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Genetically-defined lineage tracing of Nkx2.2-expressing cells in chick spinal cord

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ABSTRACT

In the spinal cord, generation of oligodendrocytes (OLs) is totally dependent on the presence of Olig2, a basic helix-loop-helix transcription factor. However, it also requires Nkx2.2 for its generation, whose expression follows the expression of Olig2. Although it is believed that oligodendrocytes originate from the pMN domain, Nkx2.2 is present in the p3 domain located ventral to the pMN domain. According to recent reports, it is possible that oligodendrocytes are directly derived from the p3 domain in addition to the pMN domain in the chick spinal cord. We examined this hypothesis in this paper.

To analyze OL development in the spinal cord, chick embryos are widely used for genetic modification by electroporation or for transplantation experiments, because it is relatively easy to manipulate them compared with mouse embryos. However, genetic modification by electroporation is not appropriate for glial development analyses because glia proliferate vigorously before maturation. In order to overcome these problems, we established a novel method to permanently introduce exogenous gene into a specific cell type. We introduced the CAT1 gene, a murine retroviral receptor, by electroporation followed by injection of murine retrovirus. By using this method, we successfully transduced murine retrovirus into the chick neural tube. We analyzed cell lineage from the p3 domain by restricting CAT1 expression by Nkx2.2-enhancer and found that most of the labeled cells became OLs when the cells were labeled at cE4. Moreover, the labeled OLs were found throughout the white matter in the spinal cord including the most dorsal spinal cord. Thus p3 domain directly generates spinal cord OLs in the chick spinal cord.

Introduction

In the developing neural tube, neurons and glial cells are generated from neural progenitor cells that are located in the ventricular area. These progenitor cells are divided into several 'domain structures' depending on the expression of transcription factors (Helm and Johnson, 2003; Tanabe and Jessell, 1996). It is known that these domain structures define the subtypes of neurons, such as motoneurons and many classes of interneurons. Recently, it was reported that astrocytes also have positional identity depending on the domain structure, and are subdivided into several subtypes (Hochstim et al., 2008). These observations indicate that progenitor cells are from a heterogenous population through the neurogenic to the gliogenic phase. Oligodendrocytes (OLs) develop from ventral spinal cord as well as more dorsal spinal cord (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). The majority of oligodendrocytes arise from ventral spinal cord and the Olig2 gene, which is present in the pMN domain, is indispensable for their generation (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Nkx2.2, a homeodomain transcription factor, defines the p3 domain that is located ventral to the pMN domain and is also required for the development of OLs in collaboration with Olig2 (Qi et al., 2001; Xu et al., 2000). Oligodendrocyte progenitor cells (OPCs) in the ventral spinal cord are thought to arise from the pMN domain, because of the lineage tracing analysis using Olig2-CreER mice (Masahira et al., 2006) or the absence of OLs in Olig2-deficient mice (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). However, it has been reported that the OPC marker, O4 or PDGFRα, is present in the Nkx2.2-expressing p3 domain, especially in chick spinal cord (Soula et al., 2001). Finally, it has been proposed that OPCs migrate out from the ventricular zone as Olig2$^+$/PDGFRα$^+$/Nkx2.2$^+$ cells from the p* domain and as Olig2$^-$/PDGFRα$^-$/Nkx2.2$^+$ cells from the p3 domain in the chick spinal cord. The latter population is considered to be Olig2$^+$ after arriving at the white matter (Fu et al., 2002). These observations suggest that the origin of OPCs in the ventral spinal cord might be

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heterogeneous and OLs can be generated from both the pMN and p3 domains. However, there is no direct evidence showing that p3-derived cells generate mature myelinating oligodendrocytes *in vivo*.

The chick-quail chimera system (Le Douarin, 1973), in which donor cells are directly injected into host organs, is used for lineage tracing analysis of relatively 'large areas' such as the dorsal or ventral areas. However, lineage tracing analysis in more restricted domain structures cannot be achieved by this method. Recently it became possible to introduce exogenous genes into chick embryos by electroporation (Nakamura and Funahashi, 2001). The expression of exogenous genes is transient, because introduced plasmids are diluted during cell proliferation. Therefore, it is inappropriate to analyze the glial cell lineage because they proliferate vigorously until their final maturation.

To overcome this problem, we designed a novel method by which murine retrovirus and their receptor were used in combination. The infectivity of murine retrovirus is dependent on the expression of cationic amino acid transporter 1 (mCAT1) at the plasma membrane of the cells (Albritton et al., 1989). Chick cells are thought to lack corresponding receptors, thus cannot be infected by murine retrovirus. If mCAT1 is exogenously expressed under the regulation of Nkx2.2-promoter or enhancer, murine retrovirus should transduce cells in the p3 domain that express mCAT1. We found that murine retrovirus transduced chick embryos depending on the exogenous expression of mCAT1. By using this method, we analyzed the cell lineage from Nkx2.2-expressing p3 domain at E4, a late stage of neurogenesis, and found that Nkx2.2-expressing cells in the p3 domain mainly differentiate into mature OLs in various regions of the spinal cord. Our observations presented *in vivo* evidence that cells that express Nkx2.2 are the source of mature myelinating OLs in chick spinal cord and that they distribute to all areas in the spinal cord.

Materials and methods

Vectors

A cDNA encoding the mCAT1 was amplified using *myc*-tagged reverse primers, resulting in C-terminal Myc-tagged mCAT1 and cloned into pCAG vector or p3T vector (MoBiTech, Germany). The enhancer region of Nkx2.2 (Lei et al., 2006) was amplified and cloned into pGL3-basic vector (Promega, USA). The luciferase sequence was replaced with mCAT1-*myc*, and β-globin minimal promoter was inserted between Nkx2.2 enhancer and mCAT1. pGAP-GFP that expresses membrane-targeted EGFP was used as previously described (Ono et al., 2004). A retroviral vector pLNRGW was obtained from Dr. Teoan Kim (Kwon et al., 2004), and vector encoding cPDGFRα was kindly provided by Dr. J-L Thomas (Spassky et al., 1998).

Animals and cell culture

Fertilized white leghorn eggs were obtained from the Ghen Corporation (Gifu, Japan) and incubated at 38 °C. Embryonic stages of chicks were determined according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). For BrdU injection, 25 mg/kg of BrdU was injected into the egg yolk. The DF-1 chicken fibroblast cell line and NIH3T3 mouse fibroblast cell line were maintained in DMEM containing 10% fetal bovine serum, streptomycin and penicillin. mCAT1-expressing plasmids were transfected into cultured cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

In situ hybridization, immunohistochemistry, and immunocytochemistry

The trunk region of early chick embryos was directly immersed into 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Spinal cords of late chick embryos were removed after perfusion with 4% paraformaldehyde in 0.1 M PB. After post fixation overnight with 4% paraformaldehyde/PB, the trunk or the spinal cord was immersed in 20% sucrose/PBS for 1 day. The trunk or the spinal cord was then frozen, and 20 μm sections were cut using a cryostat. *In situ* hybridization was performed as previously described (Ding et al., 2005). For immunocytochemical analysis, cells were seeded onto a glass coverslip. After transfection, coverslips were washed briefly with PBS, and incubated with 4% paraformaldehyde in 0.1 M PB for 20 min. The sections or the coverslips were incubated with phosphate buffered saline supplemented with 0.1% Triton X-100 (PBS-T), incubated with a blocking buffer composed of 1% BSA, PBS-T for 1 h and then incubated with primary antibody for 16 h at 4 °C. After washing the sections three times with PBS, the sections were processed with the ABC detection kit (Vector, USA) according to the manufacturer's protocol. The signals were visualized by peroxidase reaction with 3,3′-diaminobenzidine as the substrate. For fluorescent immunohistochemistry, brain sections were incubated with a primary antibody, followed with AlexaFluor 488-, AlexaFluor 543-, or AlexaFluor 633-labeled secondary antibody with DAPI, and observed using a fluorescence microscope (DP70 and DP71; Olympus, Japan) or confocal microscope (FV-1000; Olympus, Japan). The primary antibodies used in this study are as follows: anti-NeuN, anti-phosphohistone H3 (Millipore, USA), anti-GFAP (DAKO, USA), anti-GSTπ, anti-Myc (MBL, Japan), anti-MBP (Nichirei, Japan), rabbit polyclonal anti-GFP (Invitrogen, USA), rat monoclonal anti-GFP (Nacalai Tesque, Japan), anti-BrdU (BD Pharmingen, USA), and anti-cleaved caspase 3 (Cell Signaling Technology, USA) antibodies. Rabbit polyclonal antibody to GLAST was kindly provided by Dr. Masahiko Watanabe, monoclonal antibody to Nkx2.2 (clone 74.5A5) was obtained from Developmental Studies Hybridoma Bank (Univ. of Iowa, USA), and monoclonal anti-PLP antibody (clone AA3) was used as previously described (Yamada et al., 1999).

Preparation of EGFP-expressing high-titer retrovirus

Preparation of EGFP-expressing retrovirus producing cells or preparation of high-titer retrovirus was performed as previously described (Nanmoku et al., 2003). Briefly, the retrovirus producing cell line, ΨMP34, was transfected with pLNRGW and treated with G-418 (Nacalai Tesque, Japan) for 1 week. Stable clones were selected by a limited dilution method and stable clones that produced high-titer retrovirus were selected. The selected clones were cultured in 250 ml of DMEM supplemented with 10% FBS at 30 °C. The supernatant of virus-producing cells was concentrated 1/10,000 by two-rounds of centrifugation at 6000 *g* for 16 h at 4 °C and dissolved in Hanks' balanced salt solution (HBSS). For virus titration, NIH3T3 cells were seeded in 24-well plates at 1×10^4 cells/well together with serial dilutions of virus solution in the presence of 8 μg/ml of polybrene. The EGFP-positive cells were counted 48 h after infection, and the titer was estimated according to the following formula: titer of retrovirus (cfu/ml) = number of EGFP-positive cells/virus volume (ml) × 4 (replication factor of NIH3T3).

In ovo electroporation and virus injection

Plasmids indicated in each Figure were dissolved in distilled water at a concentration of 1.0 μg/μl for pCAG-mCAT1 or 500 ng/μl for pNkx2.2-mCAT1 and mixed with a 1/10 volume of 0.5% fast green. Approximately 0.1 μl of the mixed solution was injected into the neural tube of HH stage 17 to 19 chicken embryos (3 days after starting the incubation). Needle type electrodes were placed near the embryo, and a 30 V, 30 ms pulse was applied three times using an electronic stimulator (SEN-3310; Nihon Kohden, Japan). For the retroviral injection, approximately 0.1 μl of virus solution was injected into neural tube 24 h after electroporation unless otherwise indicated.