Dorsal Spinal Cord Inhibits Oligodendrocyte Development

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Contents

Abbreviations

bFGF	basic fibroblast growth factor
BCIP	X-phosphate/ 5-bromo-4-chloro-3-indolyl-phospahate
BMP	bone morphogenetic protein
CNS	central nervous system
DAB	3,3'-diaminobenzidine tetrahydrochrolide
DIG	digoxigenin
DIV	day(s) *in vitro*
DMEM	Dulbecco modified Eagle's medium
E	embryonic days
ECM	extracellular matrix
EGF	epithelial growth factor
FCS	fetal calf serum
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
IGF	insulin-like growth factor
MAP2	microtubule-associated protein 2
NBT	4-nitro blue tetrazolium chloride
NT-3	neurotrophin 3
O2A progenitor	oligodendrocyte-type2 astrocyte progenitor

OL	oligodendrocyte
P	postnatal day
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PDGFRα	alpha subunit of the receptor for PDGF
PLP	myelin proteolipid protein
Shh	sonic hedgehog
SVZ	subventricular zone
T3	triiodothyroxine
T4	thyroxin
TGFβ	transforming growth factor beta
VZ	ventricular zone

Abstract

Oligodendrocytes are the myelinating cells of the vertebrate central nervous system. In the mouse spinal cord, oligodendrocytes are generated from strictly restricted regions of the ventral ventricular zone. To investigate how they originate from these specific regions, I established an explant culture system of E12 mouse cervical spinal cord and hindbrain, named flat whole-mount preparation. Alpha subunit of the receptor for platelet-derived growth factor (PDGFRα) mRNA-expressing cells, which are the major progenitors of oligodendrocytes in the spinal cord, were first detected in the ventral half of the explant near the ventral midline of the cervical spinal cord, and subsequently distributed dorsally. When I cultured the ventral and dorsal cord separately, a robust increase in the number of PDGFRα positive cells was observed in the ventral fragment in comparison to the ventral region of the untreated cervical spinal cord. This increase was thought to due to the removal of inhibitory regulation from dorsal spinal cord and increase in the number of early stage oligodendrocytes. The number of proliferating cells, mainly Musashi positive neuroepithelial cells also increased in the ventral fragment, suggesting that factors from the dorsal spinal cord suppress a step switching the cells from proliferating stage to differentiating stage. Furthermore, the numbers of the positive cells for O4, a marker for immature and mature oligodendrocytes, and O1, a marker for mature oligodendrocytes, were also increased in the ventral fragment. These results suggest that dorsal spinal cord also inhibit oligodendrocyte differentiation

and maturation. Bone morphogenetic protein 4 (BMP4), a strong candidate for this factor that is secreted from the dorsal spinal cord, did not affect oligodendrocyte development. Previous studies demonstrated that signals from the notochord and ventral spinal cord, such as sonic hedgehog and neuregulin, promote the ventral region-specific development of oligodendrocytes. Our present study demonstrates that the dorsal spinal cord negatively regulates oligodendrocyte development

Introduction

Oligodendrocytes (OLs) are the myelin forming cells of the vertebrate adult CNS. For long, OLs have been considered to be the last cell type to be generated during CNS development. However, recent description of markers specific for OL precursors has led to the proposal that, in fact, the generation of OL precursor cells occurs either simultaneously with or following closely upon the emergence of the first neurons. In the mouse spinal cord, motoneurons are born between E9 and E12, whereas OL precursors appear at the ventricular surface at least on E12.5 (Pringle et al., 1996, Richardson et al., 2000).

Descriptive and experimental studies have shown that OLs originate from early segregating precursors characterized by the expression of two molecular markers, *pdgfrα* (Pringle and Richardson, 1993; Hall et al., 1996) and *plp/dm20* (Timsit et al., 1995; Spassky et al., 1998, Perez Villegas et al., 1999) (Fig. 1). Alpha subunit of the receptor for platelet-derived growth factor (PDGFRα) is a receptor tyrosine kinase implicated in a variety of developmental processes including the survival and proliferation of OL progenitors (Noble et al., 1988; Richardson et al., 1988; Hart et al., 1989a, b; Soriano, 1997). *plp* encodes two alternatively spliced products: PLP and DM-20, which are the major protein components of higher vertebrate CNS myelin (Griffiths et al., 1998). As illustrated by double labeling experiments, although both *pdgfrα* and *plp/dm-20* expressing cells have been shown to generate OLs, *pdgfrα* and *plp/dm-20*

are not co-expressed at the cellular level. This has led to the proposal of the existence of two different OL precursor cells: one is defined by the expression of *pdgfrα*, another characterized by expression of *plp/dm-20*. The latter is independent of PDGF-AA for its proliferation and survival (Spassky et al., 1998).

In contrast to what is observed in the above description, a series of experimental data suggest that *plp/dm-20* expressing cells in the spinal cord are not OL precursors, but differentiated OLs (Ivanova et al., in preparation; for a review: Spassky et al., 2000, Richardson et al., 2000). Instead, when PDGFRα$^+$ cells were purified from the E16 rat spinal cord by immunoselection and cultured in defined medium, they all differentiated into OLs (Hall et al., 1996). The PDGF-A knockout mouse showed profound reductions in the number of PDGFRα$^+$ cells and OLs (Fruttiger et al., 1999). In these respects, the spinal cord can be considered as a territory with a single population of OL precursors belonging to the lineage from the PDGFRα$^+$ precursors.

A specific feature of OL precursors is their restricted localization in the developing neural tube. Elegant experiments, performed by the group of R. Miller have shown that, in the rat spinal cord, OLs have a restricted ventral origin (Warf et al., 1991; Fig. 2), which is in agreement with the observation that PDGFRα$^+$ precursors form a ventrally-localized column of cells on either side of the central canal (Pringle and Richardson, 1993). This ventral localization of spinal PDGFRα$^+$ OL precursors, restricted to one or two cell layers on one coronal section, raised the question of the mechanisms responsible to achieve such a strict positional regulation of OL emergence. The neuroepithelial origin of PDGFRα$^+$ OL progenitors in the cervical spinal cord has

been mapped to the same region that generates somatic motoneurons (SM), that is, to the lower part of the Pax6 gradient, dorsal to the nkx2.2$^+$ domain that generates visceral motoneurons (VM; Sun et al., 1998; Fig3A). There are also several types of interneurons (V3, V2, V1 and V0) identified by some marker gene expression in the spinal cord (Ericson et al., 1996, 1997; Briscoe et al., 1999; Fig. 3A). The specific and restricted pattern of generation of these ventral neurons along the dorso-ventral axis, is under the influence of both ventralizing (including Shh) and dorsalizing extrinsic signals. A wide range of ventral neural cell types is eliminated after surgical removal of the notochord at early stages (van Straaten and Hekking, 1991; Yamada et al., 1991; Ericson et al., 1992), in mutant mice and zebrafish that lack notochord and floor plate (Bovolenta and Dodd, 1991; Beattie et al., 1997), in Shh null mutant mice (Chiang et al., 1996), or after blockade of Shh signaling with specific antibodies (Marti et al., 1995; Ericson et al., 1996, 1997). Recently, it has been reported that humoral factors secreted from the ventral neural tube promote OL differentiation (Fig. 3B). Shh secreted from the notochord or the floor plate induces OL development as well as motoneuron development (Poncet et al., 1996; Pringle et al., 1996). Shh does not promote the proliferation of progenitor cells, but functions in the determination of OL cell fate at an early stage (Orentas et al., 1996, 1999). Neuregulins secreted by differentiated motoneurons may also promote OL differentiation (Vertanian et al., 1999).

There have been reported about regulation of neuron from dorsal spinal cord. Some evidence for a role of TGFβ-related proteins in the control of neuronal differentiation in the CNS has emerged in part from studies of cell patterning in the dorsal spinal cord

(Basler et al., 1993; Liem et al., 1995). Especially, BMPs strongly regulate the dorsal neuronal development (Liem et al, 1997). GDF7, a BMP family member has potential to induce two dorsal sensory interneuron classes, D1A and D1B neurons (Lee et al., 1998). To my knowledge, however, no reports have questioned a possible signal from dorsal spinal cord on OL development. To address this question, I have applied and modified an explant culture system of E12 mouse cervical spinal cord (C1-C4) and caudal rhombencephalon, named flat whole-mount preparation, which has been used to investigate axonal pathfinding event *in vitro* (Fig. 4; Shirasaki et al., 1995). Flat whole-mount preparation maintains the dorso-ventral axis, such as the floor plate and roof plate, region specific differentiation, and cell-cell contact more intact than that in dissociated cultures. Here, using this culture system, I have investigated the presence of activities inhibiting the development of OLs released from the dorsal spinal cord and characterized their function. To examine the effect of removing the dorsal spinal cord on the OL development, the ventral region of the flat whole-mount preparation, where some PDGFRα^+ cells were being generated at the time of seeding, was isolated and cultured. The numbers of OL progenitors and OLs were increased in the fragment containing the isolated ventral midline in comparison to control ventral region. This suggests a significant inhibitory signal from the dorsal portion. This was confirmed by culturing an isolated midline in close contact with a dorsal portion. Ectopic dorsal signaling could inhibit the OL development. Altogether, my results provide evidence that OL development is not only regulated by factors from the ventral spinal cord, such as Shh and neuregulin, but is also strongly controlled by inhibitory signals from the

dorsal region.

EXPERIMENTAL PROCEDURES

Materials:

ICR mice were obtained from Japan SCL (Tokyo, Japan). The day of insemination was designated embryonic day zero (E0). Mouse *plp* cDNA cloned into pSPT18 (BamHI-EcoRI fragment of 1.4 kb containing entire coding region of PLP) was used for *in situ* hybridization study (Kagawa et al., 1994). Rat *pdgfrα* cDNA cloned into pGEM-1 (HindIII-EcoRI fragment of 1.5 kb corresponding to the extracellular domain of PDGFRα cDNA) was provided from Dr. W.D. Richardson in University College London (England, UK). Monoclonal O4 and O1 antibodies (Sommer and Schachner, 1981) were gifts from Dr. S. Pfeiffer in the University of Connecticut Medical School (CT, USA). Anti-neurofilament antibody 2H3 was provided from Developmental Studies Hybridoma Bank in the University of Iowa (IA, USA). Monoclonal anti-Musashi antibody was a gift from Dr. H. Okano in Osaka University (Osaka, Japan). Polyclonal anti-GFAP antibody (cat #L1814) and monoclonal anti-PCNA antibody (PC10; cat #M0879) were purchased from DAKO (CA, USA). Biotin conjugated anti-mouse IgM antibody (μ-chain specific; cat #BA2020) and ABC Elite standard kit™ (cat #PK-6100) were purchased from Vector Laboratories (CA, USA). Biotin conjugated anti-mouse IgG antibody (Fc region specific; cat #515-065-008) was purchased from Jackson Immuno Research Laboratories (PA, USA). Fluorescein conjugated anti-mouse IgM (cat#55519) was purchased from ICN (OH, USA). Monoclonal mouse anti-MAP2

(HM-2; cat #M4403), DMEM (cat #D5796) and glutaraldehyde (cat #G5882) were purchased from Sigma (MO, USA). F12 (cat #21700-075), formamide (cat #15515-018) and blocking regent (cat #1096 176) were purchased from Gibco BRL. DIG labeling mix (cat #1277 073), Proteinase–K (cat #1373 196), Alkaline Phosphatase conjugated anti-DIG antibody (Fab fragments; cat #1093 274), NBT (cat #1383 213) and BCIP (cat #1383 221) were purchased from Boehringer Mannheim (Gmbh, Germany). General reagents were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Affi-Gel Blue Gel Beads (cat #60177B) was purchased form Bio-Rad (CA, USA). Transwell™ (12 mm, Polycarbonate Membrane, ø4 μm pore; cat #3402) was purchased from Costar (Cambridge, MA, USA). Cryogenic vial™ (2 ml; cat #5000-0020) was purchased from Nalgene (NY, USA). 4 well culture dish (cat #176740) was purchased from Nunc (NY, USA). Purified BMP4 and Noggin were provided from Dr. N. Ueno in National Institute for Basic Biology (Okazaki, Japan; Natsume et al., 1997).

Procedures:

Flat whole-mount preparation of embryonic mouse brain

I established the flat whole-mount preparation system by the modification of the previous method (Shirasaki et al., 1995). The region from the medulla to the cervical spinal cord (C1-C4) of E12 mice was dissected out from the surrounding tissue, opened along the dorsal midline, and flatted on the Transwell™ with the superficial surface up (Fig. 4). Dissection procedure was performed in the ice-cold DMEM. The explants were

cultured in a 5% CO_2 incubator (cat #BNA-121D, ESPEC Co Ltd., Osaka, Japan) at 37°C for one or three days (1 or 3 DIV) in DMEM / F12 (1:1) with N2 supplement (5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite; Bottenstein and Sato, 1979), 30 ng/ml T3, 40 ng/ml T4, and 2 ng/ml bFGF. Fig. 5 indicates a view of flat whole-mount preparation from the top: The middle part of flat-whole mount preparation corresponds to the ventral, and the both edges correspond to the dorsal spinal cord and hindbrain, respectively.

In situ hybridization

Distribution of mRNA expression was detected by *in situ* hybridization using DIG-labeled cRNA antisense probes. Mouse *plp* cDNA cloned into pSPT18 and rat *pdgfrα* cDNA cloned into pGEM-1 were used for riboprobe synthesis (Fig. 6). Samples were fixed overnight in 4% PFA / 0.1 M phosphate buffer (pH 7.4) (4% PFA) at 4°C and washed 2 times for 10 min in PBS containing 0.1% Tween-20 (PBT). They were dehydrated through 50% methanol/PBT once and 100% methanol twice, and then, were rehydrated through 75%, 50% and 25% methanol /PBT each once for 5 min, and washed with PBT twice. For better penetration of probes, the samples were treated with 10 μg/ml Proteinase–K for 20 min at room temperature. The samples were post-fixed in 4% formamide / 0.1% glutaraldehyde for 20 min at room temperature. After washing with PBT, the samples were transferred into 2ml Cryogenic vial™ and were rinsed with 1:1 mixture of PBT and hybridization buffer that was consisted of 50% formamide, 1.3x SSC (750 mM NaCl, 75 mM sodium citrate; pH 5), 50 μg/ml yeast RNA, 100 μg/ml

heparin and 0.2% Tween-20. The samples were washed once more with the complete hybridization buffer and prehybridized in the hybridization buffer for 1 hour at 65°C. Hybridization was performed with 100 ng/ml DIG-labeled cRNA probe in the hybridization buffer at 65°C for overnight. After washing with pre-warmed hybridization buffer for 20 min at 65°C twice, the samples were washed with MABT (0.1 M malate, 0.15 M NaCl and 0.1% Tween-20; pH 7.5) for 30 min at room temperature twice. The samples were preincubated in the blocking solution (2% blocking regent™ with 20% heat-inactivated sheep serum in MABT) for 1 hour, and incubated with 1/2000 diluted Alkaline Phosphatase conjugated anti-DIG antibody (Fab fragments) in the blocking solution at 4°C for overnight. After washing with MABT for 1 hour for three times, the samples were treated with NTMT (0.1 M NaCl, 0.1 M Tris-HCl [pH9.5], 0.1 M $MgCl_2$, and 0.1% Tween-20) for 20min twice. The color reaction was performed in 0.45 μl/ml of 100 mg/ml NBT and 3.5 μl/ml of 50 mg/ml BCIP in NTMT for 2 or 3 overnights. Stained samples were mounted in 80% glycerol on the slide glass and observed with a microscope (BX50, Olympus, Tokyo, Japan).

Immunostaining

O4 staining with fluorescence

The region which corresponds to flat whole-mount preparation was dissected from ICR mouse at E12 or E14. The brains were incubated with O4 hybridoma supernatant for 30 min and washed with pre-warmed DMEM for 30 min. The brains were incubated with 1/200 diluted Fluorescein conjugated anti-mouse IgM antibody in DMEM for 30 min

and washed with pre-warmed DMEM for 30 min. These incubation and washing procedures were performed in a 37°C CO_2 incubator. The stained brains were fixed with 4% PFA at room temperature for 30 min and observed with a fluorescence microscope (IX70, Olympus).

O4 and O1 staining with DAB

The same volume of O4 or O1 hybridoma supernatant as the culture medium was added to the cultured explants or whole brains of embryo. After incubation in a CO_2 incubator for 1 hour, the flat whole-mount preparations were fixed with 4% PFA at 4°C for overnight. The samples were washed with PBS for 8 hours or overnight, and bleached with 0.3% H_2O_2/ methanol for 30 min at room temperature. The samples were washed with PBST (PBS containing 0.1% Triton-X100) for 8 hours at room temperature. The explants were incubated with 1/200 diluted biotin conjugated anti-mouse IgM antibody (μ-chain specific) for overnight at 4°C, washed with PBST for 8 hour at room temperature. The explants were incubated with AB complex (ABC Elite standard kit™) at room temperature for 3 hours. The samples were washed with PBST for 6 hours at room temperature. The samples were stained with the solution containing 0.5 μg/ml DAB and 0.015% H_2O_2 in PBT under a dissection microscope and the reaction was stopped with water. The samples were fixed with 4% PFA and mounted on glass slides with a small volume of 80% glycerol.

Neurofilament staining

Anti-neurofilament antibody (1/5 diluted conditioned medium of 2H3 hybridoma) was added to the flat whole-mount preparation that had been fixed with 4% PFA and treated

with 0.3% H_2O_2/ methanol. Secondary antibody used was 1/200 diluted biotin conjugated anti-mouse IgG antibody (Fc region specific).

Operation of the flat whole-mount preparation

VM fragment culture

The E12 caudal hindbrain and the cervical spinal cord (C1-C4), which are the same region used for the flat whole-mount preparation, was subdivided longitudinally into 3 fragments (see Fig. 7A). One-third fragment that contained ventral midline (VM fragment) was cultured for 3 DIV and subjected to histochemical stainings. The same culture medium described above was used. The average and standard error (s.e.) of the cells positive for the OL markers were calculated by Microsoft Excel (Microsoft, CA, USA).

In vitro transplantation of VM fragment

The VM fragment was settled besides the another flat whole-mount preparation on E12 (see Fig. 7B), and cultured for 3 DIV. The same culture medium described above was used.

Ablation of the dorsal cervical spinal cord

The E12 ICR mouse hindbrain and the cervical spinal cord (C1-C4) were dissected and flat-mounted on Transwell™. The dorsal region in the left side was ablated from the flat whole-mount preparation using a tungsten needle (see Fig. 7C). The rest of explant was cultured for 3 DIV. The same culture medium described above was used.

Quantification of the neural cells in the culture of flat whole-mount preparation

After 3 DIV, the VM fragment or ventral region of control flat whole-mount preparation were treated with 0.25% trypsin for 15 min at 37°C in a rotary shaker (Type RC8, Miyamoto Riken Inc Co. Ltd., Osaka, Japan) at 300 rpm. Single cells were prepared by gentle pipetting, plated on poly-L-lysine coated dish (4 well) at $5x10^4$ cells/cm^2 and cultured in DMEM containing 10% FCS for 7 hours at 37°C in a CO_2 incubator. These cells were fixed with 4% PFA and immunostained with mouse monoclonal anti-PCNA (PC-10), mouse monoclonal anti-MAP2 (HM-2), or rabbit polyclonal anti-GFAP antibodies.

BMP4 Treatment

The dissociated cells from the E12 mouse medulla and spinal cord were plated on poly-L-lysin coated slide glass at $5x10^5$ cells/cm^2 and incubated in DMEM containing 10% FCS for overnight at 37°C in a CO_2 incubator. The culture medium was changed to the chemical defined medium containing 0, 10, or 100 ng/ml BMP4 and cultured for another 3 DIV. The cells were fixed with 4% PFA and immunostained with mouse monoclonal anti-MAP2 (HM-2), rabbit polyclonal anti-GFAP, or mouse monoclonal anti-PCNA antibodies.

The VM fragments were isolated from E12 ICR mouse and cultured on Transwell™ without or with 10, or 100 ng/ml BMP4 in the culture medium as described above for 3 DIV. After staining with O4 antibody, the $O4^+$ cell number /cm^2 was

counted by NIH image (Version 1.62, National Institutes of Health).

Treatment of Noggin

Affi-Gel Blue beads were soaked with 20 nM purified mouse Noggin in the chemical defined medium for 30 min at room temperature. Beads soaked with the chemical defined medium were used as a control. The soaked beads were implanted in the flat whole-mount preparations at the beginning of the culture and cultured for 3 DIV (Fig. 8).

Results

Distribution of OLs and their progenitors in the flat whole-mount preparation

The earliest OLs in the CNS were detected from E12 mouse by the staining with O4 antibody, which recognizes pre-OL antigen. These $O4^+$ cells were found in the ventral region of the cervical spinal cord, where most OLs were derived from $PDGFR\alpha^+$ cells (Fig. 9A; Hall et al., 1996; Fruttiger et al., 1999); subsequently, the distribution of OL expanded dorsally (Fig. 9B). The distribution change of $PDGFR\alpha^+$ and $PLP/DM20^+$ cells were well investigated in the spinal cord region. $PDGFR\alpha^+$ cells appeared from E12.5 in the cervical spinal cord and distributed in the whole spinal cord on rat E15 (Pringle et al., 1996, Calver et al., 1998). $PLP/DM20^+$ cells appeared from E14.5 in the restricted ventral ventricular zone of the cervical spinal cord and distributed in the superficial ventral tract of the spinal cord at P1 (Timsit et al., 1995). In order to study the regulation mechanisms of OL development, I applied an explant culture system, named flat whole-mount preparation (Shirasaki et al., 1995). The hindbrain and the cervical spinal cord (C1-C4) from E12 ICR mouse embryos was used for this study. I first assessed how the *in vivo* developmental profile of OL can be reproduced in this culture system. $PDGFR\alpha^+$ cells were detected in the ventral half of the cultured cervical spinal cord on E12+1 day *in vitro* (DIV) (Fig. 10A). $PDGFR\alpha^+$ cells were distributed dorsally on E12+3 DIV, but no $PDGFR\alpha^+$ cells were observed around the ventral

midline area on this day (Fig. 10B). The disappearance of PDGFRα$^+$ cells in the ventral region was different from *in vivo* distribution pattern (Pringle et al., 1996, Calver et al., 1998, Ivanova et al. in preparation). I detected a small number of cells stained with O4 antibody in the ventricular surface along the ventral midline of the cervical spinal cord at E12+1 DIV (Fig. 11A). The presence of O4$^+$ cells increased around the ventral half of the spinal cord at E12+3 DIV (Fig. 11B). The distribution pattern of O4$^+$ cells in the flat whole-mount preparation was consistent with that observed *in vivo* (compare with Fig. 9).

Distribution pattern of PLP/DM20$^+$ cells was similar to that of O4$^+$ cells. PLP/DM20$^+$ cells appeared along the ventral midline at E12+1 DIV (Fig. 12A) , and then increased their number at E12+3 DIV (Fig. 12B). This similar distribution patterns of O4$^+$ and PLP/DM20$^+$ cells are consistent with the idea in which PLP/DM20$^+$ cells are not OL progenitors but already immature OLs in the cervical spinal cord.

Increase in the number of PDGFRα$^+$ cells in the VM fragment culture

I next examined the function of dorsal spinal cord involved in OL development. The flat whole-mount preparation was subdivided longitudinally into 3 fragments at E12 and the one-third containing the ventral midline (VM fragment) was cultured for 3 DIV (Fig. 7A). The number of PDGFRα$^+$ cells in the VM fragment increased dramatically (compare Fig. 13A and C, Table 1). The number of PDGFRα$^+$ cells in the dorsal portion (Fig. 13B) decreased in comparison to the control (Fig. 13D), suggesting that PDGFRα$^+$ cell migration from the ventral to dorsal region was interrupted, however, the increased

number in the VM fragment was significantly larger than the total number of PDGFRα$^+$ cells in the entire explant. Hence, there appear to be additional effects that accounts for this great increase in the number of PDGFRα$^+$ cells: some factors, which are normally secreted from the dorsal region and inhibit OL development, may have been removed by dissection.

To ascertain the existence of activities inhibiting OL development, the VM fragment prepared from an E12 mouse embryo was placed beside another flat whole-mount preparation potentially influencing from the dorsal spinal cord (*in vitro* transplantation of VM fragment; Fig. 7B). The number of the PDGFRα$^+$ cells in the half of the VM fragment that was proximal to the other flat whole-mount preparation was significantly less than that in the distal half (Fig. 14A, B). In the next experiment, one side of the dorsal region was ablated in an E12 sample and cultured for 3 days (Ablation of cervical spinal cord; Fig. 7C). Again, PDGFRα$^+$ cells in the ablated side were induced 2.1-fold in comparison to that in the control side (Fig. 14C, D). These two results strongly indicate the existence of inhibitory activities that reduced the PDGFRα$^+$ cell number released from the dorsal region.

Increase of O4$^+$ cell number in the VM fragment

To investigate whether the inhibitory activities from the dorsal region affected OL differentiation, I immunostained the cells with O4 antibody. In the VM fragment culture, O4$^+$ cell number also increased (Table 1) in the VM fragment (Fig. 15C) in comparison to that in the control (Fig. 15A). No cells were detected in the dorsal region of both

cases (Fig. 15B, D). In the *in vitro* transplantation of VM fragment, $O4^+$ cell number in the proximal half of the VM fragment decreased to half of that in the distal half (Fig. 16A, B). Upon ablation of the dorsal cervical spinal cord, the number of $O4^+$ cells in the ablated side was 1.8-times greater than that in the control side (Fig. 16C, D).

Increase of PLP/DM20$^+$ cell number in the VM fragment

To examine whether removal of the dorsal region also increase the number of PLP/DM20$^+$ cells, *in situ* hybridization study of VM fragment was performed with PLP/DM20 cRNA probes. At E12+1 DIV PLP/DM20$^+$ cells distributed along the ventral midline (Fig. 17A). The number of PLP/DM20$^+$ cells in VM fragment (Fig. 17C) also greatly increased compared to the control (Fig. 17B, Table 1). It suggests that the dorsal region also inhibits the appearance of PLP/DM20$^+$ cells.

Increase of O1$^+$ cell number in the VM fragment

To determine whether inhibitory activities from the dorsal region influenced OL maturation, I immunostained the flat whole-mount preparation with O1 antibody, which recognizes galactocerebroside, a marker for mature OLs. There was a substantial increase in the number of $O1^+$ cells observed in the VM fragment (compare Fig. 18A and B, Table 1), suggesting that the dorsal region normally inhibited OL maturation as well.

Effects of dorsal spinal cord

The increase of $O4^+$ and $O1^+$ cell numbers in the VM fragment can be explained by the removal of inhibitory activities on the maturation steps from dorsal spinal cord. On the other hand, there are three possible causes for the increase in the number of PDGFRα^+ progenitor cells after the removal of dorsal spinal cord: (1) induction of the stem cell proliferation, (2) progenitor cell proliferation, or (3) promoted differentiation of PDGFRα^+ cells from stem cells. To determine which of the above steps was induced by the dorsal removal, neuron, astrocyte, neuroepithelial cell and proliferating cell numbers were counted using specific markers. The VM fragment cultures and ventral regions of control untreated flat whole-mount preparations were trypsinized on E12+3 DIV, replated on poly-D-lysine coated dishes at $5x10^4$ cells/cm^2, and incubated in a CO_2 incubator at 37°C for 7 hours. The cells were subsequently immunostained. Cells positive for PCNA-staining, a marker for proliferating cells, increased drastically (Table 2). Cells positive for Musashi, a marker for neuroepithelial cell (Sakakibara et al., 1996), also increased drastically (Table 2). The increase in the number of $PCNA^+$ cells may be mostly caused by the increase of $Musashi^+$ neuroepithelial cells since the change of the percentage of $PCNA^+$ cells and $Musashi^+$ cells in the control and the VM fragment culture are similar. The percentage of cells positive for MAP2-staining, a neuronal marker, in the VM fragment was markedly reduced (Table 2). In the control flat whole-mount preparation, a few cells positive for GFAP, a marker for astrocyte, were already detectable. The $GFAP^+$ cells in the VM fragment increased slightly from that in the control (Table 2). These results indicated that the dorsal factors inhibit the proliferation

of stem cells or OL progenitors. In addition, PDGFRα$^+$ cells and O1$^+$ cells in the VM fragment increased drastically (Table 1). Taken together, the dorsal factors are suggested to (1) promote the neuronal differentiation from neuroepithelial cells; (2) inhibit the differentiation step from neuroepithelial cells to OL progenitors; and (3) inhibit the maturation of OLs from the O4$^+$ to O1$^+$ cells (Fig. 19).

Axons seem normal in the VM fragment

There was another possibility to explain the increase of OL progenitors in the VM fragment, since it has been reported that certain signals from axon influence the OL development; axons may inhibit the proliferation of OL precursors and/or suppress the maturation of OLs (Barres and Raff, 1993a; Barres et al., 1993b; Barres and Raff, 1996; Wang et al., 1998; see also discussion). The axons could have degenerated as a result of VM fragment dissection, thus alleviating the suppression of OL differentiation, with a consequent increase in the number of PDGFRα$^+$ cells. Anti-neurofilament staining (2H3; Dodd et al., 1988) revealed the presence of axons along the rostro-caudal axis in the control flat whole-mount preparation (Fig. 20A), and no great differences were observed in the staining pattern of the *in vitro* transplantation of VM fragment (Fig. 20B). The presence of intact axons in the *in vitro* transplantation of VM fragment indicated that the above possibilities are less likely.

No effect of BMP4 on O4$^+$ cells

BMPs belong to members of TGF β gene superfamily. It is well known that BMPs have

dorsalizing inductive activities during early neural development. It has also been reported that BMPs induce astrocyte differentiation from O2A progenitor cells (Mabie et al., 1997) as well as from SVZ cells of the forebrain (Gross et al., 1996). When BMP4 was added to the dissociation culture from E12 mouse medulla and cervical spinal cord region, the percentage of $MAP2^+$ cell was almost same as that in the control culture (table 3). BMP3 might effect at earlier stages of neurogenesis. However, the percentage of $GFAP^+$ cell within the cultured cells increased after 4 DIV (Table 3) and the percentage of $PCNA^+$ cell decreased (Table 3) as predicted from the previous reports. Therefore BMP4, which is secreted from the dorsal neural tube, remained a good candidate for inhibitory factor of OL development. In the VM fragment culture, I expected to observe decrease in $O4^+$ cell number if the BMPs were the major component of the dorsal factors inhibiting OL development. However, no significant changes in $O4^+$ cell numbers were observed upon the addition of 10 ng/ml or 100 ng/ml BMP4 to the VM fragment culture (Table 4).

Noggin is a suppressor for BMP2 and 4 (Zimmerman et al., 1996). To examine BMP activities on OL development, I applied noggin in the flat whole-mount preparation. Affi-Gel Blue beads were soaked in 20 nM noggin in 0.1% BSA/PBS, embedded into the flat whole-mount preparation (Fig. 8), and cultured for 3 DIV. No change was detected between the culture with noggin-beads (Fig. 21B) and that with control-beads (Fig. 21A), which were soaked with 0.1% BSA/PBS. Thus, it is unlikely that BMP4 to be the major dorsal activities inhibiting OL development in the cervical spinal cord.

Discussion

OL development in the spinal cord region is regulated through a mechanism similar to that governing neuronal development.

Many subclass of neurons are generated along the dorso-ventral axis in the spinal cord. Shh, which is a typical ventralizing factor in the cervical spinal cord, promotes the generation of motoneurons and ventral interneurons (SM, VM and V0-3 in Fig. 26; Roelink et al., 1995; Ericson et al., 1996; Briscoe et al., 1999) depending on its concentration. Development of dorsal interneurons in the cervical spinal cord is induced by dorsalizing signals such as BMPs (D1A, 1B and D2 in Fig. 22; Liem et al., 1995, 1997; Lee et al., 1998). In this thesis, development of OL is shown to be regulated by both the promoting factors from ventral region (Shh and neuregulin; Orentas et al., 1996, 1999; Pringle et al., 1996; Vertanian et al., 1999) and the inhibitory signals from dorsal region (Figs 13, 14, 15, 16, 17, 18, 22). The glial and neuronal development may be controlled by a similar mechanism.

Establishment of culture system of flat whole-mount preparation

In this thesis, I established a culture system named flat whole-mount preparation to study mouse OL development. While the manipulation of chick embryos is useful for investigating early OL development (Pringle et al., 1998), mouse embryos, on the other hand, are recalcitrant to manipulation *in utero*. The flat whole-mount preparation

enabled us to observe cell migration sequentially and to dissect desired regions in culture. These were difficult procedures even with the chick embryos. Using the flat whole-mount preparation, I became able to stain immature OLs in the ventral cervical spinal cord with O4 antibodies at as early as an E13 equivalent stage (Fig. 11A). Moreover, in previous studies, the earliest $O4^+$ cells were found at E14.5 in the mouse medulla (Hardy et al., 1996), and in the chick embryonic spinal cord at stage 26 (Ono et al., 1995). The process of fixation may damage the O4 antigen, and thus render O4 staining of the sections difficult. In the dissociation culture, the detectable number of $O4^+$ cells is minimal, however, I detected $O4^+$ cells at E12 at the beginning of the culture along the floor plate of the cervical spinal cord (Fig. 9A).

Discrepancy between PDGFRα^+ cell localization in the flat whole-mount preparation versus that observed *in vivo*

The distribution of OL precursors changes from a restricted pattern to a widely spread pattern. This is generally thought to be due to cell migration. In the chick optic nerve, OL progenitors migrate from the diencephalon near the third ventricle towards the retina (Ono et al., 1997a). In the chick spinal cord (Ono et al., 1995) and medulla (Ono et al., 1997b), progenitors migrate from the ventral half to the dorsal region.

In the flat whole-mount preparation, OLs originated from the ventral region as observed *in vivo*; however, the distribution pattern of PDGFRα^+ cells on E12+3 DIV was slightly different from that *in vivo*. No PDGFRα^+ cells were found in the ventral region of the flat whole-mount preparation (Fig. 10A, B), whereas they were observed

throughout the spinal cord *in vivo* (Pringle et al., 1993). This discrepancy may be caused by the absence of the notochord in the flat whole-mount preparation. The dorsalizing signals may be predominant over the ventralizing signals in the absence of the notochord. PDGFRα$^+$ cells in the ventral region could no longer be generated and the preexisting cells migrated to the dorsal region. While it was quite intriguing to analyze whether all or merely a sub-population of the PDGFRα$^+$ cells migrated from the ventral to dorsal region and then differentiated into OLs, I have never detected O4$^+$ cells in dorsal region within 3 DIV (Fig. 11B). In order to answer this question, establishment of a longer culture will be necessary.

Effects of inhibitory signals from the dorsal region on OL development

How do the dorsal inhibitory signals inhibit OL development? Previous studies demonstrated that the proliferation of OL progenitors promoted by PDGF and bFGF consequently inhibited OL differentiation (Gard and Pfeiffer, 1993). In my case, the removal of dorsal signals (1) promoted the differentiation process from Musashi$^+$ cells to PDGFRα$^+$ cells; (2) promoted the maturation process from O4$^+$ to O1$^+$ OLs; and (3) increased Musashi$^+$ neuroepithelial cells by inhibiting the differentiation process from Musashi$^+$ cells to MAP2$^+$ cells (Table 2; as explained below). The correlation between the reduction of MAP2$^+$ cell content (-23.2%) and the increase of Musashi$^+$ cell content (+27.6%) in the VM fragment indicates that the dorsal signals potently promote the neuronal differentiation. The large changes of their ratio suggested that the dorsal signals actively regulate neuronal populations. Removal of dorsal signals increased the

number of GFAP$^+$ cells 1.8-fold, which could be a secondary effect of the 2.7-fold increase of the Musashi$^+$ cells (Table 2). Thus, any processes which leads to astrocyte generation from neuroepithelial cells may not have been affected by the dorsal removal. In contrast, 15.5-fold increase in the number of PDGFRα^+ cells is far above the 2.7-fold increase of the Musashi$^+$ cells (Table 1, 2). Thus, the generation of OL lineage from neuroepithelial cells is promoted by the removal of dorsal region. O1$^+$ cells, representing mature OLs, were rarely seen in the control flat whole-mount preparation, whereas they clearly appeared in the VM fragment culture. Therefore, maturation of OL is also promoted by the removal of dorsal region.

Candidates for the factors inhibiting OL differentiation

There were several reports describing signals from the axons regulating OL differentiation: (1) The proliferation of OL precursors is positively regulated by electrical activity in axons (Barres and Raff, 1993a); (2) OL differentiation is promoted by humoral factors such as PDGF, IGF and NT-3 produced by neurons (Barres et al., 1993b; Barres et al., 1994; Barres and Raff, 1996); (3) OL differentiation is inhibited by the activation of the Notch1 pathway. OLs and their precursors in the developing rat optic nerve express Notch1 receptors and retinal ganglion cells express Jagged1, a ligand of Notch1, along their axons. Jagged1 expression is developmentally down-regulated, the disappearance of Jagged1 becomes a trigger of OL differentiation (Wang et al., 1998). Therefore, removal of these axonal factors by the axonal degeneration could have caused the induction of OL lineage. There are many kinds of axons in the

spinal cord and medulla region. For example, circumferentially growing axons, which are containing the posterior commissure axons and the alar plate axons (Shirasaki et al., 1996), could be damaged in the VM fragment. However, in the *in vitro* transplantation of VM fragment, there was no difference in the appearance of axons between both sides (Fig. 20B), though the OL numbers in the half proximal to the dorsal region were fewer than those in the distal half (Fig. 14A, 16A). Thus, it is less likely the axonal degeneration to be the cause for the increase in OL number after the removal of dorsal region in the flat whole-mount preparation.

bFGF (Bögler et al., 1990; McKinnon et al., 1990) and PDGF (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988) promote the proliferation of O2A progenitor cells, which are known to be the common progenitor cells of OLs and type 2 astrocytes *in vitro*, and consequently inhibit OL differentiation. However, it is unlikely that these factors are specifically secreted from a roof plate.

There were several dorsalizing factors reported in the spinal cord. Wnt1 (Roelink and Nusse, 1991) and Dorsalin-1 (Basler et al., 1993) are expressed in the dorsal spinal cord. BMP family, which was reported to induce dorsal interneurons in the spinal cord and cerebral cortex (Kalyani et al., 1998; Li et al., 1999), was the strongest candidate that influenced OL development, since it induced astrocyte differentiation from EGF-expanded forebrain SVZ cells and O2A progenitor cells (Gross et al., 1996; Mabie et al., 1997). Most importantly, BMPs always inhibited the generation of OLs in the all earlier studies (Sasai, 1998). In my experiments, BMP4 increased the number of astrocytes and inhibited the cell proliferation in the dissociation culture obtained from the same region

and developmental stage as the flat whole-mount preparation (Table 3). However, BMP4 did not affect the number of OLs in the VM fragment (Table 4). Noggin, which is a suppressor of BMP2 and 4, is supposed to promote OL differentiation and inhibits neuronal differentiation (Mabie et al., 1999), if BMPs act on OL development. However, no effects were observed in the Noggin-treated culture (Fig. 21), further strengthening my view that the BMPs are not the major component of the dorsal activities.

It has been shown through several different experiments that BMPs always decrease the number of OL in culture (Gross et al., 1996; Mabie et al., 1997; Mabie et al., 1999), while it had no effects in my VM fragment culture. There are several possibilities that can explain this discrepancy. First, the regions and developmental stages of the cells are different. In the earlier works, the OL lineage cells were mostly obtained from cerebral cortex of much older animals. We have shown that the OL lineages between the cortex and spinal cord are different (Ivanova et al., in preparation), and therefore, it is possible that the OL precursor cells responded to the BMPs in a distinct manner. Second, in the present flat whole-mount preparation, neuroepithelial cells or the OL precursor cells is locally exposed to a high concentration of OL-inducers, such as Shh and Neuregulins, while in the dissociation culture employed in the earlier studies they were not. Lastly, in the flat whole-mount preparation ECM is present. Many soluble factors adsorb to the ECM and change their properties. It is well known that bFGF requires heparin for exerting its activity (Burgess et al., 1989). The action of netrin-1 toward the growing axons is even reversed upon binding to laminin-1 (Höpker et al., 1999). Therefore, it is not surprising that BMPs did not affect number of OL in

the present flat whole-mount preparation. Several lines of evidence support the last explanation. First, the dorsal region has to be attached to the VM fragment to exert its inhibitory effects on OL development. When the VM fragments were cultured together with the flat whole-mount preparation (including the dorsal region) but without attachment, there were no effects on OL development in the VM fragment. Second, the inhibitory effect of the dorsal region on OL development cannot pass through the ventral midline (Fig. 14A, 16A). It always gives very sharp contrast at the ventral midline. All of these observations indicate that the activities from dorsal spinal cord are not secreted into the medium, but may spread by binding to the ECM. It is also possible that cells generated in the dorsal spinal cord migrate into the ventral region secreting factors inhibiting OL development. Thus the major component of the activities is less likely BMPs; at least not the BMPs secreted into the medium. It is intriguing to understand why BMPs did not influence OL development in my flat whole-mount preparation, which should tell us how the BMP activity is regulated *in vivo*.

The factors from dorsal spinal cord inhibiting OL development is not secreted into the medium, At present, however, I do not know the active component of the dorsal signals.

The Position of PLP/DM20$^+$ cells in the OL lineage

It was reported that PDGFRα^+ cells differentiated into OL through a PLP/DM20$^+$ stage in the spinal cord (Fruttiger et al., 1999). PLP/DM20$^+$ cells detected by *in situ* hybridization showed nearly the same distribution pattern as O4$^+$ cells in the my flat

whole-mount preparation. PLP/DM20$^+$ cells appeared along the ventral midline on E12+1 DIV (Fig. 12A, 17A) and increased in number on E12+3 DIV (Fig. 12B, 17B). In the culture of the cervical spinal cord, >70% of the O4$^+$ cells express PLP/DM20 (Spassky et al., 1998). In my unsectioned control culture, the detected number of O4$^+$ cells were more than that of PLP/DM20$^+$ cells (Fig. 11, 12). This result suggests the possibility that the population of PLP/DM20$^+$ cells is not completely the same with that of O4$^+$ cells. The increase of PLP/DM20$^+$ cells was more than that of O4$^+$ cells by the VM fragment dissection (Table 1). This result indicated that the dorsal spinal cord influenced the expression of *plp/dm20* gene and O4 antigen in different manner: The responsiveness of PLP promoter is more sensitive than the upregulation of O4 antigen.

Therapy for demyelinating disease

The number of OLs is significantly increased by the removal of dorsal signals inhibiting OL development in the flat whole-mount preparation (Fig. 13C, 15C). This effect was much stronger than that induced by positive regulatory factors that have been previously reported. In the adult brain, the presence of progenitors for OL as well as neural stem cells has been demonstrated. It is highly likely that the inhibitory signals also exist in the adult brain and suppress excess differentiation of OLs from these immature cells. The release of these immature cells from the effects of inhibitory signals, therefore, would be paramount in increasing the number of OLs in therapeutic approaches for demyelinating disease. The identification of these factors will greatly facilitate the development of methods to block their inhibitory effects, such as the utilization of

neutralizing antibodies, in the treatment of demyelinating diseases.

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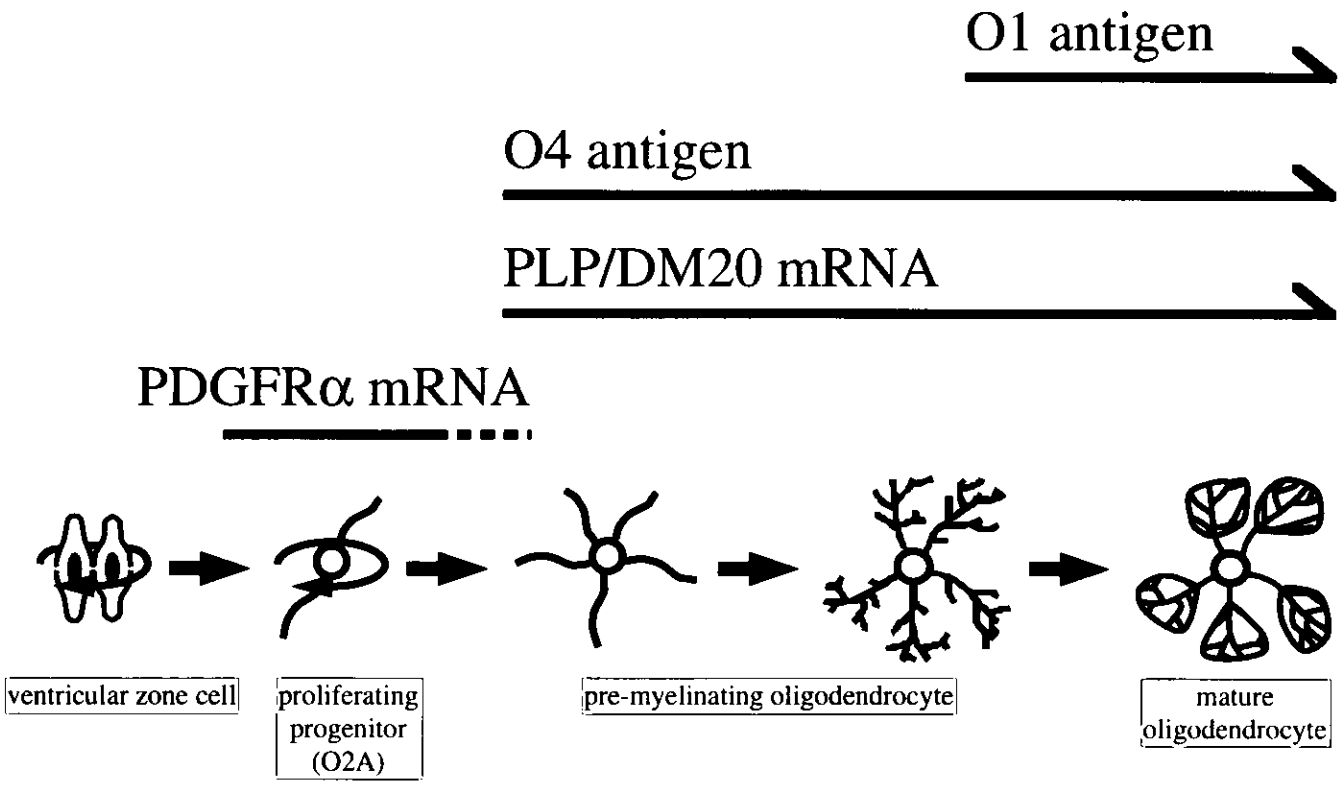
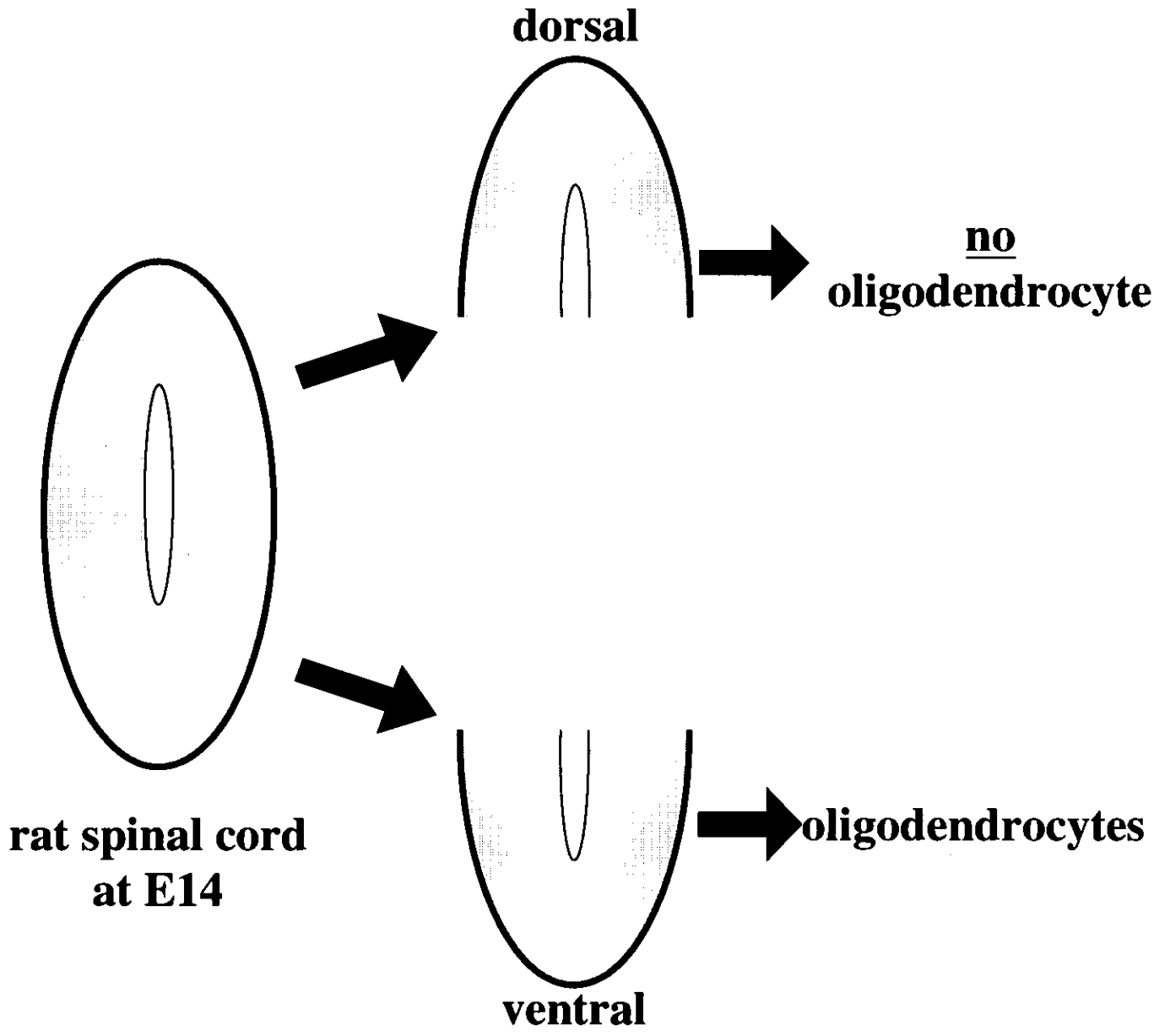

O1 antigen
O4 antigen
PLP/DM20 mRNA
PDGFRα mRNA
ventricular zone cell
proliferating progenitor (O2A)
pre-myelinating oligodendrocyte
mature oligodendrocyte

Fig. 1 Markers for OL lineage cells in the spinal cord. PDGFRα mRNA is expressed by proliferating progenitor cells. After downregulation of the PDGFRα gene expression, O4 antigen and PLP/DM20 mRNA start to be expressed, and their expression are kept till mature OL. O1 antigen can be seen only in mature OLs.



At E18, oligodendrocytes appear from both sides.

Warf et al., 1991

Fig. 2 OL generation from the ventral spinal cord in early embryos. When the rat E14 ventral and dorsal spinal cord were cultured separately, OLs appeared only from the ventral spinal cord. In contrast, both the ventral and dorsal spinal cords from E18 rat gave rise to OLs.

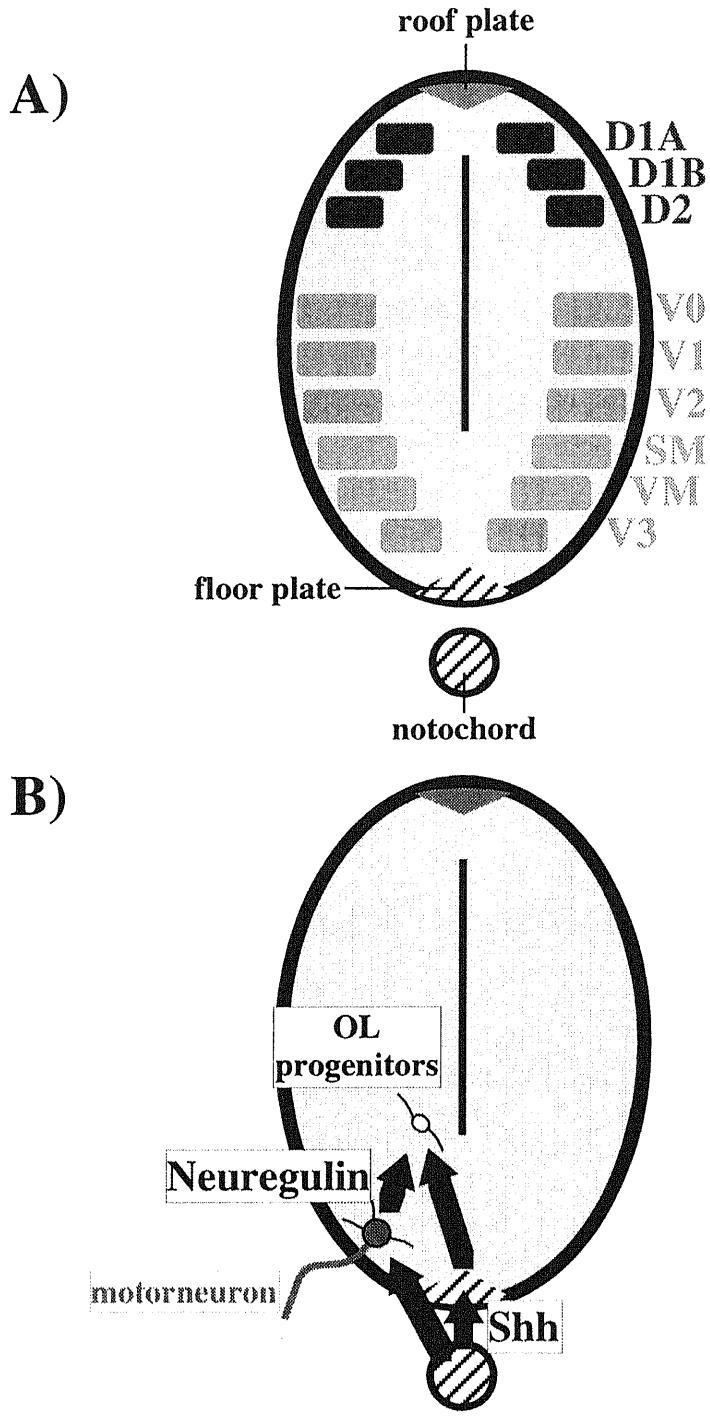

A)
roof plate
D1A
D1B
D2
V0
V1
V2
SM
VM
V3
floor plate
notochord
B)
OL
progenitors
Neuregulin
motorneuron
Shh

Fig. 3 Specification of neuronal subtype and regulation of OL development in the spinal cord. (A) Neuronal subtypes are generated along dorsoventral axis regulated by the dorsal and ventral signals in the spinal cord. Motoneurons (VM; visceral motoneuron and SM; somatic motoneuron) appear from ventral spinal cord. Ventral interneurons (V0, 1, 2) appear from regions more dorsal to those from which the motoneurons. Dorsal interneurons (D1A, D1B and D2) appear from dorsal spinal cord. (B) Oligodendroglial development is induced only in the ventral spinal cord. Shh from notochord and floor plate induce the generation of OL lineage cells and ventral neurons. Neuregulin from motoneurons may induce OL development and/or support OL survival.

E12 ICR mouse

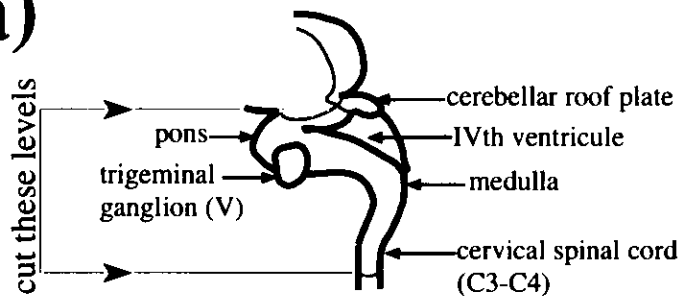
a) Dissect the hindbrain and the cervical spinal cord

b) Cut the dorsal midline

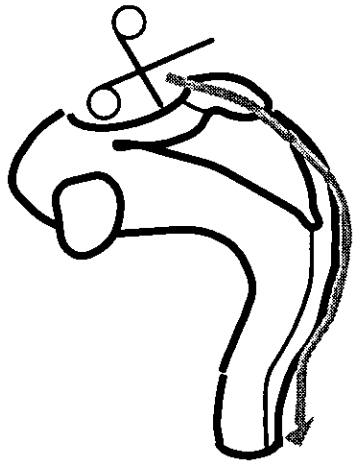
c) Put the tissue on Transwell^{TM}, ventricular side down

Culture for 1 or 3 DIV in Bottenstein/Sato's medium

a)



b)



c)

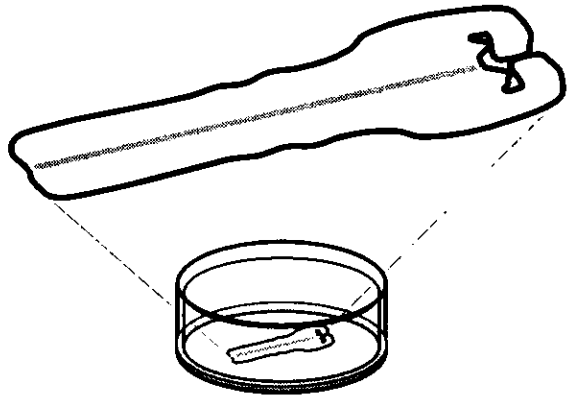


Fig. 4 The method for flat whole-mount preparation. After dissection of hindbrain and the cervical spinal cord out from E12 ICR, the dorsal midline of the brain and cord were cut. Opened brain was placed on Transwell™, ventricular side down, and cultured for 1 or 3 DIV in the chemical defined medium (see materials and methods).

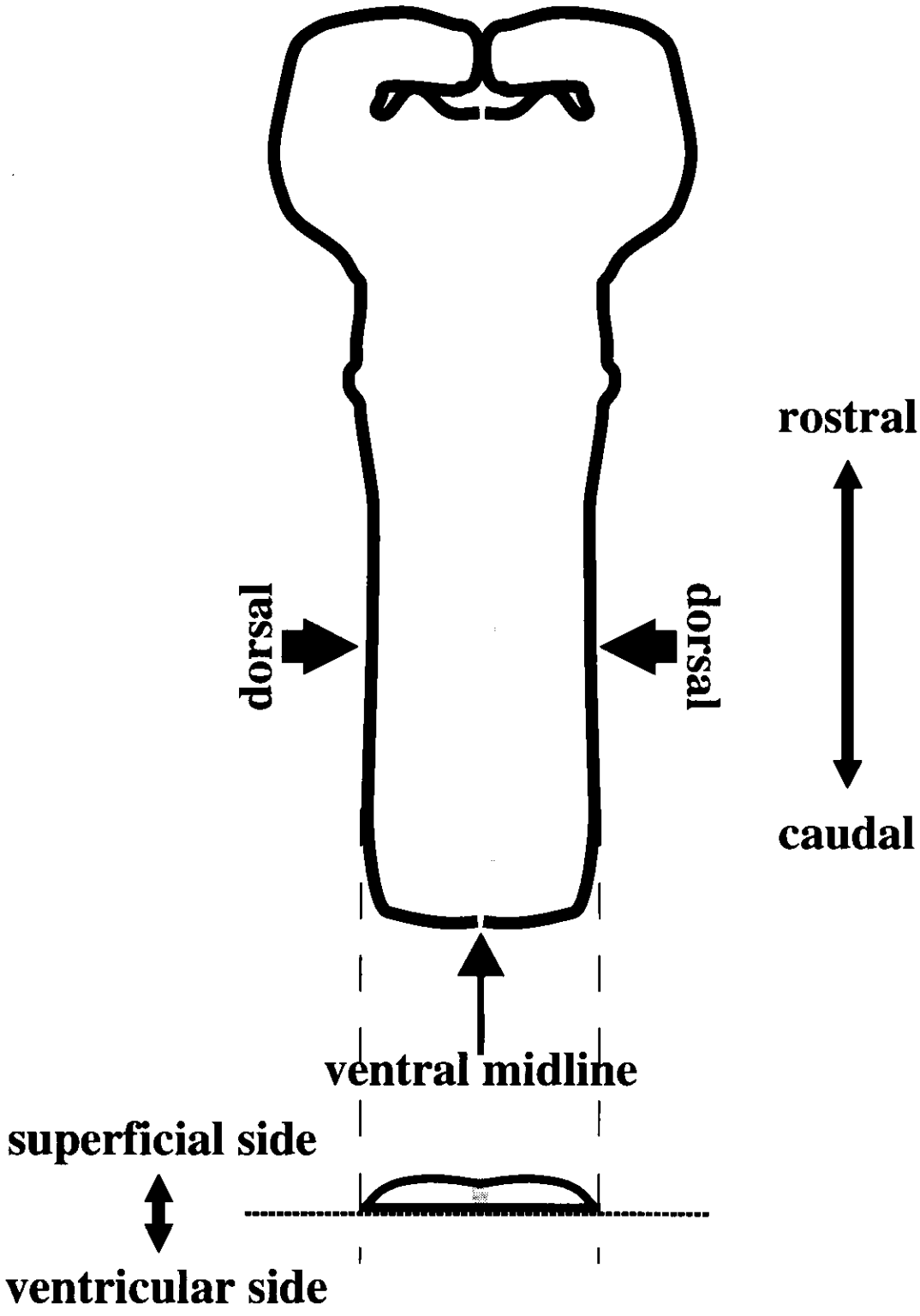
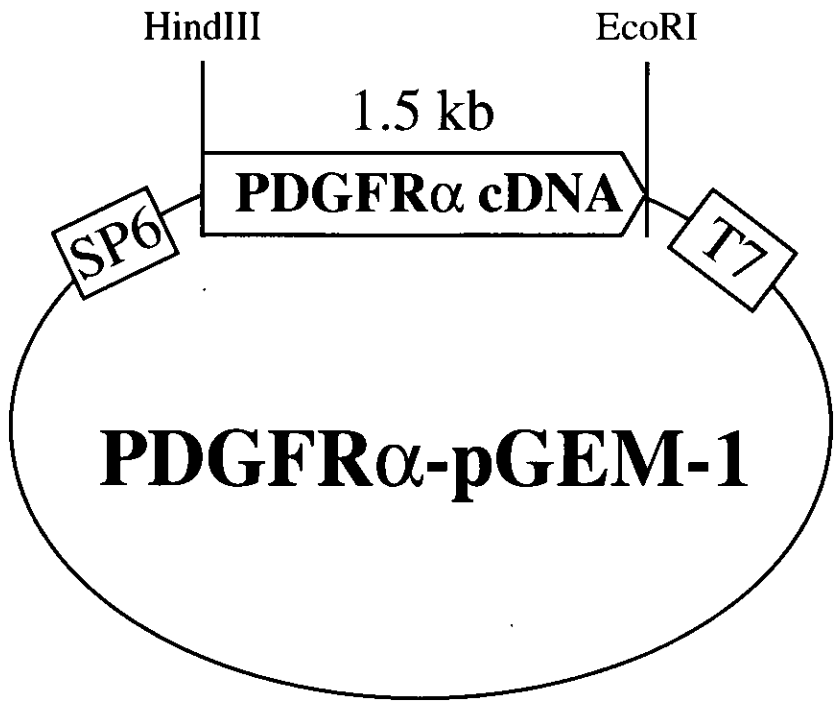

rostral
caudal
dorsal
dorsal
ventral midline
superficial side
ventricular side

Fig. 5 The top and lateral view of the flat whole-mount preparation. Ventral region is at the middle and dorsal regions locate both sides of the flat whole-mount preparation. Upper side of the top view is rostral and lower side is caudal. The side that contacts with the membrane of Transwell™ is ventricular side of the flat whole-mount preparation and the opposite is superficial side.

A)


HindIII
EcoRI
1.5 kb
PDGFRα cDNA
SP6
T7
PDGFRα-pGEM-1

B)

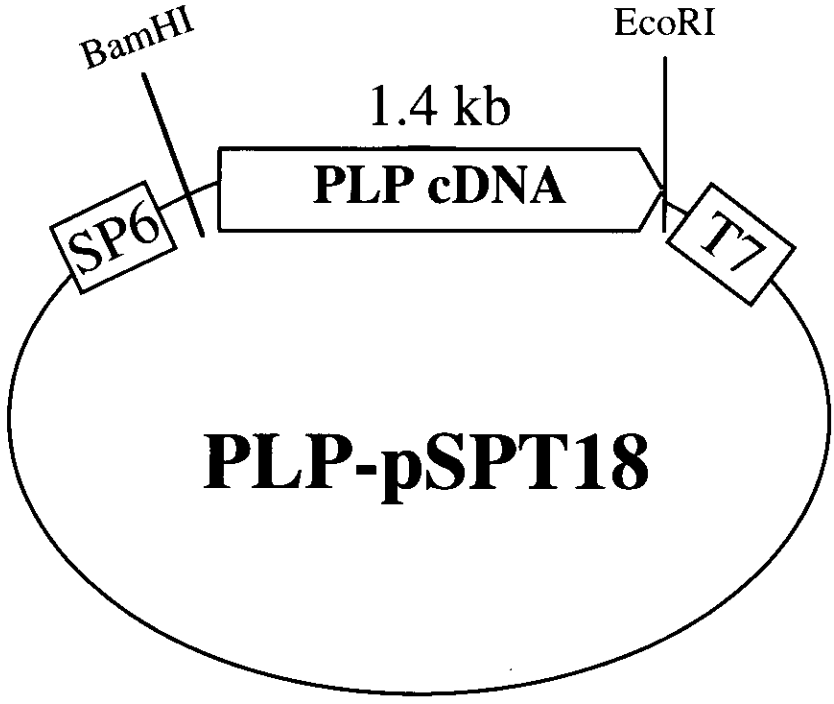
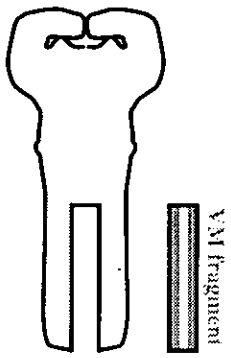

BamHI
EcoRI
1.4 kb
PLP cDNA
SP6
T7
PLP-pSPT18

Fig. 6 The constructions of cDNA for PDGFRα (A) and PLP/DM20 (B) for preparing *in situ* hybridization probes.

A) VM fragment culture



Isolate the ventral region
from E12 flat whole-mount preparation

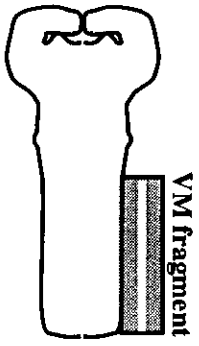
↓

Culture for 3DIV

↓

Perform immunostaining
or *in situ* hybridization

B) *In vitro* transplantation of VM fragment



Put the VM fragment
besides another explant
from E12 flat whole-mount preparation

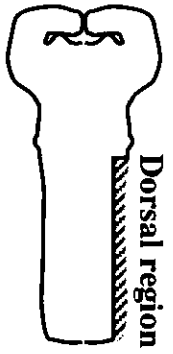
↓

Culture for 3DIV

↓

Perform immunostaining
or *in situ* hybridization

C) Ablation of dorsal spinal cord



Ablate the dorsal region
from E12 flat whole-mount preparation

↓

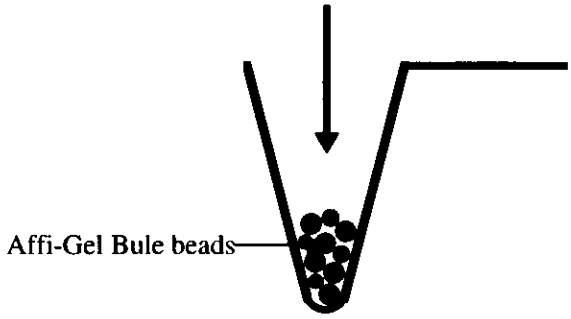
Culture for 3DIV

↓

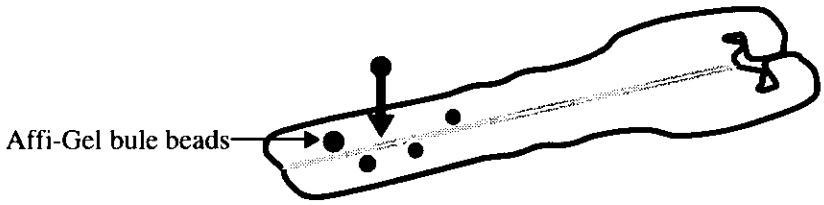
Perform immunostaining
or *in situ* hybridization

Fig. 7 Operations of the flat whole-mount preparation. (A) VM-fragment culture. E12 flat whole-mount preparation was subdivided longitudinally into 3 fragments. One third fragment that contains ventral midline was defined as VM-fragment and cultured for 3 DIV. (B) *In vitro* transplantation of VM fragment. VM fragment was settled besides another flat whole-mount preparation on E12 and cultured for 3 DIV. (C) Ablation of the dorsal cervical spinal cord. The dorsal part in the left side was ablated from the flat whole-mount preparation by using tungsten needle and cultured for 3 DIV.

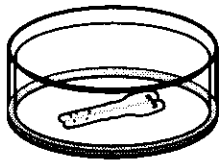
20 nM Noggin/0.1% BSA



Soak Affi-Gel Blue beads in 20 nM Noggin solution.



Embed beads on the flat whole-mount preparation.



Culture flat whole-mount preparation for 3 DIV.

Stain explants with O4 antibody.

Fig. 8 Preparation of the noggin-treated culture. Affi-Gel Blue beads were soaked in 20 nM noggin in 0.1% BSA/PBS and embedded in the E12 flat whole-mount preparation. After 3 DIV, the explants were immunostained with O4 antibody.

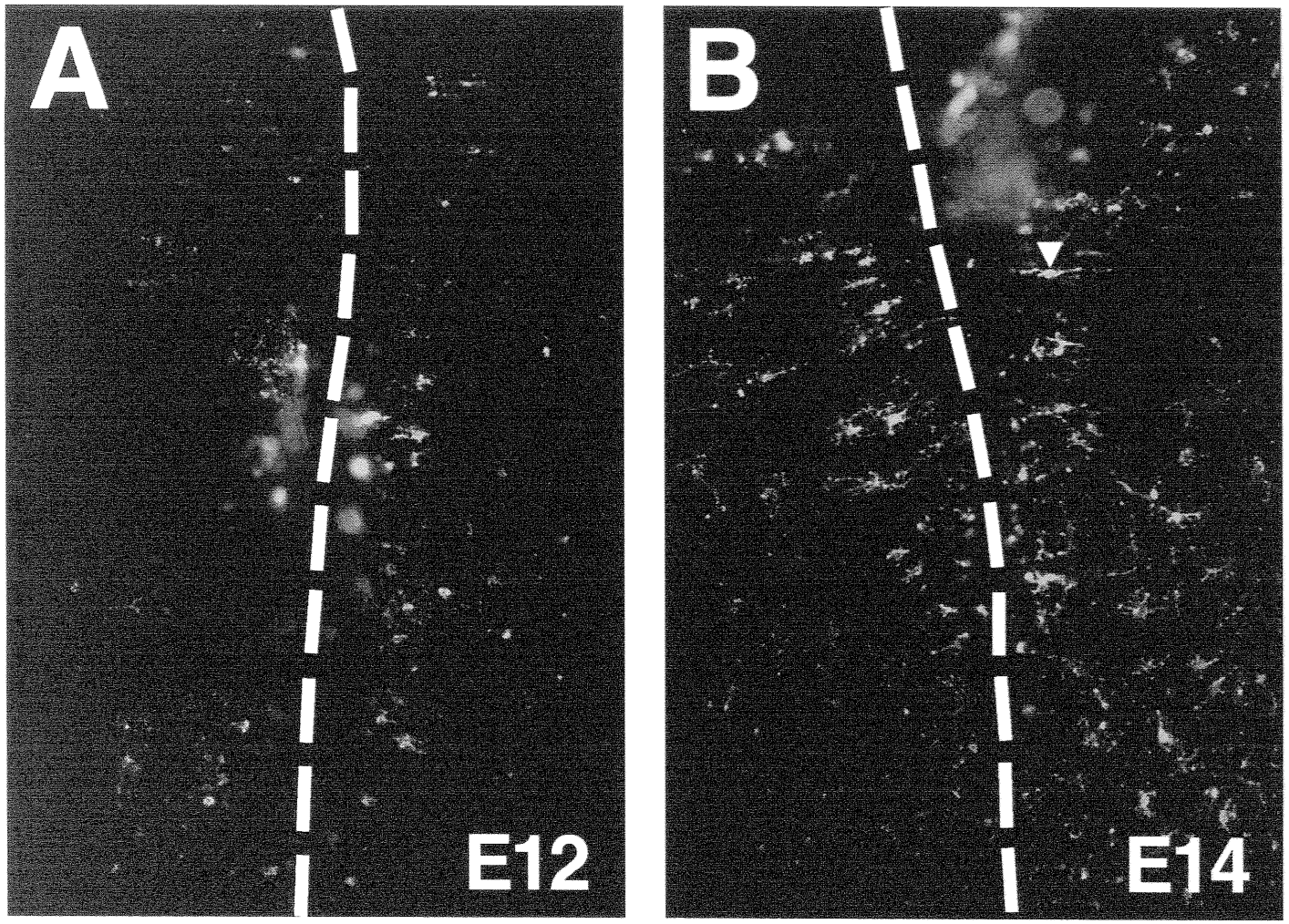

A
E12
B
E14

Fig. 9 Detection of $O4^+$ cells *in vivo*. I detected $O4^+$ cells appeared along the ventral midline of the cervical spinal cord from E12 *in vivo*. (A) On E12, $O4^+$ cells appeared from ventral spinal cord along the midline on the ventricular surface. (B) On E14, the number of $O4^+$ cells increased and expanded their area to dorsal. Arrowhead indicates a radial-shaped $O4^+$ cell, suggesting its migration to the dorsal region. White broken lines indicate the ventral midline.

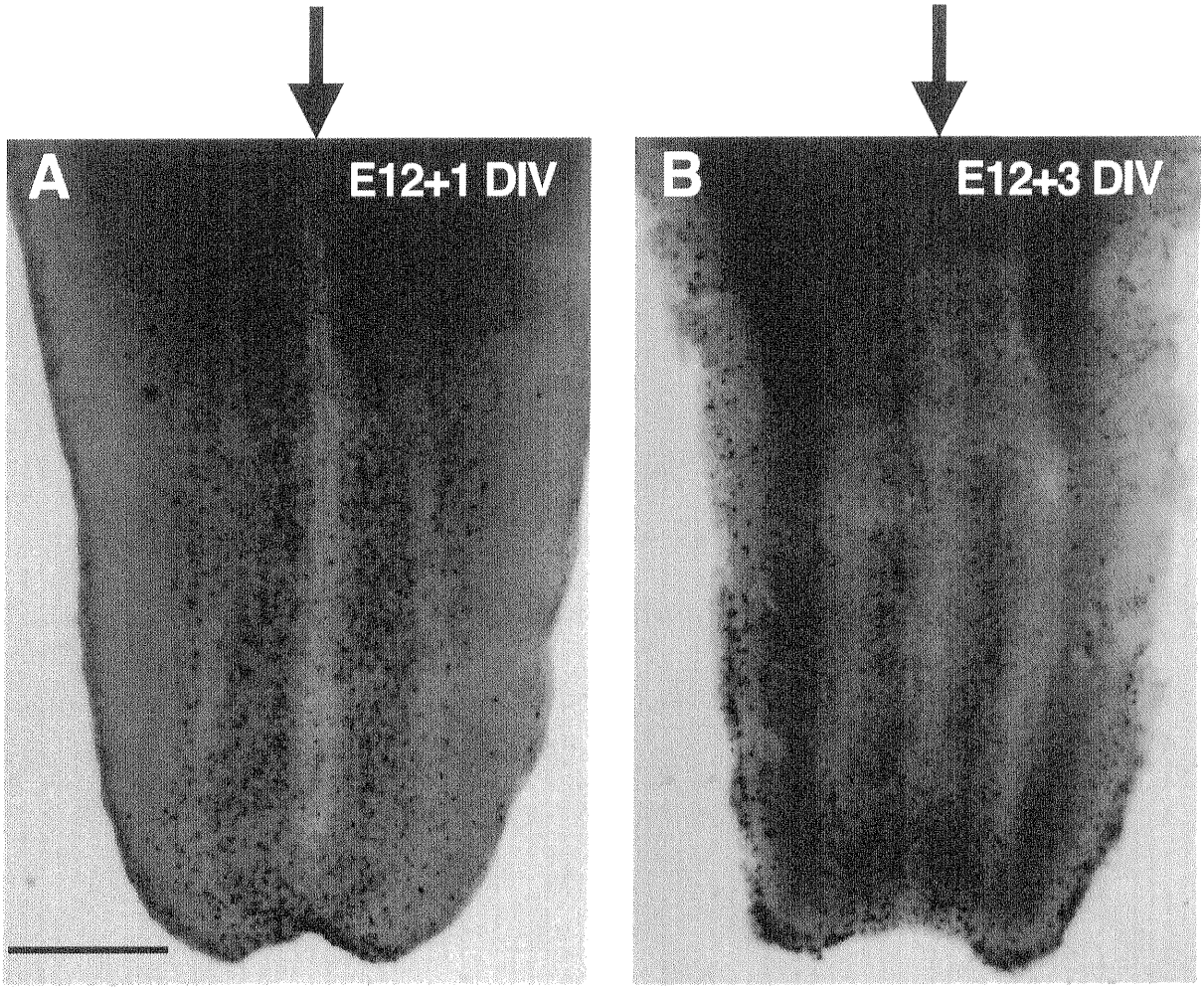

A
E12+1 DIV
B
E12+3 DIV

Fig. 10 Distribution change of PDGFRα$^+$ cells in the flat whole-mount preparation on 1 day (A) and 3 days (B). Positive cells were detected as small dots in the ventral half of the cervical spinal cord on E12+1 DIV (A), and then, distributed dorsally. No positive cells were observed around ventral midline area on 3 DIV (B). Arrows upon the pictures indicate the ventral midlines. Scale bar, 500 μm.

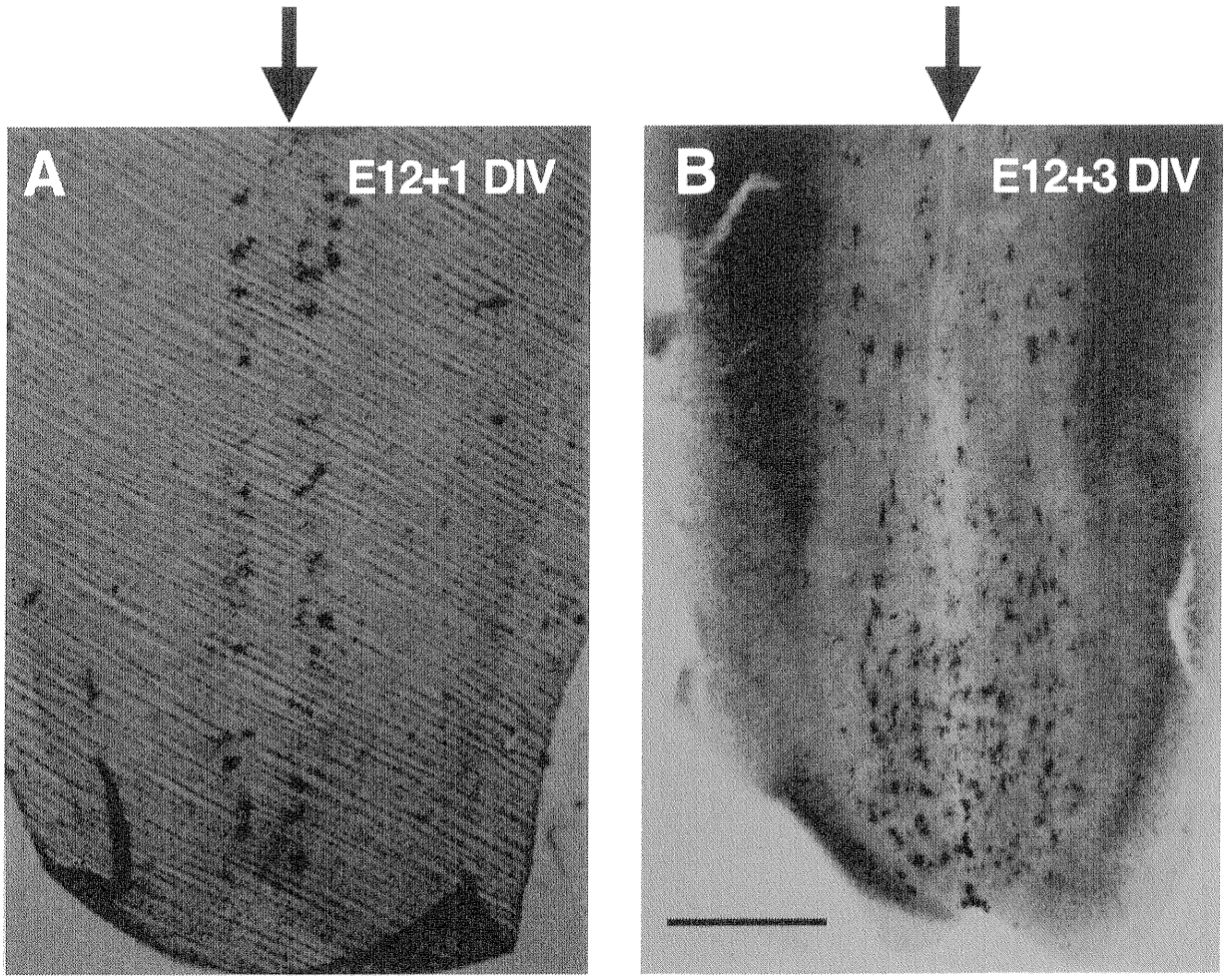

A
E12+1 DIV
B
E12+3 DIV

Fig. 11 Distribution change of the $O4^+$ cells in the flat whole-mount preparation on 1 day (A) and 3 days (B). Small number of $O4^+$ cells was detected in two lines longitudinally besides the ventral midline of the cervical spinal cord (A). $O4^+$ cells increased their number in the ventral half of the spinal cord on 3 DIV (B). Arrows upon the pictures indicate the ventral midlines. Scale bar, 500 μm.

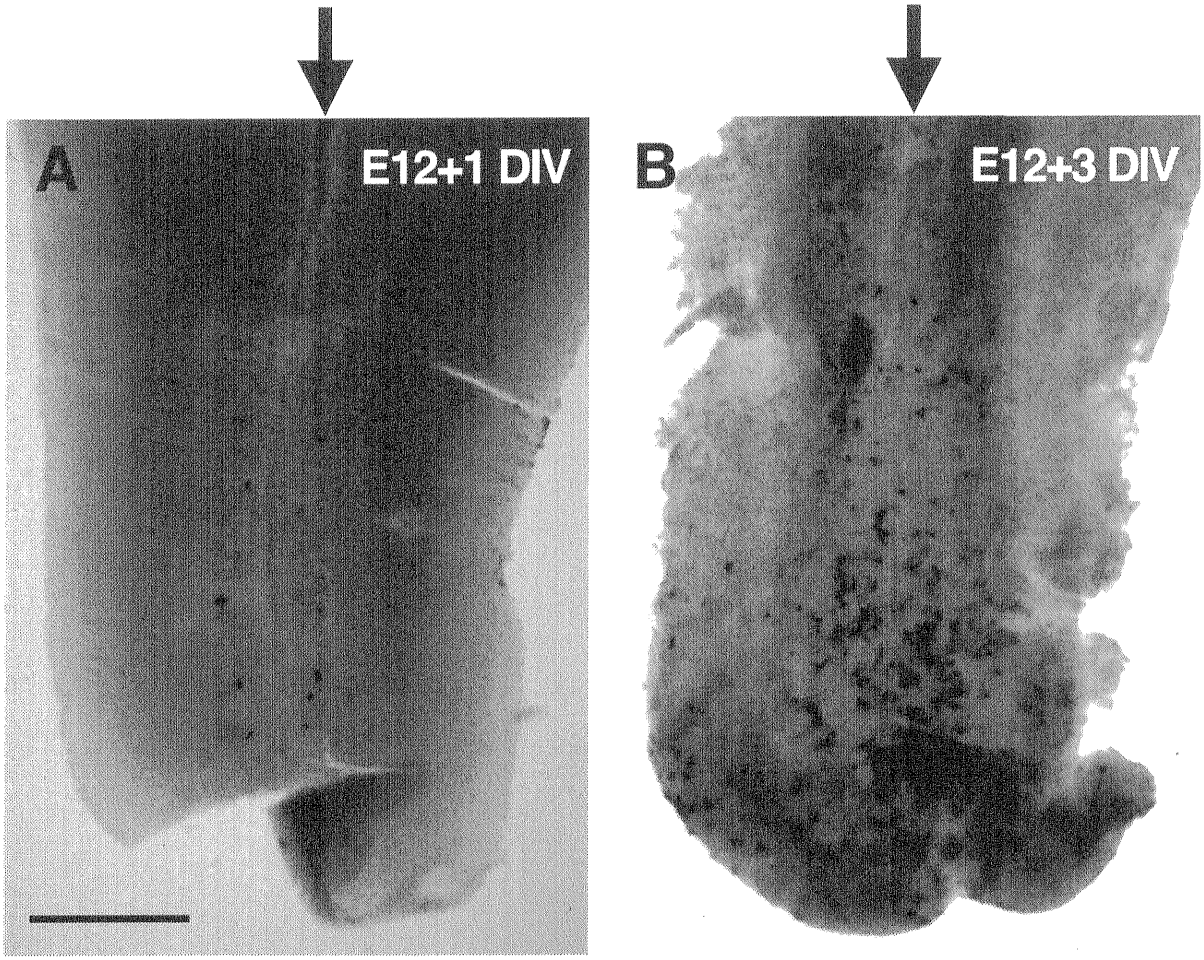

A
E12+1 DIV
B
E12+3 DIV

Fig. 12 Distribution change of PLP^+ cells in the flat whole-mount preparation on 1 day (A) and 3 days (B). The distribution pattern of *plp/dm-20* mRNA expressing cells was similar to that of $O4^+$ cells. $PLP/DM20^+$ cells appeared along ventral midline on E12+1 DIV (A) and increased around ventral midline on E12+3 DIV (B). Arrows upon the pictures indicate the ventral midlines. Scale bar, 500 μm.

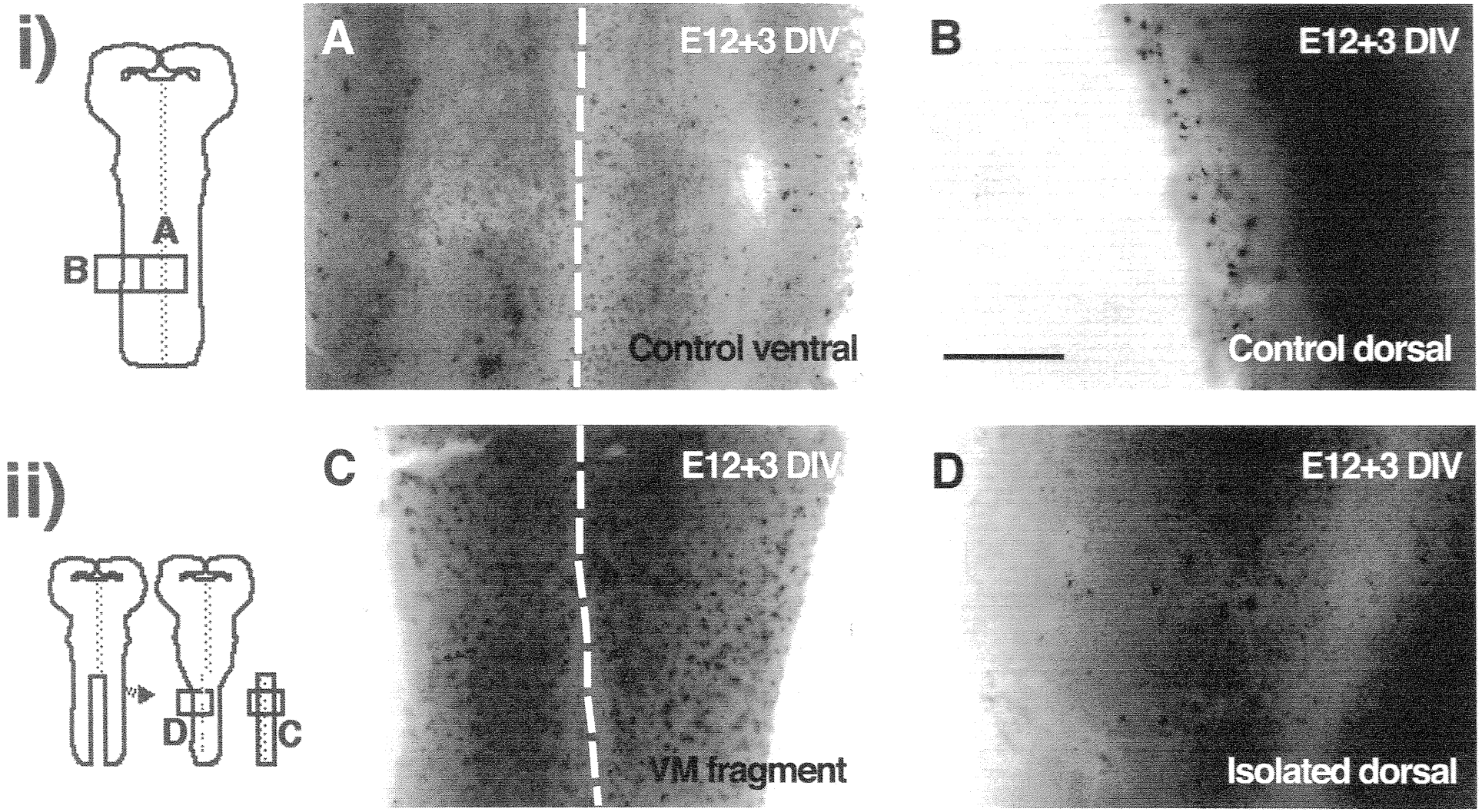

i)
A
B
A
E12+3 DIV
Control ventral
B
E12+3 DIV
Control dorsal
ii)
D
C
C
E12+3 DIV
VM fragment
D
E12+3 DIV
Isolated dorsal

Fig. 13 Robust increase in the number of PDGFRα$^+$ cells in the VM fragment of the cervical spinal cord. VM fragment of the cervical spinal cord (C1-C4) were dissected from E12 ICR mice and cultured for 3 days. The each boxed area in schematic drawings ((i) and (ii)) corresponds to the each photograph. In the control flat whole-mount preparation, PDGFRα$^+$ cells were present in the dorsal region (B) but not in the ventral region (A) on 3 DIV, shown previously in Fig. 10B in low magnification. The drastic increase in number of PDGFRα$^+$ cells were observed in the VM fragment (C), whereas PDGFRα$^+$ cells in the dorsal part decreased (D: In some cases, both sides of the dorsal parts after removing the VM fragment fused each other. see also scheme in (ii)). White broken lines in A and C indicate the ventral midline. Scale bar, 200 μm.

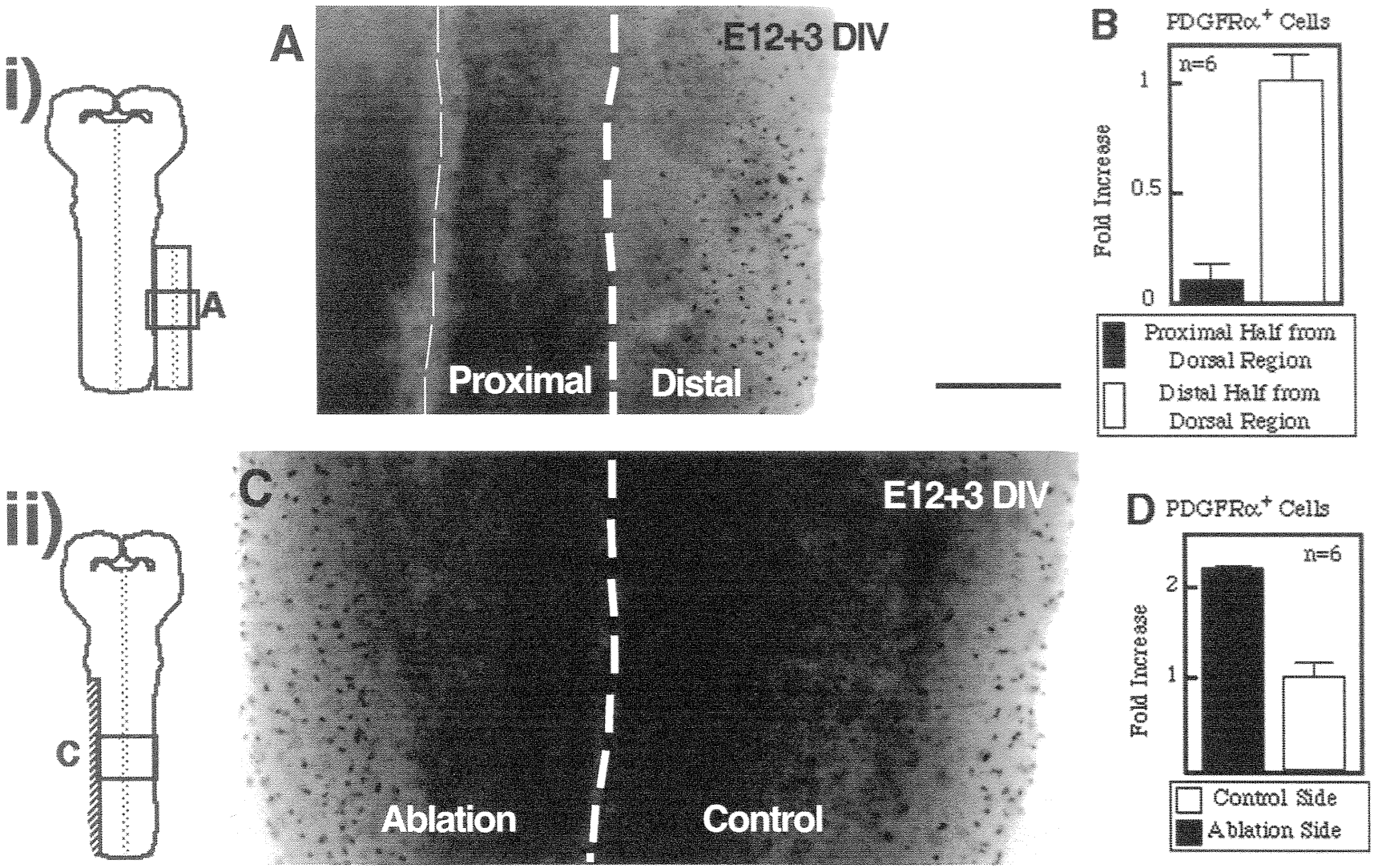

i)
A
A
E12+3 DIV
Proximal
Distal
B
PDGFRα+ Cells
n=6
Fold Increase
1
0.5
0
Proximal Half from Dorsal Region
Distal Half from Dorsal Region
ii)
C
C
E12+3 DIV
Ablation
Control
D
PDGFRα+ Cells
n=6
Fold Increase
2
1
Control Side
Ablation Side

Fig. 14 The number of PDGFRα$^+$ cells was reduced by the ectopic dorsal spinal cord and induced by removal of the dorsal region. When the VM fragment was placed besides the another flat whole-mount preparation and cultured for 3 DIV (Fig. 8B), the number of PDGFRα$^+$ cells in the proximal half (proximal) was decreased 1/10-fold (s.e.=0.04) compared to the distal half (distal) (A, B). When the dorsal region in the left side was ablated and cultured for 3 DIV (Fig. 8C). The number of the PDGFRα$^+$ cells in the ablated side (ablation) increased about 2-folds (s.e.=0.03) compared to the control right side (control) (C, D). Thin white broken line in A indicates the border between the VM fragment (right) and another flat whole-mount preparation (left). Thick white broken lines in A and C indicate the ventral midlines. Scale bar, 200 μm.

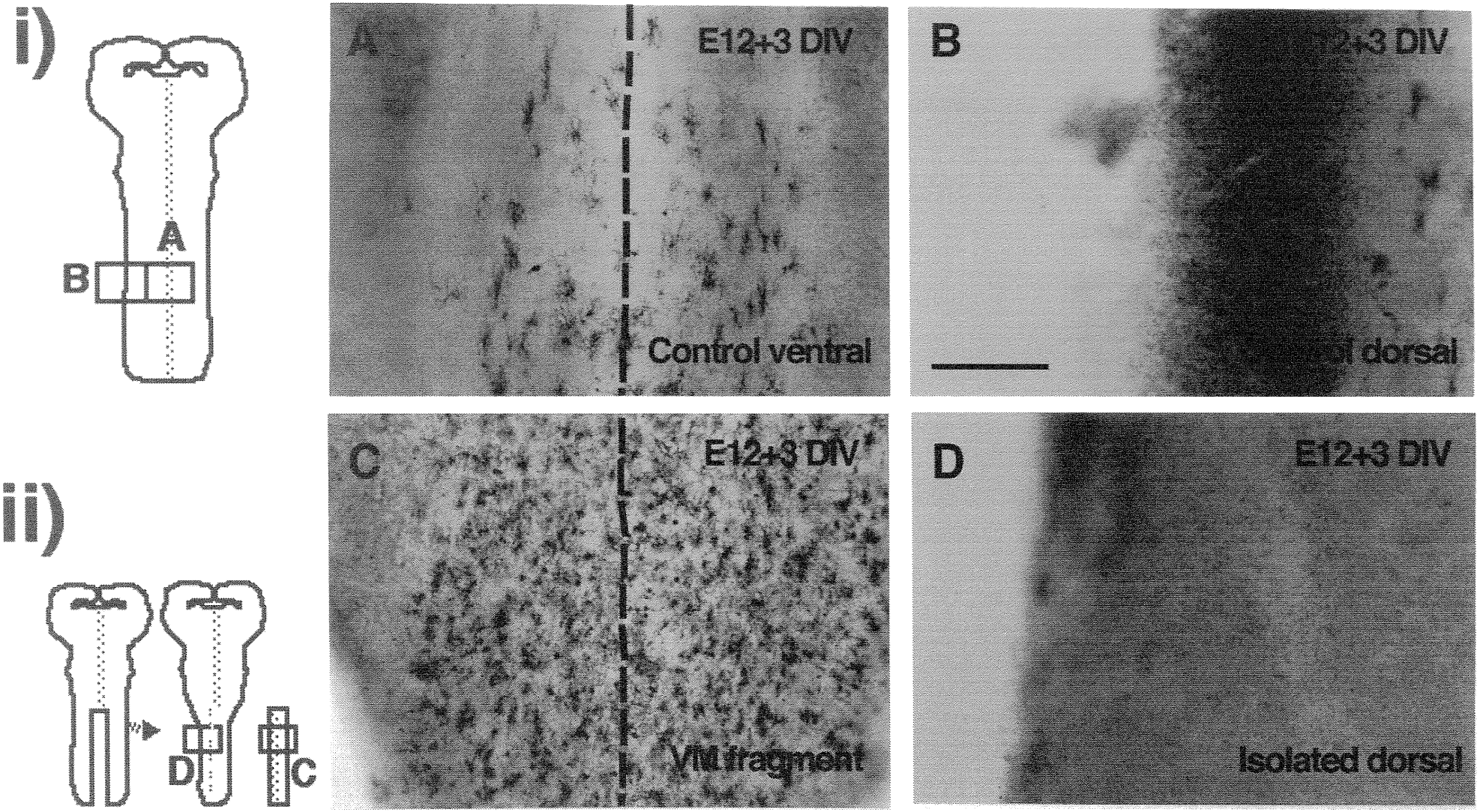

i)
A
B
A
E12+3 DIV
Control ventral
B
2+3 DIV
dorsal
ii)
D
C
C
E12+3 DIV
VM fragment
D
E12+3 DIV
Isolated dorsal

Fig. 15 Increase in number of $O4^+$ cells in the VM fragment. The each boxed area in schematic drawing ((i) and (ii)) corresponds to the each photograph. As previously shown in Fig. 11B, $O4^+$ cells distributed in the ventral region (A) but not in the dorsal region (B). The number of $O4^+$ cells was increased in the VM fragment (C) comparing to non-treated ventral (A) on E12+3 DIV. No cell was observed in the dorsal part (D: In some cases, both sides of the dorsal parts after removing the VM fragment fused each other. see also the drawing of (ii)). Black broken lines in A and C indicate the ventral midline. Scale bar, 200 μm.

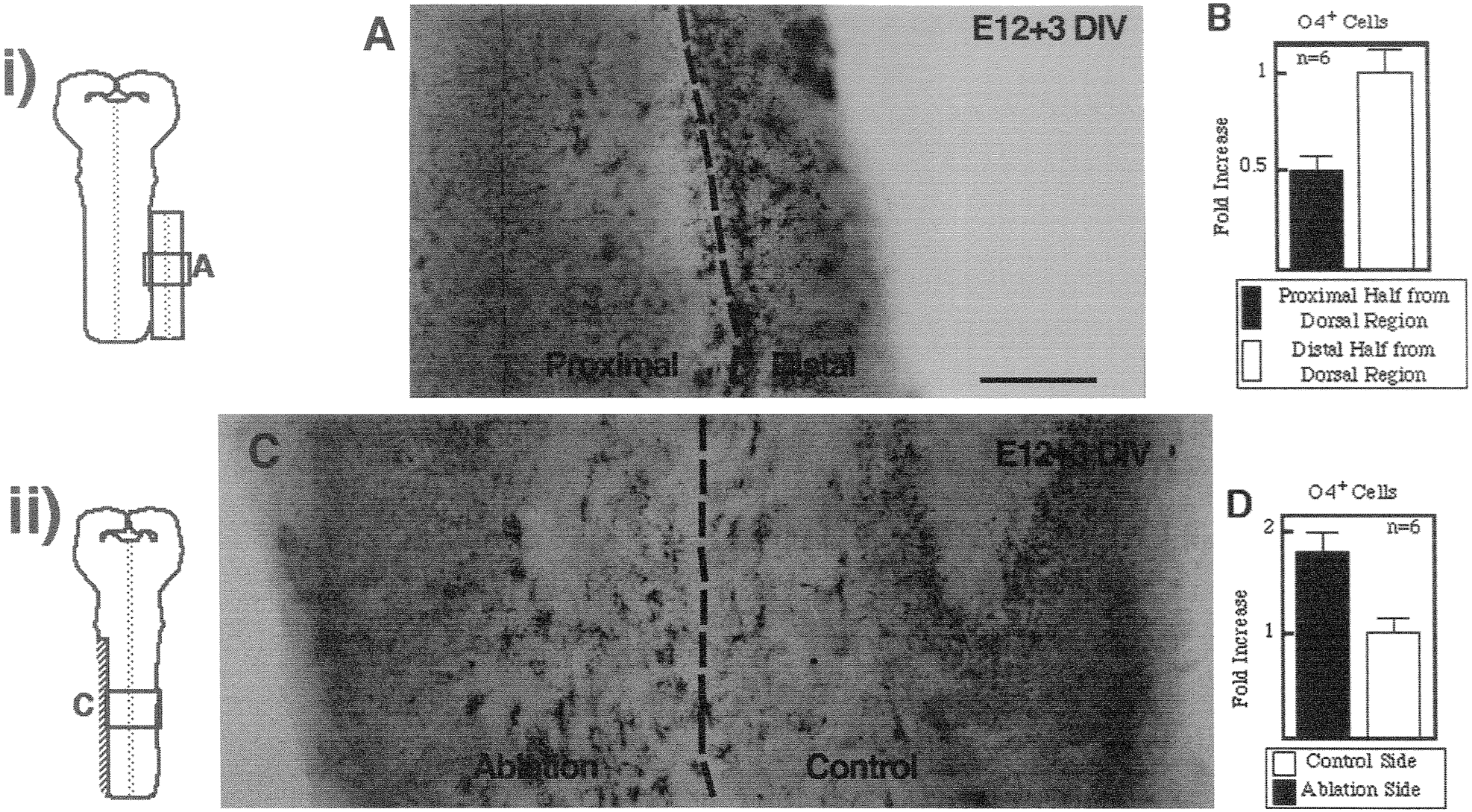

i)
A
A
E12+3 DIV
Proximal
Distal
B
O4+ Cells
n=6
1
0.5
Fold Increase
Proximal Half from Dorsal Region
Distal Half from Dorsal Region
ii)
C
C
E12+3 DIV
Ablation
Control
D
O4+ Cells
n=6
2
1
Fold Increase
Control Side
Ablation Side

Fig. 16 The number of $O4^+$ cells was downregulated by the dorsal signals. In the *in vitro* transplantation experiment, the number of $O4^+$ cells in the proximal half of the VM fragment (proximal) decreased 1/2 (s.e.=0.01) of that in the distal half (distal) (A, B). In the dorsal ablation experiment, the number of $O4^+$ cells in the ablated side (ablation) was 1.8-times (s.e.=0.43) more than that in the control side (control) (C, D). Thin broken line in A indicates the border between the VM fragment and another flat whole-mount preparation. Thick black broken lines in A and C indicate the ventral midline. Thin black broken line in A indicates the borderline between VM fragment and another explant culture. Scale bar, 200 μm.

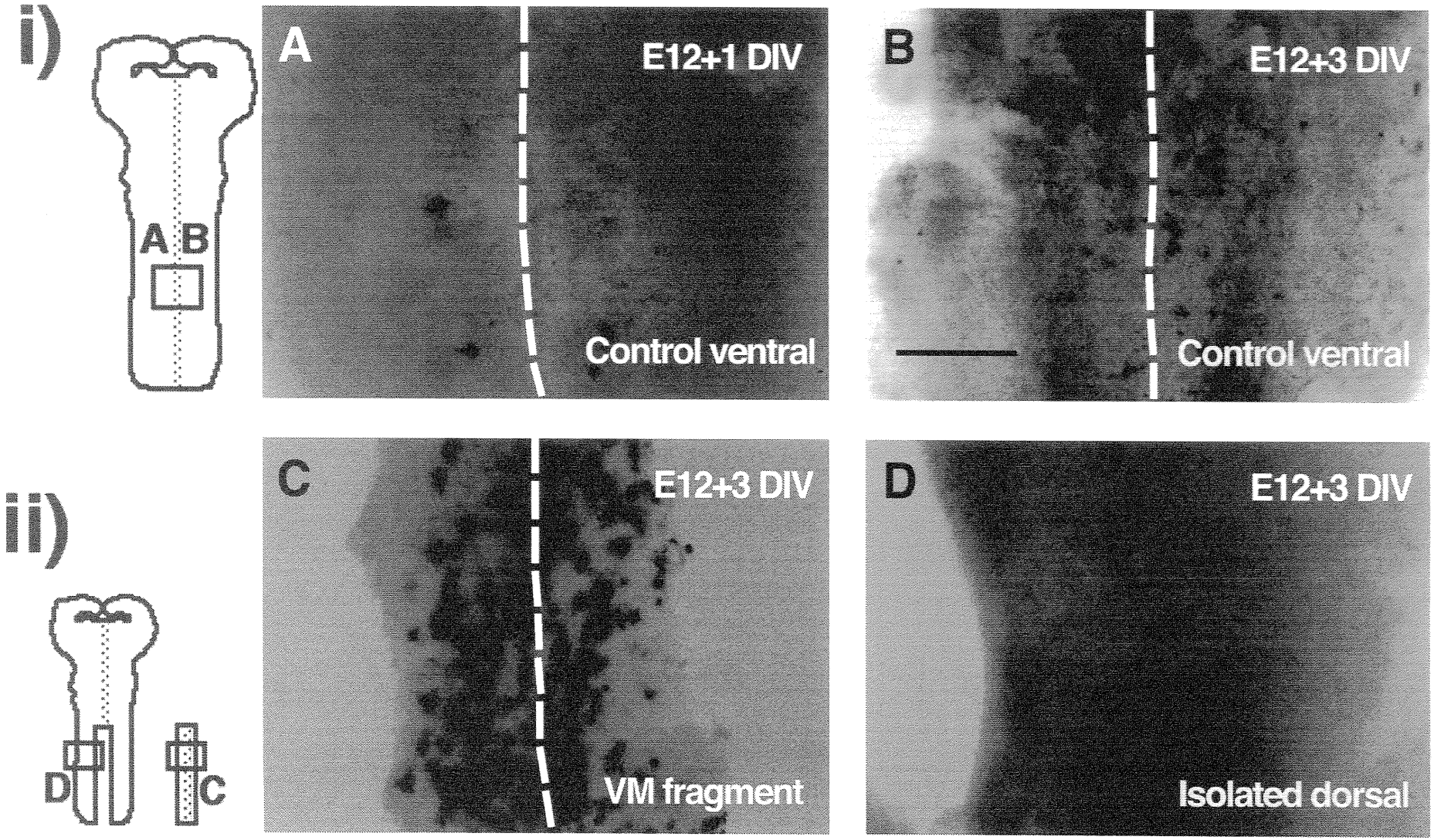

i)
A
B
A
E12+1 DIV
Control ventral
B
E12+3 DIV
Control ventral
ii)
D
C
C
E12+3 DIV
VM fragment
D
E12+3 DIV
Isolated dorsal

Fig. 17 The number of PLP/DM20^{+} cells was increased in the VM fragment. A and B are high magnification picture of Fig. 11A and B. The number of PLP^{+} cells was increased in the VM fragment (C) comparing to non-treated ventral (B) on E12+3 DIV. No cell was observed in the dorsal part (D). Thick white broken lines in A, B and C indicate the ventral midline. Scale bar, 200 μm.

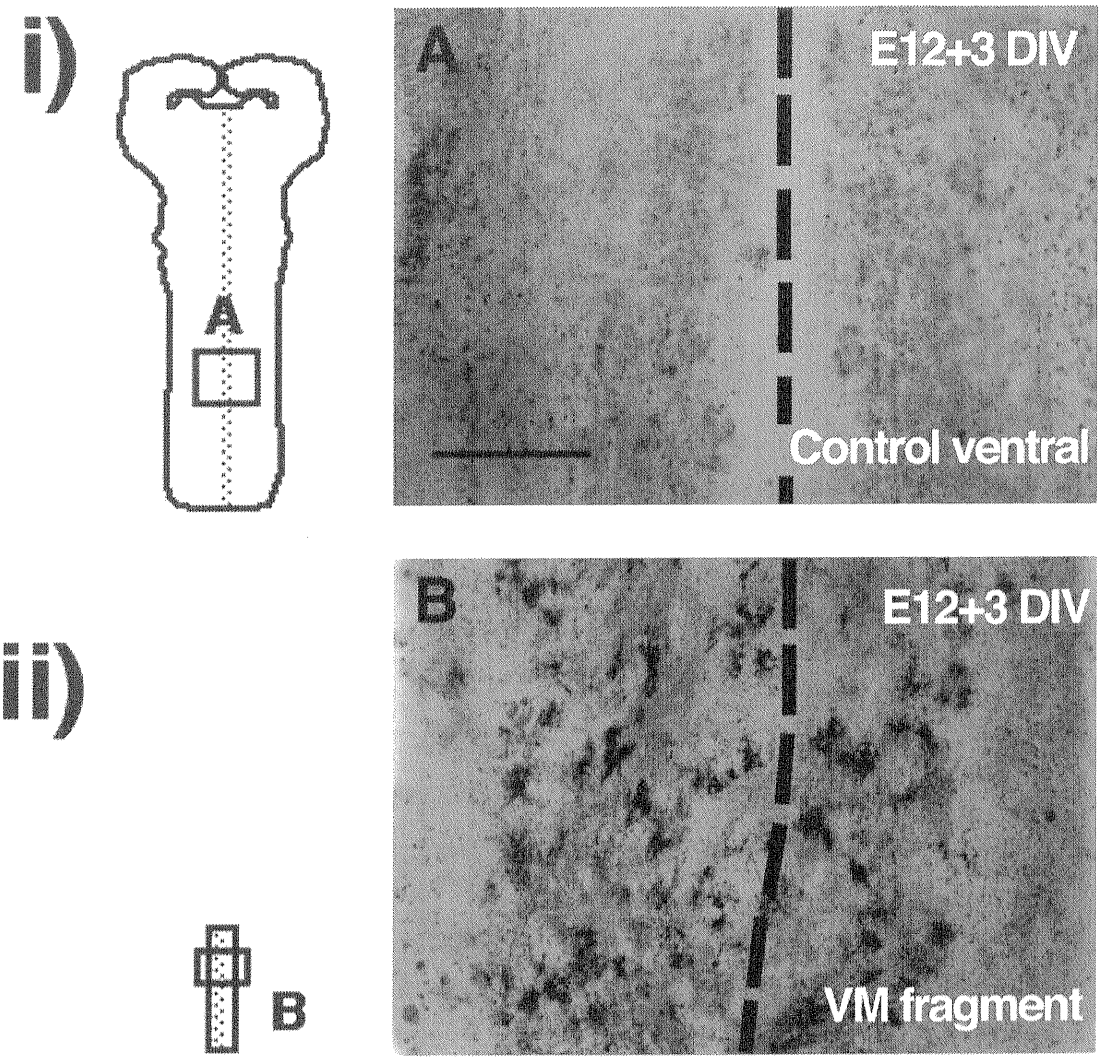

i)
A
A
E12+3 DIV
Control ventral
ii)
B
B
E12+3 DIV
VM fragment

Fig. 18 The number of mature OLs was increased in the VM fragment. The each boxed area in the schematic drawing ((i) and (ii)) indicates the position of each photograph. No positive cell for O1 antibody was found in the middle part of the control flat whole-mount preparation on E12+3 DIV (A), though very few $O1^+$ cells existed in the most caudal region (data not shown). Robust increase in number of $O1^+$ cells was observed in the VM fragment (B), where a few $O1^+$ cell was in the control. Thick black lines in A and B indicate the ventral midlines. Scale bar, 200 μm.

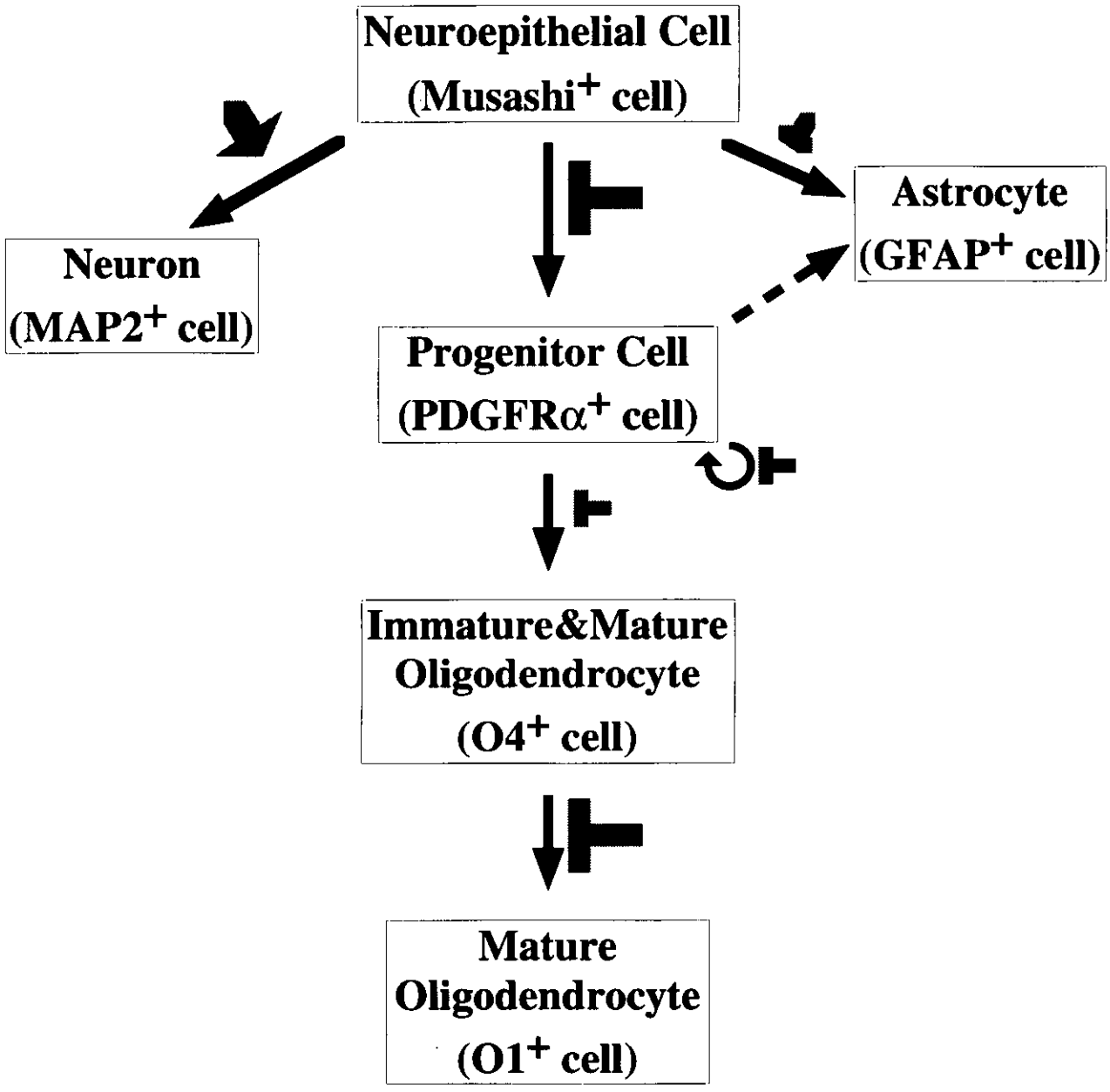

Neuroepithelial Cell
(Musashi+ cell)
Neuron
(MAP2+ cell)
Astrocyte
(GFAP+ cell)
Progenitor Cell
(PDGFRα+ cell)
Immature&Mature
Oligodendrocyte
(O4+ cell)
Mature
Oligodendrocyte
(O1+ cell)

Fig. 19 The functions of the dorsal activities. Red bars indicate certain pathways inhibited by the signals from dorsal spinal cord confirmed by series of my studies. A differentiation step from neuroepithelial cell to OL progenitor and late maturation steps of OL, and a switching of cell fate from neuroepithelial to neuron or glial cell are strongly inhibited by the dorsal spinal cord. Instead, the differentiation steps from neuroepithelial cell to neuron are induced by the dorsal spinal cord. Blue bars are the other possible pathways inhibited by the dorsal spinal cord.

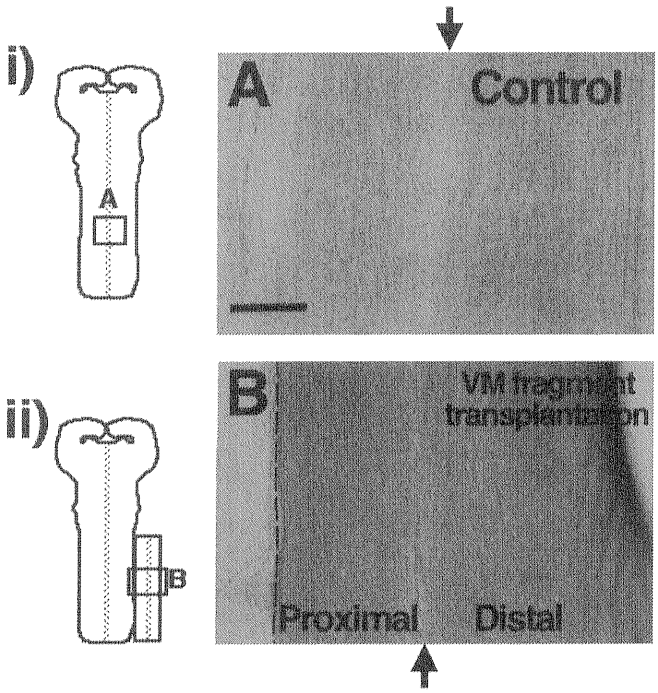

i)
A
A
Control
ii)
B
B
VM fragment transplantation
Proximal
Distal

Fig. 20 Axons appeared normal in the VM fragment culture. The each boxed area in schematic drawing ((i) and (ii)) corresponds to the each photograph. In the control culture, axonal streams stained by anti neurofilament antibody were mainly observed along rostro-caudal axis (A). In the VM fragment (B) placed besides another flat whole-mount preparation, axons running along rostro-caudal direction were likely normal in both the proximal half (proximal) and distal half (distal) on E12+3 DIV. The broken line in B indicates the border between the VM fragment (right) and another flat whole-mount preparation (left). The less stained regions in the middle of A and B correspond to the ventral midlines. No remarkable reduction of commissural axons was observed in the VM fragment. Arrows indicate the ventral midline. Scale bar, 200 μm.

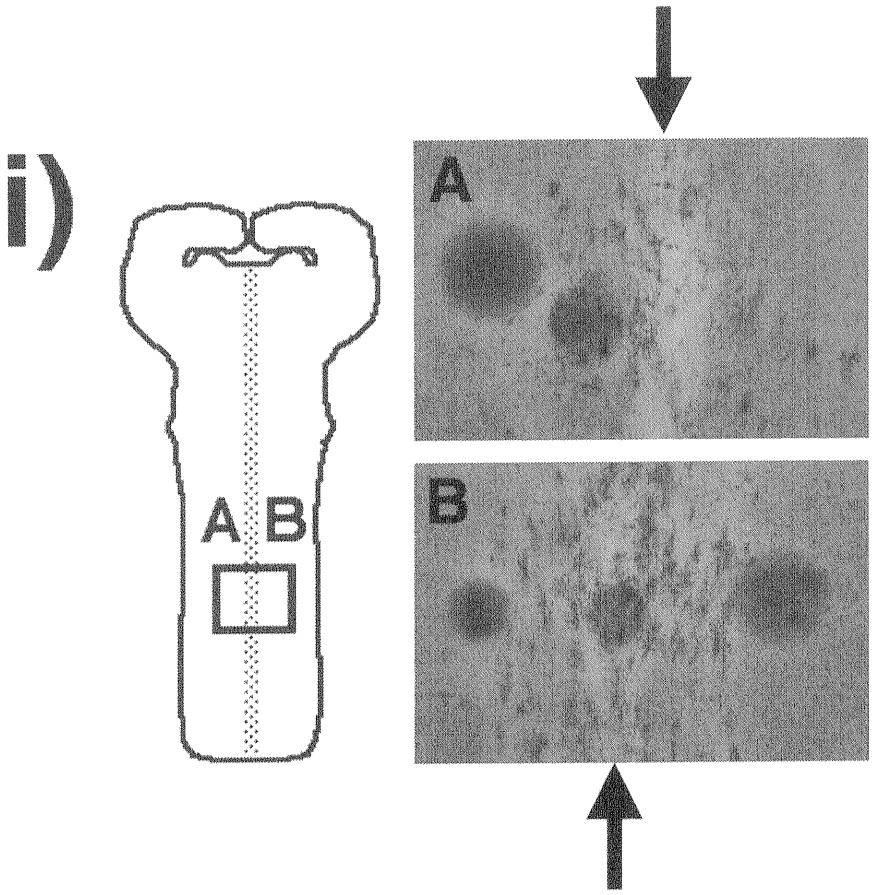

i)
A
B
A
B

Fig. 21 The number of $O4^+$ cells was not changed by the addition of noggin. The boxed area in schematic drawing i) corresponds to the each photograph. The Affi-Gel Blue Beads can be seen as blue balls in A and B. No difference was detected between the cells around Noggin-beads (B) and that around control-beads (A). Arrows indicate the ventral midlines.

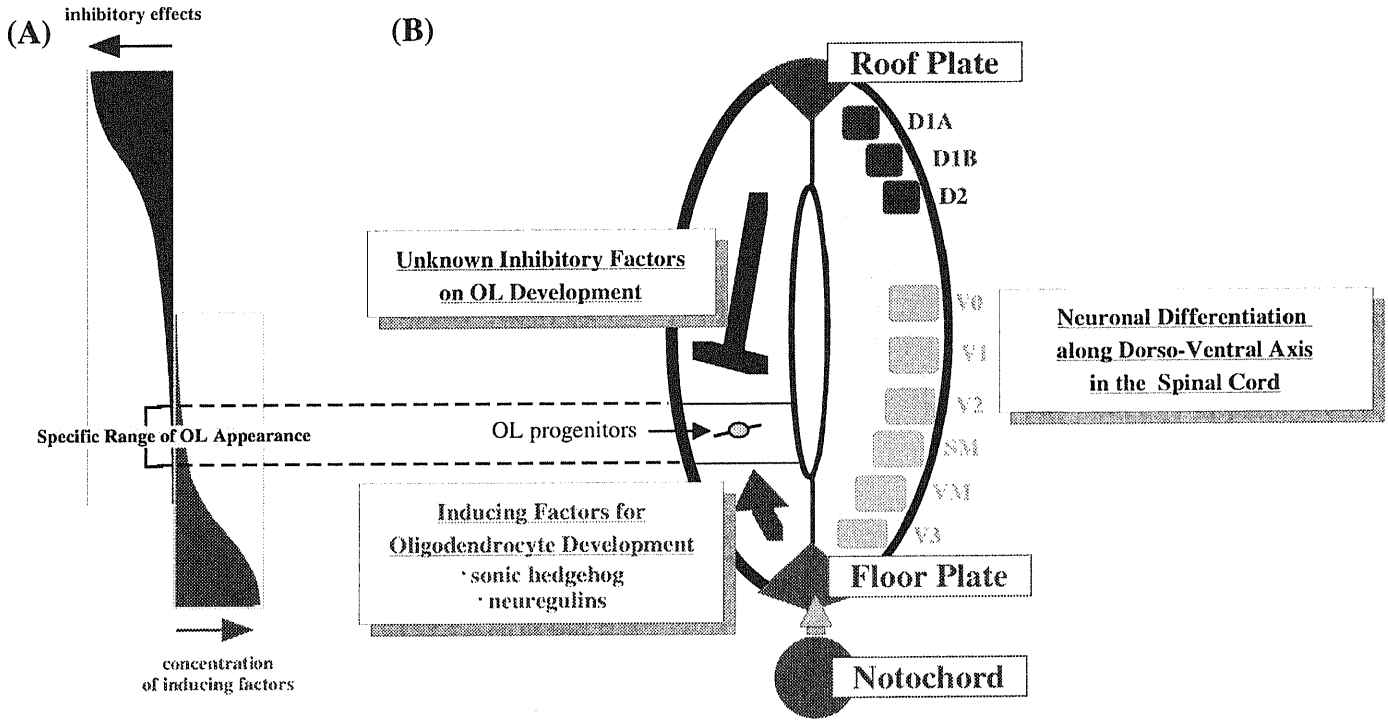

(A)
inhibitory effects
Specific Range of OL Appearance
concentration
of inducing factors
(B)
Roof Plate
D1A
D1B
D2
Unknown Inhibitory Factors
on OL Development
V0
V1
V2
MN
V3
OL progenitors
Neuronal Differentiation
along Dorso-Ventral Axis
in the Spinal Cord
Inducing Factors for
Oligodendrocyte Development
· sonic hedgehog
· neuregulins
Floor Plate
Notochord

Fig. 22 A new model for regulation mechanism of OL development. (A) Blue area indicates the effect of the inhibitory factors on OL development from dorsal spinal cord. It has stronger influence on the cells in the dorsal part, but has less effect to the ventral cells. Red area indicates the effect of the inducing factors for OL development from ventral region. As reported previously, Shh, a typical ventralizing factor and a inducer of OL development, acts at a distance and has different effects depending its concentration. The specific range of concentration between inhibitory factors and inducing factors may produce the restricted region of OL appearance. (B) Left side indicates that the ventral region specific appearance of OL progenitors in the ventral spinal cord (yellow area) may be finely restricted by the balance between inducing factors from the ventral spinal cord, such as Shh and neuregulins, and some inhibitory factors from the dorsal spinal cord. Thus, OL development in the cervical spinal cord may be controlled by the mechanism that generates neuronal diversity along dorso-ventral axis in the spinal cord (B, right). VM: visceral motoneurons; SM: somatic motoneurons; V0-3: ventral interneurons; D1A, 1B, 2: dorsal interneurons.

Table 1. Robust increase in the number of the cells positive for OL markers in the VM fragment. Fold increase of the cells positive for each marker in the VM fragment by that in the equivalent ventral region of control culture were calculated.

	Control ventral region	VM fragment	
PDGFRα$^+$ cells	1	15.5±0.13	(n=6)
O4$^+$ cells	1	3.8±0.20	(n=6)
O1$^+$ cells	1	9.0±.044	(n=6)
PLP$^+$ cells	1	9.8±0.30	(n=6)

Table 2. The change of the other neural cell proportions in the VM fragment. Cells from the VM fragment or ventral region of flat whole-mount preparation on E12+3 DIV were dissociated and plated on the poly-L-lysine coated dish. The cells were labeled with anti-PCNA antibody to visualize the proliferating cells, anti-Musashi to visualize neuroepithelial cells, anti-MAP2 antibody to visualize neurons, or anti-GFAP antibody to visualize astrocytes (see Experimental procedures). Ratio of the cells positive for each marker/ total cells was calculated (n=8). The $MAP2^+$ cells in the VM fragment showed a marked reduction in number. The $GFAP^+$ cells slightly increased in the VM fragment. $PCNA^+$ cells drastically increased in the VM fragment.

	Control ventral region	VM fragment	
$PCNA^+$ cells per total cells	20.2±7.7%	40.8±9.7%	(n=8)
$Musashi^+$ cells per total cells	16.6±6.6%	44.2±7.7%	(n=8)
$MAP2^+$ cells per total cells	57.7±8.2%	34.5±14.1%	(n=8)
$GFAP^+$ cells per total cells	3.3±1.3%	6.0±1.5%	(n=8)

Table 3. Effect of BMP4 to increase the number of astrocytes in the dissociation culture. Dissociated cells from E12 mice medulla and cervical spinal cord were plated on poly-D-lysine coated slide glasses and incubated for overnight at 37°C in a CO_2 incubator. On the next day of the dissection, the cells were treated without or with 10 or 100 ng/ml BMP4 for another 3 DIV. The cells were fixed with 4% PFA and labeled with anti-MAP2, anti-GFAP, or anti-PCNA antibodies. Ratio of the cells positive for each marker / total cells was calculated (n=2). Although the number of $GFAP^+$ cells was increased by BMP4, the number of $MAP2^+$ cell was not changed significantly. The number of $PCNA^+$ cells was decreased by the BMP4. No $O4^+$ cell was observed in the all culture conditions.

	0 ng/ml BMP4	10 ng/ml BMP4	100 ng/ml BMP4	
$MAP2^+$ cells per total cells	64.1%	69.4%	70.0%	(n=2)
$GFAP^+$ cells per total cells	1.1%	4.2%	9.8%	(n=2)
$PCNA^+$ cells per total cells	27.4%	9.3%	5.0%	(n=2)

Table 4. No effect of BMP4 on $O4^+$ cells in the flat whole-mount preparation. VM fragment was cultured in the chemical defined medium without or with 10 or 100 ng/ml BMP4. After 3 DIV, explants were stained with O4 antibody. No significant difference was observed by addition of 10 or 100 ng/ml BMP4.

	0 ng/ml BMP4	10 ng/ml BMP4	100 ng/ml BMP4	
$O4^+$ cell density (cells/cm^2)	67.8±19.4	72.3±16.2	62.2±17.8	(n=6)