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Purkinje cells originate from cerebellar ventricular zone progenitors positive for Neph3 and E-cadherin

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ABSTRACT

GABAergic Purkinje cells (PCs) provide the primary output from the cerebellar cortex, which controls movement and posture. Although the mechanisms of PC differentiation have been well studied, the precise origin and initial specification mechanism of PCs remain to be clarified. Here, we identified a cerebellar and spinal cord GABAergic progenitor-selective cell surface marker, Neph3, which is a direct downstream target gene of Ptf1a, an essential regulator of GABAergic neuron development. Using FACS, Neph3$^+$ GABAergic progenitors were sorted from the embryonic cerebellum, and the cell fate of this population was mapped by culturing in vitro. We found that most of the Neph3$^+$ populations sorted from the mouse E12.5 cerebellum were fated to differentiate into PCs while the remaining small fraction of Neph3$^+$ cells were progenitors for Pax2$^+$ interneurons, which are likely to be deep cerebellar nuclei GABAergic neurons. These results were confirmed by short-term in vivo lineage-tracing experiments using transgenic mice expressing *Neph3* promoter-driven GFP. In addition, we identified E-cadherin as a marker selectively expressed by a dorsally localized subset of cerebellar Neph3$^+$ cells. Sorting experiments revealed that the Neph3$^+$ E-cadherin^{high} population in the embryonic cerebellum defined PC progenitors while progenitors for Pax2$^+$ interneurons were enriched in the Neph3$^+$ E-cadherin^{low} population. Taken together, our results identify two spatially demarcated subregions that generate distinct cerebellar GABAergic subtypes and reveal the origin of PCs in the ventricular zone of the cerebellar primordium.

Introduction

The cerebellum is composed of the cerebellar cortex, containing two glutamatergic neuronal subtypes (granule and unipolar brush cells) and six GABAergic subtypes (Purkinje, Golgi, Lugaro, globular, stellate and basket cells), and the deep cerebellar nuclei (DCN), containing two GABAergic subtypes and glutamatergic neurons (Carletti and Rossi, 2008; Hoshino, 2006; Leto et al., 2006; Simat et al., 2007; Wang and Zoghbi, 2001). Purkinje cells (PCs) provide the primary output from the cerebellar cortex to the DCN, which controls movement and posture, and loss of PCs causes severe cerebellar dysfunction (Sidman, 1983; Wang and Zoghbi, 2001; Taroni and DiDonato, 2004). Transplantation of PCs is one of the potential therapeutic approaches for cerebellar degenerative diseases.

Since the cerebellum has a simple structure that consists of a small number of cell types, the developmental mechanisms of the cerebellum have been extensively studied as a model system for progenitor expansion/differentiation, neuronal migration, network formation and tissue morphogenesis (Hatten and Heintz, 1995). Two major germinal centers, the external granular layer (EGL) and ventricular zone (VZ), are responsible for the generation of cerebellar neurons (Sotelo, 2004; Millen and Gleeson, 2008). Accumulating evidence has revealed that granule cells are produced by EGL progenitors that originate from the rhombic lip (RL) located at the dorsal-most VZ in the embryonic cerebellar primordium. A recent study revealed that glutamatergic DCN neurons and unipolar blush cells are also derived from the RL (Carletti and Rossi, 2008; Fink et al., 2006). Therefore, all the glutamatergic neurons in the cerebellum appear to originate from the RL.

The second germinal center, the VZ, has been thought to be responsible for cerebellar GABAergic neurons (Carletti and Rossi, 2008; Hoshino, 2006). A Cre-mediated genetic lineage-tracing approach revealed that all cerebellar GABAergic neurons are derived from progenitors expressing Ptf1a, which is required for their specification (Hoshino et al., 2005; Pascual et al., 2007). In addition,

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astrocytes are also derived from the VZ (Grimaldi et al., 2009). However, Ptf1a is expressed throughout the cerebellar GABAergic progenitor domains, which appear to be able to be divided into subdomains based on their gene expression profiles (Zordan et al., 2008). Furthermore, its expression in the progenitors persists during a broad range of developmental stages (Pascual et al., 2007), which is consistent with the fact that cerebellar GABAergic neurons are generated throughout the developmental stages (E11 to the early postnatal stage) (Carletti and Rossi, 2008; Hoshino, 2006). In addition, the long-range migration and absence of markers specific for the individual early GABAergic precursor subtypes make it difficult to determine the lineage relationships between progenitors and the mature GABAergic neuronal subtypes. Therefore, the spatiotemporal origins of the individual GABAergic subtypes remain to be clarified. Recent studies delineating the domain map of the developing cerebellum based on gene expression studies have suggested the progenitor domain for PCs and DCN GABAergic neurons (Chizhikov et al., 2006; Minaki et al., 2008; Zordan et al., 2008). However, their lineage relationships have not yet been directly examined by fate-mapping or lineage-tracing experiments.

In the present study, we identified a cell surface marker, Neph3, which is selectively expressed in Ptf1a^{+} GABAergic progenitors, and a PC progenitor-selective marker, E-cadherin. Fate-mapping experiments using cell sorting with these markers, together with short-term in vivo lineage-tracing experiments using transgenic mice expressing *Neph3* promoter-driven GFP, identified the origin of PCs as Neph3^{+} E-cadherin^{high} progenitors in the VZ of the embryonic cerebellum.

Materials and methods

Mice

pN3 was constructed by ligating the SV40 poly(A) signal and genomic fragments for the promoter of the *Neph3* gene into pSP73 (Promega). pN3-GFP was constructed by ligating a *GFP* cDNA into the *Kpn*I/*Not*I sites of pN3. The primers used for amplification of these fragments were as follows: *Neph3* promoter: 5′-GAG ATC GAT TAG GAG CCT ATG GTG GCA CTT GTG AG-3′/5′-GAG GGT ACC CCC GAG TGT GTG TAC CCC AAG ATC TC-3′; poly(A) signal: 5′-GAG AAG CTT TCT AGA GGG CCC GTT TAA ACC CGC TG-3′/5′-GAG CTC GAG TCT TTC CGC CTC AGA AGC CAT AGA GC-3′; *GFP* cDNA: 5′-GAG GGT ACC GCC ACC ATG GTG AGC AAG GGC GAG GAG CTG TTC-3′/5′-GAG GCG GCC GCT TAC TTG TAC AGC TCG TCC ATG CCG AG-3′. The linearized pN3-GFP construct was injected into fertilized eggs and founder embryos were collected at E12.5. The embryos were genotyped by PCR.

Cerebelless mutant embryos (Hoshino et al., 2005) were generously provided by Dr. Y. Nabeshima (Faculty of Medicine, Kyoto University). The embryos were genotyped by PCR using the following primers: *cbll*: 5′-TGG GAG TAG TCA GGA GAG GA-3′/5′-AGC CCC TTG AGC ATC TGA CT; wild-type: 5′-ATT TAT GGC CGT CCG TGA TTC TT-3′/5′-TCC CCC TTC CTT TTT CCT ATT CA-3′.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as described previously (Nakatani et al., 2004). Briefly, mouse embryos were harvested at E12.5 and immersed in 4% paraformaldehyde (PFA) in phosphate-buffered saline at 4 °C for 2 h. After fixation, the embryos were cryoprotected in 20% sucrose, embedded in OCT compound and cut into 14-μm sections. The sections were washed three times with 0.1% Triton X-100 in PBS (1× PBS-T) at room temperature for 5 min and blocked in 25% Block Ace (Dainippon-Seiyaku) at room temperature for 30 min. The sections were incubated with the primary antibodies overnight at 4 °C. After three washes with 1× PBS-T, the sections were incubated with secondary antibodies at room temperature for 1 h. After three washes with 1× PBS-T, the sections were rinsed with PBS and mounted in mounting medium. The primary antibodies used were: hamster monoclonal anti-Neph3 (1:100; Minaki et al., 2005); rabbit polyclonal anti-Corl2 (1:500; Minaki et al., 2008); hamster monoclonal anti-Ptf1a (1:10; Minaki et al., 2008); guinea pig monoclonal anti-Lbx1 (1:20000; a generous gift from Dr. T. Muller, KAN Research Institute Inc.); mouse monoclonal anti-Lhx1/5 (1:10; Developmental Studies Hybridoma Bank); rabbit polyclonal anti-Pax2 (1:500; Covance); mouse monoclonal anti-HuC/D (1:500; Molecular Probes); mouse monoclonal anti-Nestin (1:100) and mouse monoclonal anti-Brn3a (1:100) (Chemicon); goat polyclonal anti-Lhx1 (1:250) and goat polyclonal anti-RORα (1:100) (Santa Cruz Biotechnology); rat monoclonal anti-GFP (1:100; Nacalai Tesque); mouse monoclonal anti-Gad65 (1:250) and mouse monoclonal anti-Mash1 (1:500) (BD PharMingen); goat polyclonal anti-calbindin (1:500; Sigma); and rat monoclonal anti-E-cadherin (1:100; TaKaRa).

In situ hybridization was performed as described previously (Nakatani et al., 2004). *Neph3* and *GFP* cDNAs were amplified by PCR using the following primers: *Neph3*: 5′-CGG AGA GAA TTG TGT GCA GAG AGA GG-3′/5′-CTG AGT GTA CAC CAA CAG TCC TGA TG-3′; *GFP*: 5′-GAG GCG GCC GCG CCA CCA TGG TGA GCA AGG GCG AGG AGC TGT TC-3′/5′-GAG GGT ACC TTA CTT GTA CAG CTC GTC CAT GCC GAG-3′. The amplified PCR fragments were cloned into pCRII (Invitrogen) and used as templates for the transcription of DIG-labeled probes.

Cell sorting and culture

The cerebellum, myelencephalon and spinal cord were dissected from E12.5 mouse embryos and dissociated using Accumax (Innovative Cell Technologies Inc.). Cell suspensions were stained with hamster monoclonal anti-Neph3 (1:100), rat monoclonal anti-E-cadherin (1:50) or anti-PSA-NCAM (1:300; Millipore) primary antibodies and then labeled with PE- or APC-labeled secondary antibodies (eBioscience). Cell sorting was performed using a FACS Aria (BD Bioscience). Sorted cells were plated on glass chambers coated with poly-L-ornithine, laminin and fibronectin and cultured in DMEM/F12 supplemented with N2 (Invitrogen), B27 (Invitrogen) and 20 ng/ml BDNF (R&D Systems). Cells were fixed with 2% paraformaldehyde and immunostained as described previously (Nakatani et al., 2004).

Retroviruses expressing *GFP*, *Ptf1a-IRES-GFP* or *Ptf1aW298A-IRES-GFP* were prepared from 293E cells using a RetroMax Retroviral System (Imgenex). E12.5 mouse dorsal mesencephalic cells were plated on glass chambers coated with poly-L-ornithine, laminin and fibronectin and cultured for 30 min. The cells were then infected with a retrovirus using ViroMag R/L 100 (OZ Bioscience) and cultured in DMEM/F12 supplemented with N2, B27, 2 ng/ml bFGF (R&D Systems) and 20 ng/ml BDNF. After 1 day of culture, the cells were dissociated and stained with the anti-Neph3 monoclonal antibody.

For BrdU incorporation experiments, the cerebella were dissected from E12.5 mouse embryos and dissociated using Accumax. Cells were plated on glass chambers coated with poly-L-ornithine, laminin and fibronectin and cultured in DMEM/F12 supplemented with N2, B27, 20 ng/ml BDNF and 10 μg/ml BrdU. After 2 h of culture, the cells were fixed with 2% paraformaldehyde and immunostained with hamster monoclonal anti-Neph3 (1:100) and anti-BrdU (1:300; Abcam) antibodies.

Reporter assay

Reporter assays were performed as described previously (Nakatani et al., 2004). Briefly, 293E cells were transfected with 0.2 μg of luciferase reporter plasmid and 0.4 μg of expression vector together with 0.005 μg of pRL-SV40 (Promega) using the TransIT LT1 reagent (Mirus Corporation). After 48 h, cell lysates were prepared and assayed using a dual-luciferase reporter assay system (Promega). Luciferase activity was normalized by the *Renilla* luciferase activity.