Developmental Biology 338 (2010) 202–214

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

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

Eri Mizuhara [a,1], Yasuko Minaki [a], Tomoya Nakatani [a], Minoru Kumai [b], Takeshi Inoue [a,2], Keiko Muguruma [c], Yoshiki Sasai [c], Yuichi Ono [a,b,*,1]

[a] Group for Neuronal Differentiation, KAN Research Institute Inc., Kobe, Hyogo 650-0047, Japan
[b] Group for Transgenic Technology, KAN Research Institute Inc., Kobe, Hyogo 650-0047, Japan
[c] Organogenesis and Neurogenesis Group, Center for Developmental Biology, RIKEN, Kobe, Hyogo 650-0047, Japan

ARTICLE INFO

Article history:
Received for publication 30 July 2009
Revised 12 November 2009
Accepted 30 November 2009
Available online 11 December 2009

Keywords:
Neph3
E-cadherin
Ptf1a
Purkinje cell
Origin
Neuronal subtype
Cell sorting
Cell surface marker
Fate mapping

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,

* Corresponding author. KAN Research Institute Inc., Kobe MI R&D Center, 6-7-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan. Fax: +81 78 306 5920.
E-mail address: y-ono@kan.eisai.co.jp (Y. Ono).
[1] These authors contributed equally to this work.
[2] Present address: Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan.

0012-1606/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2009.11.032

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.

Neph3 promoter reporters were constructed by ligating the genomic fragments amplified using the following primers into the *Kpn*I/*Xho*I sites of pGL2-basic (Promega): −2628-luc: 5′-GAG GGT ACC CAC CAG CAG CTT GTT TGT TGC TGT CT-3′/5-GAG CTC GAG CCC GAG TGT GTG TAC CCC AAG ATC TC-3′; −740-luc: 5′-GAG GGT ACC AGG ACT CAG CAC AGC TGT TCA GTC TG-3′/5-GAG CTC GAG CCC GAG TGT GTG TAC CCC AAG ATC TC-3′; −380-luc: 5′-GAG GGT ACC ACT GCG TGG GAA CGC CCA GCT GGG CT-3′/5-GAG CTC GAG CCC GAG TGT GTG TAC CCC AAG ATC TC-3′; −358-luc: 5′-GAG GGT ACC GGC TGC ACC GGA GCT GTC AGG ACA AG-3′/5-GAG CTC GAG CCC GAG TGT GTG TAC CCC AAG ATC TC-3′. E1/2M-luc and TC1/2M-luc were constructed by PCR-mediated mutagenesis of two E-boxes and TC-boxes, respectively.

ChIP assay

The E11.5 mouse cerebellum was dissected, crosslinked in 1% formaldehyde at room temperature for 15 min and then lysed on ice in 0.5% NP40, 25 mM HEPES (pH 7.6), 50 mM KCl and 10 mM EDTA for 60 min. The nuclear pellets were sonicated in 1% SDS, 10 mM Tris–HCl (pH 8.0), 100 mM NaCl and 1 mM EDTA, and diluted 1:10 with 1.1% Triton X-100, 10 mM Tris–HCl (pH 8.0), 250 mM NaCl and 1 mM EDTA. The length of the sonicated genomic DNA ranged from 300 to 900 bp. Crosslinked protein/DNA complexes were immunoprecipitated with hamster anti-Ptf1a or hamster anti-Lmx1b (control) antibodies using protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). The immunoprecipitated DNA was purified with a QIAQuick PCR purification kit (Qiagen) and quantified by qPCR with Power CYBRGreen PCR Master Mix (ABI). The data are shown as the fold enrichment of genomic fragments immunoprecipitated by the anti-Ptf1a antibody relative to that immunoprecipitated by the control antibody. The primer sequences were as follows: *Neph3* F1/R1: 5′-CTC ACA GCT CTC TAC CAA GG-3′/5′-AGG TGT TGA TAG GTT TGG AG-3′; *Neph3* F2/R2: 5′-AGG TCC AGG AAT GTC AGA CC-3′/5′-GGA ACC GCA CAG CTT GTC CT-3′; *Foxa2*: 5′-AAC AAG ATG CTG ACG CTG AG-3′/5′-AAG CAG TCG TTG AAG GAG AG-3′.

Subtractive PCR and RT-PCR

Total RNA was prepared from Neph3$^+$ cells sorted from the E12.5 cerebellum, myelencephalon and spinal cord using an RNeasy mini kit (Qiagen). Subtractive PCR was performed as described previously (Osada et al., 2005) using the cerebellar Neph3$^+$ cells as a tester and spinal cord Neph3$^+$ cells as a driver. RT-PCR was performed using cDNA amplicons as templates. PCR amplification was carried out by denaturation at 94 °C for 30 s (2 min in the first cycle), annealing at 65 °C for 30 s and extension at 72 °C for 30 s (2 min in the last cycle) with *ExTaq* polymerase (TaKaRa). The number of cycles was 26. The primer sequences were as follows: *Neph3*: 5′-CTT CCC GTA TGC TAC CTT GTC TCC AC-3′/5′-CCA ACA GTC CTG CAT GCT TGT AAT GA-3′; *E-cadherin*: 5′-CTC CAA TGC CTG CTC TTG ATG GTA GC-3′/5′-TCT CTG TGT AGC CCT GGC TGT CCT AG-3′.

Differentiation of ES cells

Differentiation of ES cells into spinal cord neurons was performed essentially as described previously (Watanabe et al., 2005). Briefly, dissociated ES cells (5×10^4 cells/ml) in GMEM supplemented with 5% KSR, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.1 M nonessential amino acids and 1 mM pyruvate were seeded into bacterial-grade dishes. During differentiation days 3–10, 2 μM all-*trans* retinoic acid (Sigma) was added. On day 10, cell aggregates were fixed with 4% paraformaldehyde, cryoprotected in 20% sucrose, cryosectioned and immunostained. For FACS, cell aggregates were dissociated with Accumax on day 7 and stained with the anti-Neph3 monoclonal antibody.

Results

Neph3 is selectively expressed in precursors for dILA GABAergic interneurons

Previously, we reported that a cell adhesion molecule, Neph3 (also known as Kirrel2), is selectively expressed in some developing CNS regions including the spinal cord and cerebellum (Minaki et al., 2005). Since Neph3 is a cell adhesion molecule expressed on the cell surface (Minaki et al., 2005), we reasoned that Neph3 can be used as a marker for cell sorting, which enables us to perform fate-mapping experiments to determine the spatiotemporal origin of the cerebellar neurons. To this end, we first tested the accuracy of this approach using the spinal cord system, in which Neph3 is selectively expressed in a subset of nascent postmitotic precursors in the dorsal VZ (Minaki et al., 2005).

At E12.5, Neph3 expression was restricted to the dorsal part of the VZ (Figs. 1Aa, b), where two neuronal subtypes, dILA and dILB, emerge (Muller et al., 2002), suggesting that Neph3$^+$ early postmitotic precursors differentiate into these neurons. However, since these two subtypes are intermingled within one domain, it remained possible that Neph3 marks precursors for either dILA or dILB, or both. To determine the cell lineage of Neph3$^+$ precursors, we performed fate-mapping experiments using cell sorting and culture. Cell surface expression of Neph3 was detected by FACS analysis in E12.5 spinal cord cells, and the Neph3$^+$ population was successfully sorted with more than 99% purity (Fig. 1B). As expected from the Neph3 expression pattern, the vast majority of the sorted Neph3$^+$ cells expressed the VZ cell marker Nestin (Fig. 1Ca), and about 65% of the Neph3$^+$ cells expressed Sox2 (Fig. 1Cb). Most of the Sox2$^-$ Nestin$^+$ cells expressed the postmitotic neuron marker HuC/D at a low level (Figs. 1Cc, d). These findings may reflect the duration of Neph3 protein expression in differentiating cells and are consistent with the previous proposal that Neph3 is selectively expressed in nascent postmitotic precursors in the VZ (Minaki et al., 2005).

When the sorted spinal Neph3$^+$ cells were cultured for 2 days in vitro (DIV), 96% of the cells differentiated into Nestin$^-$ HuC/D$^+$ postmitotic neurons (Figs. 1Da, b) and 94% of these neurons were positive for the dIL interneuron marker Lbx1 (Muller et al., 2002) (Fig. 1Dc). Importantly, most of the Lbx1$^+$ neurons expressed the dILA markers Pax2 and Gad65 (Figs. 1Dd, e), while only less than 5% expressed the dILB marker Brn3a (Fig. 1Df), indicating that the majority of the Neph3$^+$ cells sorted from the E12.5 spinal cord differentiated into dILA GABAergic neurons. Under the same culture conditions, Neph3$^-$ NCAM$^-$ progenitors generated many Lbx1$^+$ Brn3a$^+$ dILB neurons (Supplementary materials), thereby excluding the unlikely possibility that the culture conditions caused preferential differentiation into a dILA fate. Therefore, Neph3 appears to be selectively expressed by nascent postmitotic precursors for dILA in the spinal cord.

A previous report demonstrated that the dILA neuronal fate is determined in the nascent postmitotic precursor state by the bHLH transcription factor Ptf1a, which is selectively expressed by this neuronal lineage (Glasgow et al., 2005). In the E12.5 spinal cord, *Neph3* mRNA expression highly resembled Ptf1a expression (Figs. 1Aa, c), and virtually all the sorted Neph3$^+$ cells positive for Nestin expressed Ptf1a (Fig. 1Ce), demonstrating that Ptf1a$^+$ dILA precursors can be sorted by FACS as Neph3$^+$ populations, and that Neph3 and Ptf1a are coexpressed in the dILA precursors. Taken together, our fate-mapping experiments based on cell sorting were highly consistent with previous in vivo lineage-tracing data using *Ptf1a-Cre* (Glasgow et al., 2005), thereby demonstrating the accuracy of our fate-mapping method.

At an early developmental stage (E10.5), Neph3 expression was restricted to the dI4 precursor domain, where Ptf1a was selectively expressed (Supplementary materials).

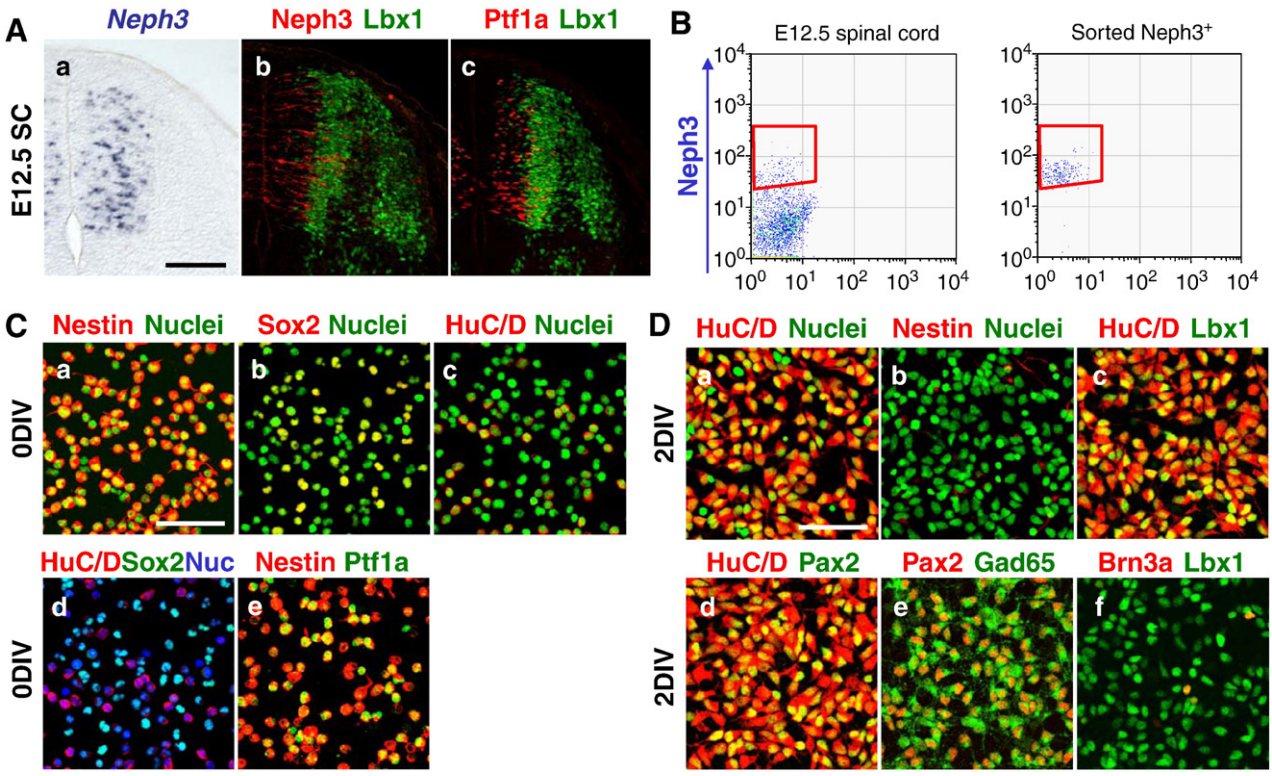


Fig. 1. Spinal cord Neph3$^+$ cells differentiate into dILA neurons. (A) Neph3 is selectively expressed by a subpopulation of VZ cells in the spinal dIL domain. Images show the dorsal spinal cord region at E12.5. (B) FACS analysis of E12.5 spinal cord cells. Neph3$^+$ cells can be sorted with more than 99% purity. (C) Spinal Neph3$^+$ cells exhibit immature neural precursor characteristics. Virtually all the sorted Neph3$^+$ cells express Nestin and Ptf1a. About 35% of the Neph3$^+$ cells express HuC/D at a low level. (D) Spinal Neph3$^+$ cells differentiate into dILA GABAergic interneurons in vitro. Most of the sorted Neph3$^+$ cells differentiate into Lbx1$^+$ Pax2$^+$ dILA interneurons at 2 DIV. Bars: A, 100 μm; C and D, 50 μm.

Neph3 is a direct downstream target gene of Ptf1a

The similarities in the expression patterns of *Neph3* and Ptf1a in the developing spinal cord led us to compare the expressions of these genes in other CNS regions. In E12.5 embryos, high levels of Neph3 expression were detected in the cerebellar primordium, dorsal myelencephalon, ventral diencephalon and retina (Fig. 2A), all of which were reported to be Ptf1a$^+$ regions (Fujitani et al., 2006; Glasgow et al., 2005; Hoshino et al., 2005; Nakhai et al., 2007), and the expression patterns of these genes were highly similar in all regions, suggesting the existence of a regulatory link between Neph3 and Ptf1a. Since Ptf1a is a transcriptional regulator, we first examined whether *Ptf1a* is required for Neph3 expression. In the *cerebelless* mutant embryos, which carry a deletion mutation within the putative enhancer region of the *Ptf1a* gene and lose *Ptf1a* expression in the cerebellum (Hoshino et al., 2005), *Neph3* expression was abolished in the cerebellum (Fig. 2B), indicating that Ptf1a is necessary for *Neph3* expression, at least in this region. Next, we examined the ability of Ptf1a to induce Neph3 expression using an ectopic expression approach. When *Ptf1a* was retrovirally transduced into dorsal mesencephalic cells that did not normally express Neph3, surface Neph3 expression was induced in a cell-autonomous manner (Fig. 2C). Taken together, these results demonstrate that *Neph3* is a downstream target gene of Ptf1a.

To further understand the mechanism of the Neph3 induction by Ptf1a, we cloned a genomic fragment of the *Neph3* gene containing 3.3 kb upstream to the initiation codon and analyzed the promoter activity. Transgenic mice were generated by injecting a construct containing the promoter fragment linked to a *GFP* cDNA (N3-GFP, Fig. 3A). Reporter gene expression recapitulated the *Neph3* gene expression in all CNS regions examined, although not all Neph3$^+$ progenitors expressed the GFP reporter in all regions, and weak leaky expression of the reporter was observed in the RL regions ($n=4$) (Fig. 3C and data not shown). These results demonstrate that the 3.3-kb promoter fragment was nearly sufficient for the control of *Neph3* expression.

To examine whether the 3.3-kb *Neph3* promoter region is responsible for the activation by Ptf1a, we performed luciferase assays using the 293E cell line and observed that Ptf1a activated the reporter by 10-fold (Fig. 3D). Similar results were obtained using the NS20Y neuroblastoma cell line (data not shown). To identify the Ptf1a-responsive elements, we created a series of deletion constructs and found that the sequences between −740 and −381 and between −380 and −359 were responsible for the activation by Ptf1a (Fig. 3D). Comparisons with the corresponding genomic sequences from several mammalian species identified a conserved E-box element in each Ptf1a-responsive region (E1 at −359 and E2 at −724) (Fig. 3B). Mutations disrupting both E1 and E2 (CAGCTG to *GT*CGTG) significantly reduced the response to Ptf1a (Fig. 3D), suggesting that Ptf1a activates the *Neph3* promoter through these two conserved E-boxes.

A previous report revealed that Ptf1a activates transcription and determines the GABAergic fate by physically interacting with RBP-J family transcription factors (Hori et al., 2008). Interestingly, TC-box elements, which can be recognized by RBP-Js, were identified near the conserved E-boxes in the *Neph3* promoter and were both highly conserved (Fig. 3B). Mutations in these TC-boxes (TTTCC to TTGAC) again reduced the response to Ptf1a (Fig. 3D), suggesting that Ptf1a cooperates with RBP-J factors to activate the *Neph3* promoter, similar to the case for pancreatic exocrine cell gene activation (Beres et al., 2006). Consistently, we observed that a Ptf1a W298A mutant, which is unable to associate with RBP-Jk and consequently fails to induce the GABAergic fate (Beres et al., 2006; Hori et al., 2008), was unable to induce endogenous Neph3 expression (Fig. 2C) or efficiently activate the *Neph3* reporter (Fig. 3D). Therefore, Ptf1a appears to cooperate

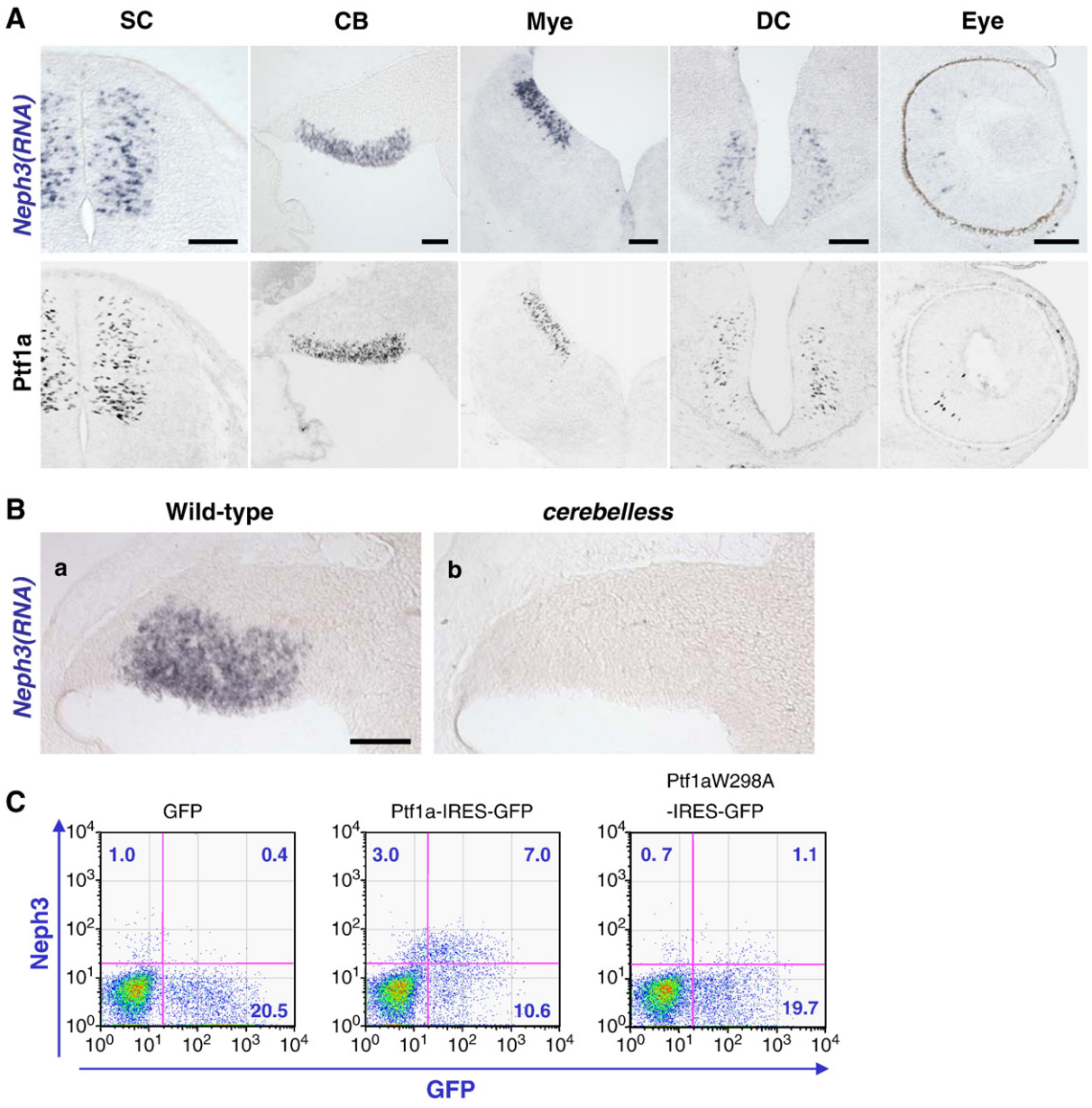


Fig. 2. Ptf1a can induce *Neph3* expression. (A) *Neph3* is selectively expressed in CNS regions positive for Ptf1a. The images show serial sections of the dorsal spinal cord (SC), cerebellum (CB), myelencephalon (Mye), diencephalon (DC) and eye regions at E12.5 stained with an anti-Ptf1a antibody or hybridized with a *Neph3* antisense riboprobe. (B) *Neph3* expression is abolished by loss of *Ptf1a*. The images show sections of the cerebellum of wild-type (a) and *cerebelless* mutant (b) embryos at E12.5 hybridized with a *Neph3* antisense riboprobe. (C) Neph3 expression is induced by exogenous Ptf1a. Dorsal mesencephalic cells were transduced with *GFP*- or *Ptf1a-IRES-GFP*-expressing retroviruses and cultured for 1 DIV. Cell surface Neph3 expression is induced by wild-type, but not by W298A mutant, Ptf1a. Bars: 100 μm.

with RBP-Js to activate the *Neph3* promoter through two conserved sets of E-boxes and TC-boxes.

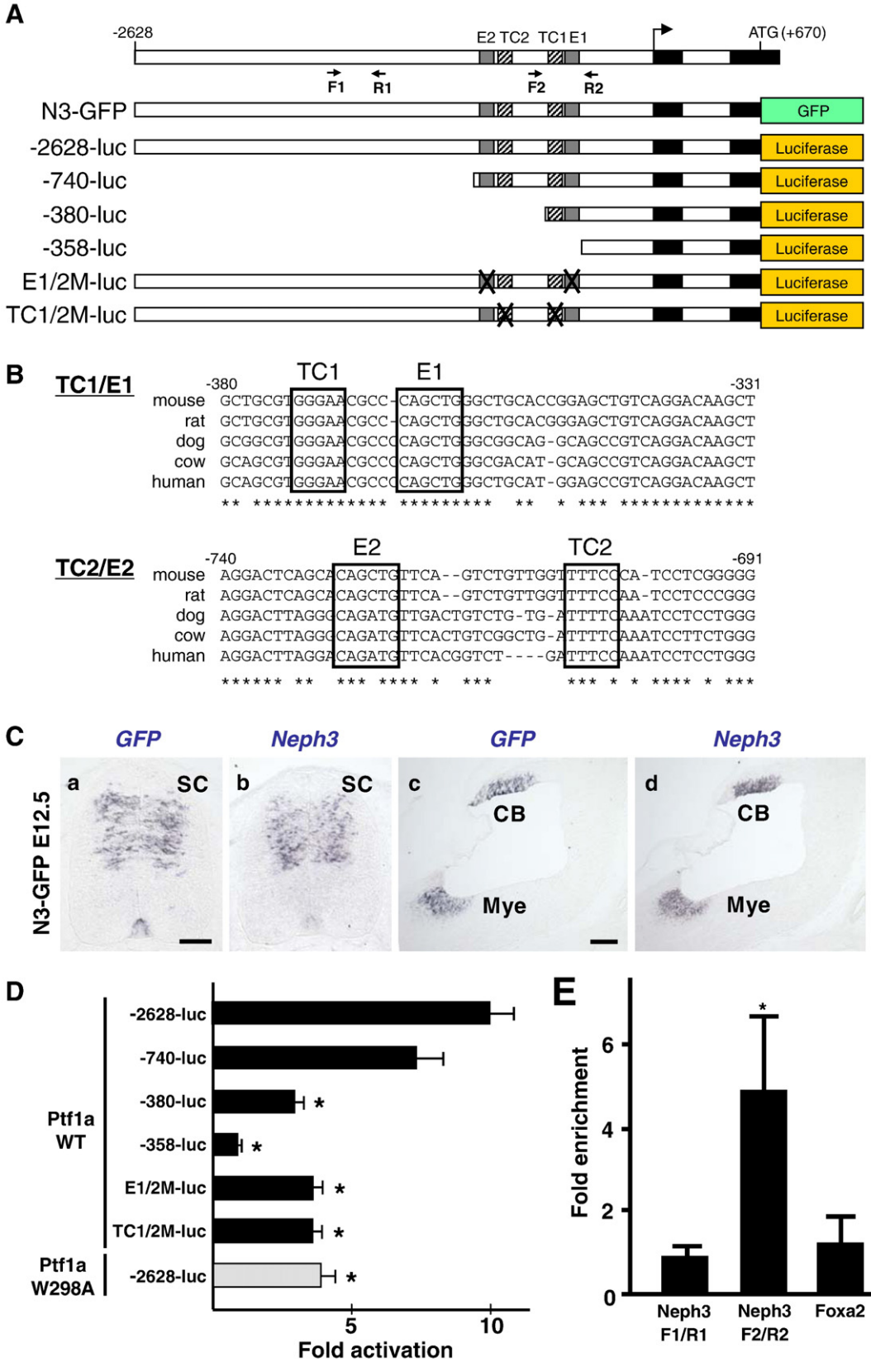
Finally, to determine whether Ptf1a directly binds to the *Neph3* promoter in vivo, ChIP experiments were performed. The *Neph3* promoter region around E1 and TC1 was efficiently immunoprecipitated with the anti-Ptf1a antibody whereas a fragment from further upstream was not precipitated compared with an unrelated genomic region (*Foxa2*) (Fig. 3E), thereby demonstrating that Ptf1a directly

Fig. 3. *Neph3* is a direct downstream target gene of Ptf1a. (A) Schematic representation of the *Neph3* promoter constructs used for transgenic mice generation and luciferase assays. E1/E2 and TC1/TC2 represent conserved E-boxes and TC-boxes, respectively, whose sequences are shown in (B). (B) E-boxes and TC-boxes are conserved within the *Neph3* promoter among mammalian species. Sequence alignments reveal conserved E-boxes and TC-boxes within the promoter region of the *Neph3* gene. (C) *GFP* reporter gene expression recapitulates the *Neph3* gene expression in N3-GFP transgenic embryos. Images show in situ hybridization analyses with *GFP* (a, c) and *Neph3* (b, d) antisense riboprobes using serial sections of the spinal cord (a, b) and cerebellum (CB)/myelencephalon (Mye) regions (c, d) of an N3-GFP embryo at E12.5. Bars: Ca, 100 μm; Cc, 200 μm. (D) Ptf1a activates the *Neph3* promoter through the conserved E-boxes and TC-boxes. 293E cells were transfected with a reporter plasmid and the indicated Ptf1a expression vectors. The results show the fold activations of the luciferase activity with the Ptf1a expression vector relative to that with the empty vector. The data are the means ± SD of four independent experiments. *$P<0.001$. (E) Ptf1a directly binds to the *Neph3* promoter in vivo. The E11.5 cerebellar primordium was crosslinked and subjected to ChIP analysis using anti-Ptf1a and anti-Lmx1b (control) antibodies. The positions of the primers used for qPCR are indicated in panel A. A *Foxa2* genomic fragment was used as an unrelated control. Data are shown as the fold enrichment of genomic fragments immunoprecipitated by the anti-Ptf1a antibody relative to that immunoprecipitated by the control antibody. The genomic fragment encompassing the E-box and TC-box is enriched while the 800-bp upstream region is not enriched compared with the control *Foxa2* fragment. *$P<0.01$, vs. the fold activation of −2628-luc by wild-type Ptf1a.

binds to the *Neph3* promoter region. Taken together, our results demonstrate that *Neph3* is a direct downstream target gene of Ptf1a in the developing CNS and suggest that Neph3 can be used as a cell surface marker for sorting Ptf1a^{+} progenitors in all CNS regions including the cerebellum.

Identification of the origin of PCs

Next, we examined the spatiotemporal expression pattern of Neph3 in the developing cerebellum. At E12.5, when PCs and DCN GABAergic interneurons emerge (Leto et al., 2006; Miale and Sidman,



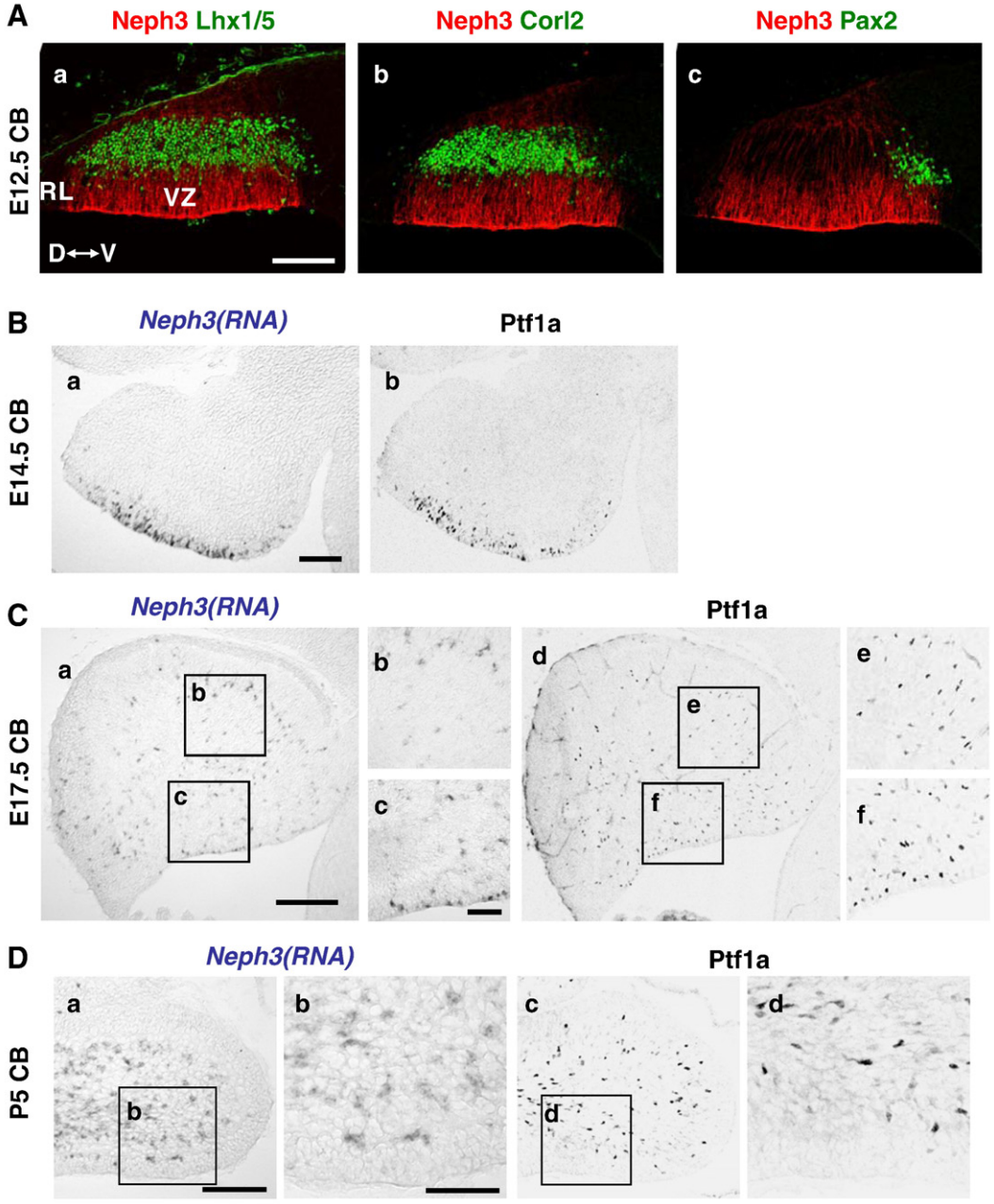


Fig. 4. Neph3 is selectively expressed in GABAergic progenitors in the developing cerebellum. Images show the cerebellum (CB) at E12.5 (A), E14.5 (B), E17.5 (C) and p5 (D). At E12.5, *Neph3* is selectively expressed in the VZ cells of the GABAergic domain in the developing cerebellum (A). The VZ-specific expression of *Neph3* is maintained at E14.5 (B). At E17.5, in addition to the expression in the VZ (Cb), scattered signals for *Neph3* are observed throughout the cerebellum (Cc). At P5, *Neph3* expression is observed in the white matter regions where progenitors for GABAergic interneurons, such as basket and stellate cells, are known to exist (D). It should be noted that the pattern of *Neph3* expression is highly similar to that of Ptf1a at all stages. Bars: A and B, 100 μm; Ca, 200 μm; Cc, 50 μm; Da, 100 μm; Db, 50 μm;.

1961; Sekerkova et al., 2004a,b), Neph3 was selectively expressed in the VZ neighboring the mantle layer (ML) containing Lhx1$^+$ GABAergic neurons (Chizhikov et al., 2006; Minaki et al., 2008) (Figs. 4Aa–c). At E14.5, when PC generation declines and cortical GABAergic interneurons start to be generated instead, *Neph3* expression was selectively detected in the VZ (Fig. 4Ba), similar to the observations at E12.5. At E17.5, *Neph3* expression in the VZ persisted (Figs. 4Ca, b), and in addition to the VZ signals, scattered *Neph3* signals were detected throughout the cerebellum (Fig. 4Cc). At P5, *Neph3* expression was observed in the white matter region where progenitors for basket and stellate cells are known to exist (Zhang and Goldman, 1996) (Figs. 4Da, b). Importantly, at all the stages examined, *Neph3* and Ptf1a showed highly similar patterns in the developing cerebellum (Figs. 2A and 4B–D). Taken together with the transcriptional link between Ptf1a and *Neph3*, these observations suggest that *Neph3* marks all the GABAergic progenitors in the cerebellum. In the present study, we focused on clarifying the origin of PCs and examined the fate of E12.5 Neph3$^+$ cells based on the birthdate of PCs.

At E12.5, in the Lhx1$^+$ domain adjacent to the Neph3$^+$ VZ, two separate neuronal populations were observed, namely Corl2$^+$ PC precursors in the dorsal part of the domain and Pax2$^+$ probable DCN GABAergic precursors in the ventral-most portion of the domain (Figs. 4Ab, c), consistent with a previous report (Minaki et al., 2008).

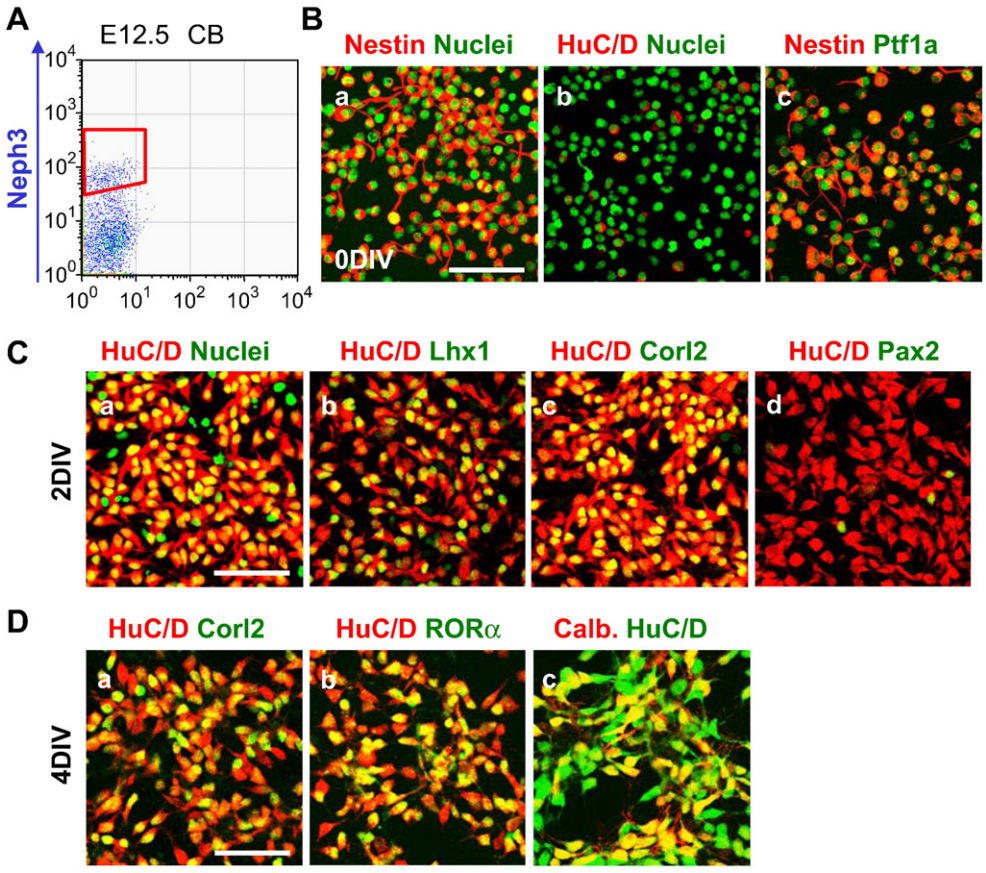


Fig. 5. Cerebellar Neph3$^+$ cells differentiate into PCs. (A) FACS analysis of E12.5 cerebellar cells. Neph3$^+$ cells can be sorted with 97% purity. (B) Cerebellar Neph3$^+$ cells exhibit neural progenitor characteristics. Virtually all the sorted Neph3$^+$ cells express Nestin and Ptf1a. About 20% of the Neph3$^+$ cells express HuC/D at a low level. (C, D) Cerebellar Neph3$^+$ cells differentiate into PCs in vitro. (C) Most of the sorted Neph3$^+$ cells differentiate into Lhx1$^+$ GABAergic neurons at 2 DIV. More than 90% of the neurons express the PC marker Corl2 and 7% express the GABAergic interneuron marker Pax2. (D) More than 82% and 69% of the neurons derived from the sorted Neph3$^+$ cells express the mature PC markers RORα and calbindin (Calb.), respectively. Bars: 50 μm.

As expected, cell surface expression of Neph3 was detected in the cerebellar precursor cells (Fig. 5A) and these Neph3$^+$ cells were successfully sorted.

In contrast to the spinal cord Neph3$^+$ cells (Minaki et al., 2005), cerebellar Neph3$^+$ cells appeared to be proliferative progenitors because cerebellar Neph3$^+$ cells efficiently incorporated BrdU both in vitro and in vivo (Supplementary materials and data not shown). Consistently, the vast majority of the sorted cerebellar Neph3$^+$ cells were positive for Nestin (Fig. 5Ba) and contained some HuC/D^{low} nascent neurons (Fig. 5Bb). Furthermore, most of the sorted cells retained Nestin expression after 24 h of culture in vitro in contrast to the case for sorted spinal Neph3$^+$ cells (data not shown). As expected, most of the cerebellar Neph3$^+$ cells expressed Ptf1a (Fig. 5Bc).

To determine the lineage of the cerebellar Neph3$^+$ cells, we performed fate-mapping experiments by culturing the sorted cells. At 2 DIV, most of the sorted cells differentiated into HuC/D$^+$ postmitotic neurons (Fig. 5Ca). The vast majority of these neurons expressed Lhx1 and Gad1 (Fig. 5Cb and data not shown), indicating that the sorted Neph3$^+$ cells were progenitors for cerebellar GABAergic neurons. These findings are consistent with previous in vivo lineage-tracing data indicating that cerebellar GABAergic neurons originate from Ptf1a$^+$ progenitors (Hoshino et al., 2005). However, previous lineage-tracing experiments could not determine the spatiotemporal distributions of the progenitors for the different GABAergic subtypes. To address this issue, we examined which subtypes of GABAergic neurons were generated from the Neph3$^+$ progenitors sorted from the E12.5 cerebellum. Pax2, which marks most cerebellar GABAergic interneurons (Maricich and Herrup, 1999; Weisheit et al., 2006), was expressed in only about 7% of the neurons generated from the sorted Neph3$^+$ progenitors in vitro (Fig. 5Cd). These Pax2$^+$ neurons are likely to be DCN GABAergic interneurons as judged from their birthdate (Leto et al., 2006; Miale and Sidman, 1961; Sekerkova et al., 2004a,b), although we could not definitively determine their identity because of the lack of markers that can distinguish among the early precursor subtypes for GABAergic interneurons. In contrast, more than 90% of the neurons derived from the Neph3$^+$ progenitors expressed the early PC precursor marker Corl2 (Fig. 5Cc). Consistently, at 4 DIV, the majority of the neurons expressed the early PC marker RORα (Fig. 5Db) and 69% of the neurons expressed the more mature PC marker calbindin (Fig. 5Dc). Under the same culture conditions, Neph3$^-$ NCAM$^-$ progenitors generated many neurons without Corl2 expression, thereby excluding the unlikely possibility that the culture conditions caused preferential differentiation into a PC fate (Supplementary materials). Therefore, most of the sorted Neph3$^+$ cells appeared to generate PCs. Taken together with the expression pattern of Neph3 in vivo and previously reported data from birthdating experiments (Miale and Sidman, 1961), our results suggest that Neph3$^+$ progenitors in the E12.5 cerebellum generate PCs.

To further confirm the origin of PCs, we performed in vivo lineage-tracing experiments using N3-GFP transgenic mice, in which the duration of GFP protein expression enabled us to trace the cell lineage in the short term. As expected, GFP expression was detected in some postmitotic neurons near the VZ (Fig. 6). Virtually all the GFP$^+$ neurons, which could be considered to be derived from Neph3$^+$ progenitors, expressed Lhx1 (Fig. 6A), and importantly, expression of Corl2 was observed in the GFP$^+$ Lhx1$^+$ neurons (Fig. 6B), supporting our conclusion from the above-described in vitro fate-mapping

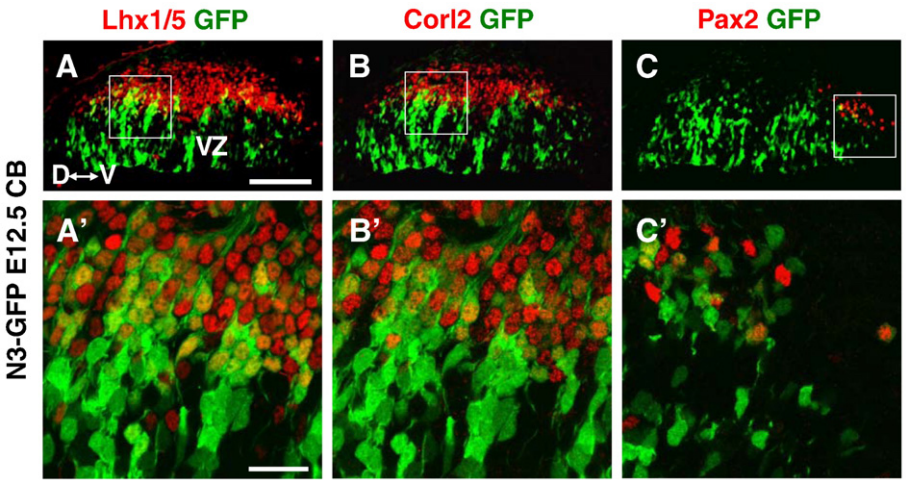


Fig. 6. Short-term lineage-tracing of cerebellar Neph3$^+$ cells in vivo. (A and B) Section of the cerebellum of an E12.5 N3-GFP transgenic embryo was stained with anti-Lhx1, anti-Corl2 and anti-GFP antibodies. The three-channel confocal image was split and recombined as red-green images. (C) The serial section was stained with anti-Pax2 and anti-GFP antibodies. In N3-GFP embryos, GFP expression is retained in some postmitotic neurons near the VZ. The coexpression of GFP with Lhx1, Corl2 and Pax2 confirms the results of the in vitro FACS-mediated fate-mapping experiments shown in Fig. 4. Bars: A, 100 μm; A′, 20 μm.

experiments. In addition, and consistent with the sorting experiment data, Pax2$^+$ probable DCN GABAergic neurons were generated from Neph3$^+$ progenitors located at the ventral-most part of the Neph3$^+$ domain (Fig. 6C).

Taken together, our observations suggest that PCs originate from dorsal Neph3$^+$ progenitors in the VZ of the developing cerebellum (see Fig. 8C).

Purkinje progenitor cells originate from Neph3$^+$ E-cadherin^{high} progenitors in the developing cerebellum

The above-described results identified Neph3$^+$ progenitors in the E12.5 cerebellum as the origin of PCs. However, since Neph3 is also expressed by progenitors other than the PC lineage, the precise spatial localization of PC progenitors within the Neph3$^+$ domain could not be determined by these FACS-mediated fate-mapping experiments. To identify a cell surface marker that distinguishes PC progenitors from other GABAergic progenitors, we searched for PC progenitor-selective genes by subtractive PCR by comparing the gene expression profile of the cerebellar Neph3$^+$ population with that of the myelencephalic Neph3$^+$ population, which did not generate PCs (Fig. 7A) as previously reported (Yamada et al., 2007).

Among several genes identified, we focused on E-cadherin because of its cell surface expression. E-cadherin was selectively expressed in Neph3$^+$ cells sorted from the cerebellum, but not in myelencephalic and spinal Neph3$^+$ populations, at both the transcript and protein levels (Figs. 7B, C) as previously reported (Shimamura and Takeichi, 1992). Importantly, E-cadherin expression was not uniform in the cerebellar Neph3$^+$ region at E12.5. Dorsally located progenitors probably generating PCs expressed E-cadherin at a high level, whereas its level of expression in ventral progenitors located near Pax2$^+$ neurons was low or below the limit of detection (Figs. 7Ca, b). These findings were further confirmed by FACS analysis because the majority of Neph3$^+$ cells in the E12.5 cerebellum expressed a high level of E-cadherin while a small subset of Neph3$^+$ cells expressed a low level of E-cadherin (Fig. 8A). At E14.5, when PC generation is completed and only GABAergic interneurons, such as Golgi cells, emerge, E-cadherin expression in the Neph3$^+$ VZ declined to a level below the detection limit (Fig. 7Ce). To examine the cell lineage of these Neph3$^+$ E-cadherin^{high} and Neph3$^+$ E-cadherin^{low} populations in the E12.5 cerebellum, we sorted and cultured these populations. At 2 DIV, most of the cells in both populations differentiated into postmitotic neurons (Fig. 8B). The vast majority of Neph3$^+$ E-cadherin^{high} cell-derived neurons expressed Corl2 (Fig. 8Ba) and only 6% of the neurons expressed Pax2 (Fig. 8Bc). In contrast, approximately 30% of neurons derived from the Neph3$^+$ E-cadherin^{low} population expressed Pax2 (Fig. 8Bd), suggesting that DCN GABAergic progenitors were enriched in the Neph3$^+$ E-cadherin^{low} fraction. Therefore, E-cadherin is selectively expressed at a high level in the PC progenitors among the Neph3$^+$ cerebellar GABAergic progenitors.

Taken together, our results identify two dorsoventrally demarcated subregions within the Neph3$^+$ GABAergic progenitor domain in the E12.5 cerebellum, namely a dorsal c2d region consisting of E-cadherin^{high} progenitors that generate PCs and a ventral c2v region consisting of E-cadherin^{low} progenitors that generate probable DCN GABAergic interneurons (Fig. 8C). Therefore, the cell fates for PCs and DCN GABAergic neurons appear to be determined at the proliferative progenitor state at distinct locations, which is consistent with previously reported observations from in vivo clonal analyses (Mathis and Nicolas, 2003) and gene expression data (Zordan et al., 2008).

Discussion

In the present study, we identified a cell surface marker, Neph3, as a direct downstream target gene of Ptf1a, thereby enabling us to sort GABAergic progenitor cells. This provided us with a tool for fate-mapping experiments to directly examine the origin of GABAergic neurons. The resulting data, together with data from GFP reporter-mediated short-term in vivo lineage-tracing experiments, answered a longstanding question in the field of cerebellar development regarding the origin of PCs.

The origin of Purkinje cells

In the developing cerebellum, two germinal centers, VZ and EGL, are responsible for constructing the mature cerebellum structure by generating all the required neuronal components (Sotelo, 2004; Millen and Gleeson, 2008). Gene expression, in vitro culture and genetic lineage-tracing studies have revealed that glutamatergic granule cells, which are the major components of the cerebellum, are generated from EGL progenitors, and that the EGL progenitors themselves originate from the dorsal-most VZ, termed the RL, which also generates other glutamatergic neurons in the cerebellum (Sotelo, 2004; Millen and Gleeson, 2008). In contrast, cerebellar GABAergic neurons have long been thought to be derived from VZ progenitors in

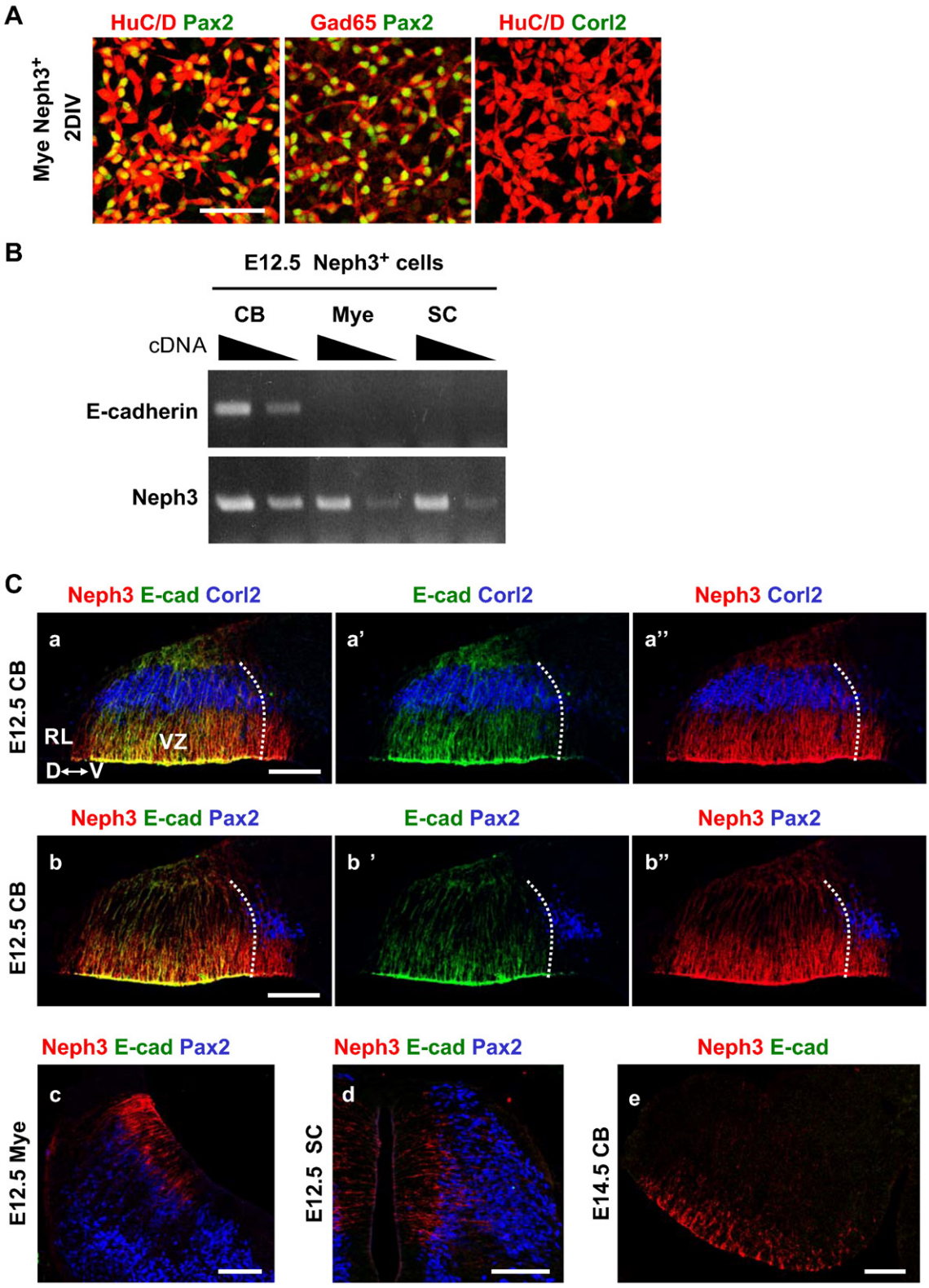


Fig. 7. E-cadherin is selectively expressed in cerebellar Neph3$^+$ progenitors. (A) Myelencephalic Neph3$^+$ cells do not differentiate into PCs in vitro. Sorted myelencephalic Neph3$^+$ cells differentiate into Pax2$^+$ Gad65$^+$ neurons but not into PCs. (B) *E-cadherin* is selectively expressed in cerebellar Neph3$^+$ cells. The results of RT-PCR analyses of *E-cadherin* and *Neph3* expression in Neph3$^+$ populations sorted from the E12.5 cerebellum (CB), myelencephalon (Mye) and spinal cord (SC) are shown. (C) E-cadherin is selectively expressed in the VZ cells in the dorsal part of the Neph3$^+$ GABAergic domain of the developing cerebellum. Images show the cerebellum (CB, a and b), myelencephalon (Mye, c) and spinal cord (SC, d) at E12.5 and the cerebellum at E14.5 (e). a′, a″, b′, b″ are two-color images of a and b. Bars: A, 50 μm; C, 100 μm.

the cerebellar plate, and a Cre-mediated genetic lineage-tracing study confirmed this theory (Hoshino et al., 2005). However, the complex birthdates of cerebellar GABAergic neuronal subtypes and different clonal relationships among these subtypes indicate that the individual GABAergic subtypes originate from spatiotemporally distinct VZ progenitor populations (Miale and Sidman, 1961; Mathis and Nicolas,

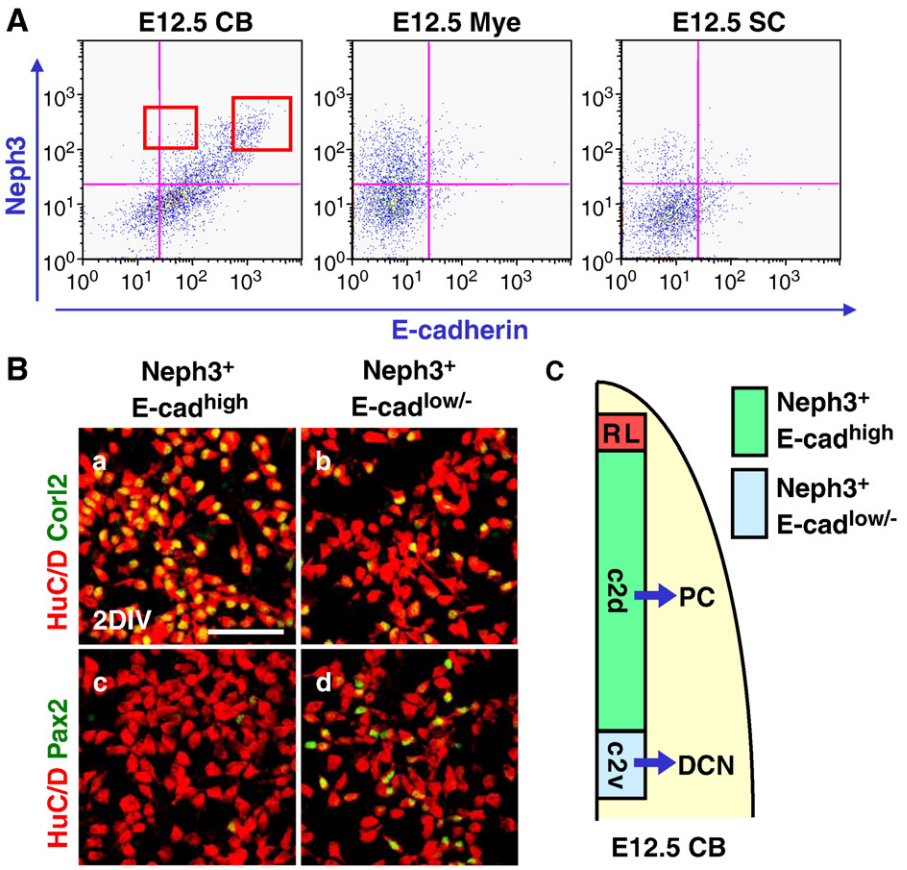


Fig. 8. PCs originate from Neph3$^+$ E-cadherin^{high} cells in the VZ of the developing cerebellum. (A) FACS analysis of E12.5 cerebellar (CB), myelencephalic (Mye) and spinal cord (SC) cells. E-cadherin expression is selectively detected in the cerebellar cells. Note that most of the cerebellar Neph3$^+$ cells express E-cadherin at a high level while some Neph3$^+$ cells show a low level or no expression of E-cadherin. (B) Sorted Neph3$^+$ E-cadherin^{high} progenitors differentiate into PCs in vitro. Virtually all the neurons derived from Neph3$^+$ E-cadherin^{high} cells express Corl2. In contrast, more than 50% of neurons differentiated from Neph3$^+$ E-cadherin$^{low/-}$ cells express Pax2. (C) Schematic model of the domain map in the cerebellar primordium at E12.5. PCs are generated from Neph3$^+$ E-cadherin^{high} progenitors in the c2d domain, while Pax2$^+$ GABAergic neurons, which are likely to be DCN neurons, are generated from Neph3$^+$ E-cadherin$^{low/-}$ progenitors in the ventral c2v domain. Bars: 50 μm.

2003), thereby making it difficult to determine the precise origins of these subtypes by genetic approaches. Our FACS-mediated fate-mapping approach is useful for both spatial and temporal identification of these origins. We took advantage of this FACS-mediated approach, together with staining for the recently identified early PC precursor marker Corl2 (Minaki et al., 2008), to spatiotemporally determine the origin of PCs and revealed that most of the Neph3$^+$ cerebellar VZ progenitors at E12.5 have the potency to generate PCs in vitro. One concern regarding this approach is the requirement for in vitro culture, during which the sorted progenitor cells may lose their progenitor-subtype identity. Indeed, previous observations have suggested that initially generated neurons may secrete signaling molecules that affect the temporal identity of the remaining progenitors (Barnabe-Heider et al., 2005; Kim et al., 2005). However, our data are highly consistent with the birthdating data, and our conclusion is further supported by the *Neph3* promoter-driven GFP-mediated short-term lineage-tracing data. Furthermore, the present FACS-mediated fate-mapping data for dILA progenitors in the spinal cord are completely consistent with previously reported genetic in vivo lineage-tracing data (Glasgow et al., 2005), and a similar FACS-mediated approach was used to reveal the origin of mesencephalic dopaminergic neurons, which was confirmed by genetic in vivo lineage-tracing data (Kittappa et al., 2007; Ono et al., 2007). Therefore, under these culture conditions, the sorted progenitor cells appeared to maintain their identity during the first 2 days of culture, representing the period required for differentiation into postmitotic neurons. In addition, and consistent with previous *Ptf1a-Cre*-mediated lineage-tracing data (Hoshino et al., 2005) and birthdating experiments (Leto et al., 2006; Miale and Sidman, 1961; Sekerkova et al., 2004a,b), Pax2$^+$ probable DCN GABAergic neurons were generated from the E12.5 Neph3$^+$ progenitors. Two distinct GABAergic neuron subtypes, namely SMI32$^+$ projection neurons and SMI32$^-$ interneurons, have been identified in the DCN (Leto et al., 2006). Since SMI32$^+$ neurons in the DCN are not derived from Ptf1a$^+$ progenitors (Hoshino et al., 2005), Pax2$^+$ GABAergic neurons derived from E12.5 Neph3$^+$ progenitors appear to be SMI32$^-$ DCN GABAergic interneurons. This population could be separated by FACS using another surface marker, E-cadherin. These observations are consistent with previous in vivo clonal analysis data showing that PCs and DCN GABAergic neurons are derived from distinct populations (Mathis and Nicolas, 2003). Taken together, our FACS-mediated fate-mapping approach and the in vivo expression patterns of the markers used for FACS reveal that PCs originate from the c2d domain in the cerebellar VZ at around E12.5 (Fig. 8C).

In most cases of neuronal development, neuronal subtype identity is determined at the proliferative progenitor state by inductive signals and downstream transcription factors (Jessell, 2000; Helms and Johnson, 2003). However, although Ptf1a has been identified to determine the GABAergic phenotype in cerebellar neurons, the mechanism for cerebellar GABAergic subtype specification has not yet been unmasked owing to the absence of identification of cell fate determinants for GABAergic subtypes. The present cell sorting data suggest that the PC and DCN GABAergic fates are determined at the Neph3$^+$ proliferative progenitor state,

and that, at least for these subtypes, dorsoventral patterning signals appear to be involved in their specification. Factors selectively expressed in each progenitor subpopulation may be involved in the specification. Our cell sorting technology may lead to the provision of a useful cell source for identifying the genes controlling PC specification/differentiation. Indeed, we have isolated several candidates by comparing the gene expression profiles among sorted Neph3$^+$ progenitor subpopulations (unpublished data). Future analyses of these genes will unmask the specification mechanism for cerebellar GABAergic subtypes.

Neph3 is a direct downstream target gene for Ptf1a

Using several experimental approaches, including gene expression, loss-of-function, gain-of-function, transgenic reporter, luciferase assay and ChIP studies, we have shown that *Neph3* is a direct downstream target gene for Ptf1a, although a requirement of Ptf1a for induction of *Neph3* has not yet been proven. This direct relationship appears to cause coincident expression of these genes in several developing brain regions and enables us to efficiently and accurately sort Ptf1a$^+$ GABAergic progenitors using Neph3 as a cell surface marker.

Several direct target genes for Ptf1a have been identified in the pancreatic lineage and analyses of their promoters have unmasked a characteristic feature of Ptf1a, namely that it requires RBP-J family members as cofactors for activating transcription (Beres et al., 2006). In vivo analyses using mice expressing a mutant Ptf1a that cannot associate with RBP-Jk revealed that the interaction between Ptf1a and RBP-Jk is essential not only for early pancreatic development but also for GABAergic differentiation (Masui et al., 2007; Hori et al., 2008). Our identification of the *Neph3* gene as a neuronal target gene for Ptf1a as well as the identification of its responsible promoter may represent useful tools for analyzing the mechanism underlying the transcriptional regulation by Ptf1a. Importantly, our promoter analyses have demonstrated that, similar to the case for *Neurogenin2*, a recently identified downstream target gene for Ptf1a in the CNS (Henke et al., 2009), and for pancreatic gene regulation, an association between Ptf1a and RBP-Jk is required for *Neph3* induction. Future detailed analyses will identify the common and/or specific mechanisms of transcriptional regulation in pancreatic and neuronal Ptf1a targets.

Possible stem cell-based regeneration therapy for cerebellar degeneration diseases

PCs are one of the major neuronal subtypes that degenerate in some cerebellar degenerative diseases. Purification of desired neuronal subtypes will be required to realize stem cell-based regenerative medicine, and trials are ongoing toward sorting of specific subtypes of neurons, such as dopaminergic neurons for the treatment of Parkinson's disease patients (Ono et al., 2007). Our cell sorting technology may provide a useful cell source for stem cell-based transplantation therapies for cerebellar neurodegenerative diseases. However, although cerebellar granule cells can be efficiently induced, a method for efficiently inducing PCs from ES cells has not yet been developed (Su et al., 2006). Since spinal cord neurons can be induced by the SFEB method with addition of all-*trans* retinoic acid (Watanabe et al., 2005), we preliminarily examined whether Neph3 can be used to isolate ES cell-derived GABAergic neurons (Supplementary materials). As expected from the finding that Neph3 is a direct target gene of Ptf1a, which is an essential regulator of GABAergic specification, the results showed that dILA precursors were successfully sorted, similar to the case for the embryonic spinal cord. Future studies on the development of an efficient method for inducing PCs from ES cells are required to test the usefulness of our cell sorting technology using Neph3 and E-cadherin for realizing stem cell-based regenerative therapies for cerebellar neurodegenerative diseases.

Acknowledgments

We are grateful to Dr. T. Imai (KAN Research Institute Inc.) for helpful comments and encouragement. We thank Dr. Y. Nabeshima (Faculty of Medicine, Kyoto University) for the *cerebelless* mutant embryos. We also thank Dr. T. Muller (Max-Delbruck-Centrum for Molecular Medicine) for the anti-Lbx1 antibody. The anti-Lhx1/5 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained at the Department of Biological Sciences, The University of Iowa, Iowa City, IA 52242.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.11.032.

References

Barnabe-Heider, F., Wasylnka, J.A., Fernandes, K.J., Porsche, C., Sendtner, M., Kaplan, D.R., Miller, F.D., 2005. Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. Neuron 48, 253–265.

Beres, T.M., Masui, T., Swift, G.H., Shi, L., Henke, R.M., MacDonald, R.J., 2006. PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. Mol. Cell. Biol. 26, 117–130.

Carletti, B., Rossi, F., 2008. Neurogenesis in the cerebellum. Neuroscientist 14, 91–100.

Chizhikov, V.V., Lindgren, A.G., Currle, D.S., Rose, M.F., Monuki, E.S., Millen, K.J., 2006. The roof plate regulates cerebellar cell-type specification and proliferation. Development 133, 2793–2804.

Fink, A.J., Englund, C., Daza, R.A., Pham, D., Lau, C., Nivison, M., Kowalczyk, T., Hevner, R.F., 2006. Development of the deep cerebellar nuclei: transcription factors and cell migration from the rhombic lip. J. Neurosci. 26, 3066–3076.

Fujitani, Y., Fujitani, S., Luo, H., Qiu, F., Burlison, J., Long, Q., Kawaguchi, Y., Edlund, H., MacDonald, R.J., Furukawa, T., Fujikado, T., Magnuson, M.A., Xiang, M., Wright, C.V., 2006. Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. Development 133, 4439–4450.

Glasgow, S.M., Henke, R.M., Macdonald, R.J., Wright, C.V., Johnson, J.E., 2005. Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. Development 132, 5461–5469.

Grimaldi, P., Parras, C., Guillemot, F., Rossi, F., Wassef, M., 2009. Origins and control of the differentiation of inhibitory interneurons and glia in the cerebellum. Dev. Biol. 328, 422–433.

Hatten, M.E., Heintz, N., 1995. Mechanisms of neural patterning and specification in the developing cerebellum. Annu. Rev. Neurosci. 18, 385–408.

Helms, A.W., Johnson, J.E., 2003. Specification of dorsal spinal cord interneurons. Curr. Opin. Neurobiol. 13, 42–49.

Henke, R.M., Savage, T.K., Meredith, D.M., Glasgow, S.M., Hori, K., Dumas, J., Macdonald, R.J., Johnson, J.E., 2009. Neurog2 is a direct downstream target of the Ptf1a-Rbpj transcription complex in dorsal spinal cord. Development 136, 2945–2954.

Hori, K., Cholewa-Waclaw, J., Nakada, Y., Glasgow, S.M., Masui, T., Henke, R.M., Wildner, H., Martarelli, B., Beres, T.M., Epstein, J.A., Magnuson, M.A., Macdonald, R.J., Birchmeier, C., Johnson, J.E., 2008. A nonclassical bHLH Rbpj transcription factor complex is required for specification of GABAergic neurons independent of Notch signaling. Genes Dev. 22, 166–178.

Hoshino, M., 2006. Molecular machinery governing GABAergic neuron specification in the cerebellum. Cerebellum 5, 193–198.

Hoshino, M., Nakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y.V., Fukuda, A., Fuse, T., Matsuo, N., Sone, M., Watanabe, M., Bito, H., Terashima, T., Wright, C.V., Kawaguchi, Y., Nakao, K., Nabeshima, Y., 2005. Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. Neuron 47, 201–213.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev., Genet. 1, 20–29.

Kim, J., Wu, H.H., Lander, A.D., Lyons, K.M., Matzuk, M.M., Calof, A.L., 2005. GDF11 controls the timing of progenitor cell competence in developing retina. Science 308, 1927–1930.

Kittappa, R., Chang, W.W., Awatramani, R.B., McKay, R.D., 2007. The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age. PLoS Biol. e325, 5.

Leto, K., Carletti, B., Williams, I.M., Magrassi, L., Rossi, F., 2006. Different types of cerebellar GABAergic interneurons originate from a common pool of multipotent progenitor cells. J. Neurosci. 26, 11682–11694.

Maricich, S.M., Herrup, K., 1999. Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. J. Neurobiol. 41, 281–294.

Masui, T., Long, Q., Beres, T.M., Magnuson, M.A., MacDonald, R.J., 2007. Early pancreatic development requires the vertebrate Suppressor of Hairless (RBPJ) in the PTF1 bHLH complex. Genes Dev. 21, 2629–2643.

Mathis, L., Nicolas, J.F., 2003. Progressive restriction of cell fates in relation to neuroepithelial cell mingling in the mouse cerebellum. Dev. Biol. 258, 20–31.

Miale, I.L., Sidman, R.L., 1961. An autoradiographic analysis of histogenesis in the mouse cerebellum. Exp. Neurol. 4, 277–296.
Millen, K.J., Gleeson, J.G., 2008. Cerebellar development and disease. Curr. Opin. Neurobiol. 18, 12–19.
Minaki, Y., Mizuhara, E., Morimoto, K., Nakatani, T., Sakamoto, Y., Inoue, Y., Satoh, K., Imai, T., Takai, Y., Ono, Y., 2005. Migrating postmitotic neural precursor cells in the ventricular zone extend apical processes and form adherens junctions near the ventricle in the developing spinal cord. Neurosci. Res. 52, 250–262.
Minaki, Y., Nakatani, T., Mizuhara, E., Inoue, T., Ono, Y., 2008. Identification of a novel transcriptional corepressor, Corl2, as a cerebellar Purkinje cell-selective marker. Gene Expr. Patterns 8, 418–423.
Muller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., Birchmeier, C., 2002. The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34, 551–562.
Nakatani, T., Mizuhara, E., Minaki, Y., Sakamoto, Y., Ono, Y., 2004. Helt, a novel basic-helix-loop-helix transcriptional repressor expressed in the developing central nervous system. J. Biol. Chem. 279, 16356–16367.
Nakhai, H., Sel, S., Favor, J., Mendoza-Torres, L., Paulsen, F., Duncker, G.I., Schmid, R.M., 2007. Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina. Development 134, 1151–1160.
Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H., Takahashi, J., Imai, T., 2007. Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. Development 134, 3213–3225.
Osada, A., Kiyozumi, D., Tsutsui, K., Ono, Y., Weber, C.N., Sugimoto, N., Imai, T., Okada, A., Sekiguchi, K., 2005. Expression of MAEG, a novel basement membrane protein, in mouse hair follicle morphogenesis. Exp. Cell Res. 303, 148–159.
Pascual, M., Abasolo, I., Mingorance-Le Meur, A., Martinez, A., Del Rio, J.A., Wright, C.V., Real, F.X., Soriano, E., 2007. Cerebellar GABAergic progenitors adopt an external granule cell-like phenotype in the absence of Ptf1a transcription factor expression. Proc. Natl. Acad. Sci. U. S. A. 104, 5193–5198.
Sekerkova, G., Ilijic, E., Mugnaini, E., 2004a. Bromodeoxyuridine administered during neurogenesis of the projection neurons causes cerebellar defects in rat. J. Comp. Neurol. 470, 221–239.
Sekerkova, G., Ilijic, E., Mugnaini, E., 2004b. Time of origin of unipolar brush cells in the rat cerebellum as observed by prenatal bromodeoxyuridine labeling. Neuroscience 127, 845–858.
Shimamura, K., Takeichi, M., 1992. Local and transient expression of E-cadherin involved in mouse embryonic brain morphogenesis. Development 116, 1011–1019.
Sidman, R.L., 1983. Experimental neurogenetics. Res. Publ.—Assoc. Res. Nerv. Ment. Dis. 60, 19–46.
Simat, M., Parpan, F., Fritschy, J.M., 2007. Heterogeneity of glycinergic and gabaergic interneurons in the granule cell layer of mouse cerebellum. J. Comp. Neurol. 500, 71–83.
Sotelo, C., 2004. Cellular and genetic regulation of the development of the cerebellar system. Prog. Neurobiol. 72, 295–339.
Su, H.L., Muguruma, K., Matsuo-Takasaki, M., Kengaku, M., Watanabe, K., Sasai, Y., 2006. Generation of cerebellar neuron precursors from embryonic stem cells. Dev. Biol. 290, 287–296.
Taroni, F., DiDonato, S., 2004. Pathways to motor incoordination: the inherited ataxias. Nat. Rev., Neurosci. 5, 641–655.
Wang, V.Y., Zoghbi, H.Y., 2001. Genetic regulation of cerebellar development. Nat. Rev., Neurosci. 2, 484–491.
Watanabe, K., Kamiya, D., Nishiyama, A., Katayama, T., Nozaki, S., Kawasaki, H., Watanabe, Y., Mizuseki, K., Sasai, Y., 2005. Directed differentiation of telencephalic precursors from embryonic stem cells. Nat. Neurosci. 8, 288–296.
Weisheit, G., Gliem, M., Endl, E., Pfeffer, P.L., Busslinger, M., Schilling, K., 2006. Postnatal development of the murine cerebellar cortex: formation and early dispersal of basket, stellate and Golgi neurons. Eur. J. Neurosci. 24, 466–478.
Yamada, M., Terao, M., Terashima, T., Fujiyama, T., Kawaguchi, Y., Nabeshima, Y., Hoshino, M., 2007. Origin of climbing fiber neurons and their developmental dependence on Ptf1a. J. Neurosci. 27, 10924–10934.
Zhang, L., Goldman, J.E., 1996. Generation of cerebellar interneurons from dividing progenitors in white matter. Neuron 16, 47–54.
Zordan, P., Croci, L., Hawkes, R., Consalez, G.G., 2008. Comparative analysis of proneural gene expression in the embryonic cerebellum. Dev. Dyn. 237, 1726–1735.