131:364–377.

Sakakibara S, Imai T, Hamaguchi K, Okabe M, Aruga J, Nakajima K, Yasutomi D, Nagata T, Kurihara Y, Uesugi S, Miyata T, Ogawa M, Mikoshiba K, Okano H. 1996. Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. Dev Biol 176:230–242.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256.

Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbutt G, van der Kooy D. 2004. Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22:1115–1124.

Shah NM, Groves AK, Anderson DJ. 1996. Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. Cell 85:331–343.

Sieber-Blum M, Grim M, Hu YF, Szeder V. 2004. Pluripotent neural crest stem cells in the adult hair follicle. Dev Dyn 231:258–269.

Stemple DL, Anderson DJ. 1992. Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell 71:973–985.

Takashima Y, Era T, Nakao K, Kondo S, Kasuga M, Smith AG, Nishikawa S. 2007. Neuroepithelial cells supply an initial transient wave of MSC differentiation. Cell 129:1377–1388.

Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD. 2001. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 3:778–784.

Toma JG, McKenzie IA, Bagli D, Miller FD. 2005. Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells 23:727–737.

Tomita Y, Matsumura K, Wakamatsu Y, Matsuzaki Y, Shibuya I, Kawaguchi H, Ieda M, Kanakubo S, Shimazaki T, Ogawa S, Osumi N, Okano H, Fukuda K. 2005. Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. J Cell Biol 170:1135–1146.

Uccelli A, Moretta L, Pistoia V. 2008. Mesenchymal stem cells in health and disease. Nat Rev Immunol 8(9):726–736.

Wong CE, Paratore C, Dours-Zimmermann MT, Rochat A, Pietri T, Suter U, Zimmermann DR, Dufour S, Thiery JP, Meijer D, Beermann F, Barrandon Y, Sommer L. 2006. Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. J Cell Biol 175:1005–1015.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370.

Yamauchi Y, Abe K, Mantani A, Hitoshi Y, Suzuki M, Osuzu F, Kuratani S, Yamamura K. 1999. A novel transgenic technique that allows specific marking of the neural crest cell lineage in mice. Dev Biol 212:191–203.

Yoshida S, Shimmura S, Nagoshi N, Fukuda K, Matsuzaki Y, Okano H, Tsubota K. 2006. Isolation of multipotent neural crest-derived stem cells from the adult mouse cornea. Stem Cells 24:2714–2722.

Zirlinger M, Lo L, McMahon J, McMahon AP, Anderson DJ. 2002. Transient expression of the bHLH factor neurogenin-2 marks a subpopulation of neural crest cells biased for a sensory but not a neuronal fate. Proc Natl Acad Sci USA 99:8084–8089.

Mini Review

Regenerative medicine for spinal cord injury: Current status and open issues

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Spinal cord injuries result in devastating loss of function, because spinal cord of human beings never regenerates after injury. People believed in this dogma for a long time. There is an emerging hope for regeneration-based therapy of the damaged spinal cord due to the progress of neuroscience and regenerative medicine including stem cell biology. In this review, we have summarized recent studies aimed at the development of regeneration-based therapeutic approaches for spinal cord injuries, including therapy with transplantation of neural crest stem cells and induction of axonal regeneration, and the establishment of new method for evaluating injured and regenerated axonal fibers by MRI.

Rec.12/24/2008, 1/16/2009, pp198-203

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Key words spinal cord injury, neural crest stem cell, chondroitin sulfate proteoglycan, semaphorin 3A, diffusion tensor tractography

Introduction

Neural stem cell transplantation is a promising regenerative medicine strategy for the treatment of spinal cord injury (SCI). We previously investigated the optimum timing of neural stem cell transplantation from the perspective of microenvironments within the injured spinal cord[1-3], and successfully transplanted rat neural stem cells into the injured spinal cord of adult rats[4] and human neural stem cells into the injured spinal cord of common marmosets[5], thereby promoting functional recovery. We believe these findings represent a significant step toward the clinical application of neural stem cell transplantation. However, because of various problems, it has not yet been possible to use neural stem cells clinically. Herein, we present basic studies that have been conducted to address various barriers against the realization of regenerative medicine for SCI. These problems include: (1) ethical issues related to the use of aborted fetal tissues, (2) axonal growth inhibitors within the injured spinal cord, and (3) insufficient methods for evaluating damaged and regenerated axons within the spinal cord.

Ethical issues related to the use of aborted fetal tissues

Aborted fetus-derived cells were used in our above-mentioned studies on neural stem cell transplantation for treating SCI. Ethical issues related to the clinical use of such cells have long been discussed at the relevant councils of the Ministry of Health,

Labour and Welfare. However, even the guidelines on the use of human stem cells for clinical research, published in 2006, do not include a definite stance on the validity of using aborted fetus-derived cells (Fig.1). Because of these problems and uncertainties, we have recently focused on neural crest cells derived from the patient's own tissue as a source of somatic stem cells, rather than on stem cells from aborted fetal tissue.

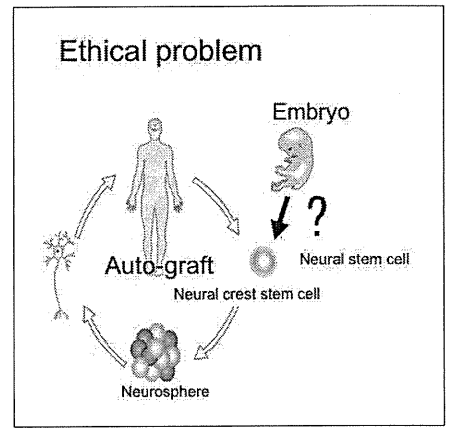

Fig.1 Avoidance of ethical problems associated with aborted tissue-derived neural stem cells by using neural crest cells

1) What is a neural crest cell?

Neural crest cells, which are induced at the border of the epidermal ectoderm and neural plate during development, move to the surrounding tissue immediately after closure of the neural tube. The migratory neural crest cells have diverse differentiation potentials, and are able to differentiate into neurons and glia of the sensory and autonomic systems, adrenal medulla, pigment cells, cranial skeleton (bone and cartilage), teeth (odontoblasts), arterial smooth muscle, and other cell types. Neural crest cells play an important role in many aspects of organ development and therefore are called the "fourth germ layer" [6]. Migratory neural crest cells differentiate into diverse tissues, depending on their environment. However, a portion of these cells remains undifferentiated and latent within various tissues while retaining their multipotential nature, even in adult organs. These neural crest stem cells have recently been attracting close attention as a potential cell source for autologous transplantation, because of their capacities of self-renewal and multipotential[7].

2) Isolation and identification of neural crest stem cells

Neural crest stem cells exist in many tissues, including the skin, intestine, heart, and corneas of adult mice[8-11]. Using transgenic PO-Cre and Wnt1-Cre/FLoxed-EGFP mice[12-14], we demonstrated that neural crest stem cells are also present in the dorsal root ganglia and bone marrow of adult mice[15]. Although the presence of stem cells (serving as a source of neural cells) in bone marrow has been shown in many reports, their embryological origin and differentiation potentials have been regarded as questionable. There are no reports that explain how the bone marrow-derived cells, of mesodermal origin, can differentiate into neural cells, which are of ectodermic origin. In addressing these questions, we found that neural crest stem cells move via the blood into the bone marrow during early embryonic development and remain latent in the bone marrow until adulthood, when they can produce neurons and glia. Furthermore, when we compared the properties of neural crest cells derived from different tissues (i.e. dorsal root ganglion, skin, and bone marrow) of adult mice, we found striking differences in their differentiation potential and gene expression profile . This result indicated that the neural crest stem cells latent in each adult tissue are not uniform, but rather retain properties that depend on their tissue of origin.

3) Transplantation of neural crest stem cells

Several recent transplantation studies have used skin-derived neural crest stem cells as a cell source. Miller et al. reported that skin-derived neural crest stem cells transplanted into demyelinated regions of central and peripheral axons differentiate into Schwann cells, which subsequently engage in remyelination[16]. Furthermore, these authors reported that skin-derived neural crest stem cells transplanted into injured spinal cords also differentiate into Schwann cells, and their differentiation is followed by axonal growth and the accumulation of endogenous Schwann cells, leading to a recovery in locomotor function[17]. However, in all these reports, the neural crest stem cells were obtained from neonatal mice. There are significant differences in the properties of neonatal and adult neural crest stem cells[18].

To achieve the goal of clinical application, it is important to evaluate the potential usefulness of neural crest stem cells derived from various adult tissues. In another study, neural crest stem cells derived from adult mouse skin were transplanted into injured mouse spinal cord. Some of the transplanted cells survived, but no improvement in locomotor function was described[19]. Further studies are needed to determine the effectiveness of this approach. Since neural crest stem cells are found in a variety of adult tissues, and since their characteristics depend on their tissue of origin, it will be essential to select the type of neural crest

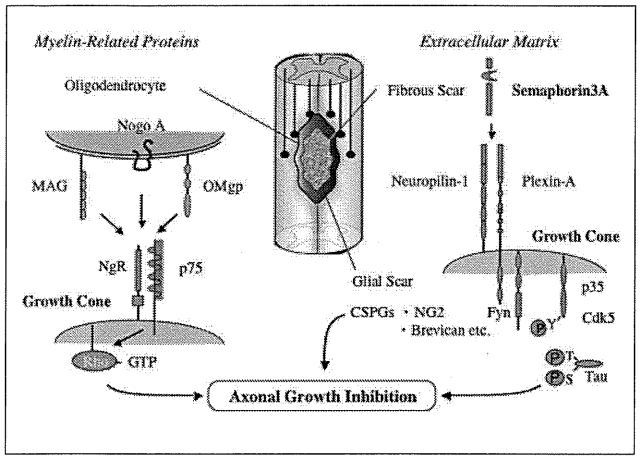

Fig.2 Axonal growth inhibitors in the injured spinal cord

stem cell for transplantation that will yield optimal results. In any event, neural crest stem cells are somatic stem cells that can be used for autologous transplantation. Given these features, in terms of both ethics and safety, neural crest stem cells are a promising source for transplantation in clinical cases.

Overcoming axonal growth inhibitors

Axonal growth does not occur in the injured central nervous system, although it can take place in the injured peripheral nervous system. One explanation for this is the presence of factors that inhibit axonal regeneration in the central nervous system. Even if effective stem cell transplantation for acute or sub-acute SCI can be achieved, it will still be difficult to establish valid regenerative treatments for chronic SCI unless the effects of the axonal growth inhibitors in the central nervous system can be overcome.

The axonal growth inhibitors found to date in the central nervous system can be roughly divided into myelin-associated proteins present in the myelin sheath (Nogo, MAG, and OMgp), and extracellular matrix components present in glial scar tissue, such as chondroitin sulfate proteoglycan (CSPG) and semaphorin 3A (Sema 3A) (Fig.2). In recent studies, animal models of SCI were treated with Nogo receptor antagonists (NEP1-40)[20], chondroitinase ABC (an enzyme involved in the degradation of CSPG)[21], and Rho signal- suppressing drugs (C3 and Y-27632)[22,23]. These methods are anticipated to be of value in treating spinal cord injuries.

We developed a Sema 3A inhibitor and applied it to the subarachnoid cavity of rats for 4 weeks after complete transection of the thoracic spinal cord. This agent stimulated axonal regeneration, induced vascularization, and promoted the migration of Schwann cells into the injured area, thus facilitating the recovery of leg locomotor function in the rats[24]. We also induced a thoracic contusive SCI in rats and administered chondroitinase ABC into the subarachnoid cavity of each rat for one week, beginning one week after injury. The CSPG level in the injured spinal cord decreased to a normal level after this treatment. In the same study, neural stem cell transplantation, applied in combination with chondroitinase ABC, exerted synergistic effects, and induced more marked axonal regeneration than either treatment given alone[25]. These results indicated that the regeneration of injured axons can be induced by combining the use of axonal extension inhibitors with neural stem cell transplantation. This important finding opens the door for effective treatments for chronic SCI.

Establishment of a method for evaluating spinal cord regeneration

The realization of regenerative medicine for the spinal cord requires the establishment of an evaluation method. Needless to say, axonal regeneration in the spinal projection tract is important for achieving spinal cord regeneration. However, the absence of an established method for evaluating axonal regeneration has made it clinically difficult to evaluate the responses of the injured spinal cord to cell transplantation.

To address this need, we have focused on a particular imaging technique, diffusion weighted imaging (DWI), which yields images based on the diffusion of water molecules. Two DWI methods, diffusion tensor imaging (DTI) and diffusion tensor tractography (DTT), have especially attracted our attention. We have applied these methods to the visualization of long tracts within injured spinal cords.

1) Anisotropy and the FA map

How water molecules diffuse in the living body varies depends on the nature of the local environment, and this variation is called, "anisotropic diffusion." For example, the white matter fibers constituting the spinal cord are highly anisotropic, and visualization of their anisotropy should delineate axonal arrangements. FA (fractional anisotropy) provides an indicator of the magnitude of anisotropy. FA ranges from 0 to 1, where it is 0 in cases with isotropic diffusion, and approaches 1 as the diffusion becomes more anisotropic. An image representing anisotropy two-dimensionally is called an "anisotropy map" or an "FA map." In a color FA map, different colors are assigned to different axes; thus, fibers can be distinguished from each other by using dif-