Immortalizing oncogenes subvert the establishment of granule cell identity in developing cerebellum

Wei-Qiang Gao* and Mary E. Hatten
The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, USA
*Present Address: Department of Neuroscience, Genentech, Inc., South San Francisco, CA 94080, USA

SUMMARY

After implantation into the external germinal layer of early postnatal cerebellum, primary external germinal layer progenitor cells gave rise exclusively to granule neurons. In contrast, all major classes of cerebellar cells were observed following implantation of embryonic day 13 cerebellar precursor cells into the external germinal layer. These results suggest that granule cells arise from precursors with a restricted potential. In contrast to results with the primary external germinal layer population, cell lines established from external germinal layer cells, by infection with a retrovirus containing the SV40 large T-antigen oncogene, gave rise to several cerebellar cell types upon implantation. These included granule neurons, one subclass of stellate interneurons, Golgi cells, Bergmann glia and astrocytes. From these results, we conclude that early postnatal external germinal layer progenitors are normally fated to a granule cell identity and that expression of the SV40 large T-antigen oncogene subverts mechanisms that control granule neuron fate.

Key words: granule neuron, cerebellum, oncogene, cell lines, transplantation

INTRODUCTION

Current models of vertebrate neural fate specification derive from the hematopoietic system, where cell fate is established by the proliferation of pluripotent stem cells (Spangrude et al., 1988; Till and McCulloch, 1961), partial commitment of precursor cells to sublineages, amplified division of partially committed cells (Metcalfe, 1987), and final differentiation of cells within a given sublineage (Nicola and Johnson, 1982; Ogawa et al., 1983). Evidence for a hemopoiesis-like model for vertebrate neural cells has been obtained in studies on the neural crest cell population, including the sympathoadrenal sublineage (reviewed by Anderson, 1989). A general role for local signals in the establishment of CNS neural identity has been inferred from studies on neurological mutant mice (Heintz et al., 1993; Sidman, 1972), from CNS precursor cell transplantation studies (McConnell and Kaznowski, 1991), from the identification of diffusible factors that influence cell specification (Jessell and Melton, 1992) and from in vitro studies showing that close appositions among CNS precursor cells promote precursor cell division and restrict cell fate (Gao et al., 1991).

The cerebellar cortex is perhaps the best studied region of the CNS. For nearly a century, all of the cerebellar cell types have been recognized and their pattern of synaptic connections known. Much of this wealth of information was described by Ramon y Cajal (1911), with information on the development (Altman and Bayer, 1978; Miale and Sidman, 1961; Altman and Bayer, 1985), anatomy (Palay and Chan-Palay, 1974), fiber tracts (Brodal, 1993) and circuitry (Llinas and Hillman, 1969) of the cerebellum emerging over the past several decades. The basic plan of the cerebellar cortex includes three layers—two neuronal layers, the Purkinje cell layer (PCL) and the internal granule cell layer (IGL), and a superficial, plexiform layer, the molecular layer (ML). The PCL consists of a single row of Purkinje neurons situated at the upper margin of the IGL. The Purkinje neuron is easily distinguished by its large cell soma, elaborate, ascending dendrites, and single, descending axon. The IGL contains a vast number of granule neurons, calculated in the human cerebellar cortex to number 10^11 (Kandel et al., 1991). The numerous, small granule neurons elaborate short, radiating dendrites in the IGL and project a unique ‘T-shaped’ axon up into the ML. The granule cell axons, termed parallel fibers, are densely stacked through the depth of the ML.

Within each of three cerebellar layers, interneurons can be discerned by their location and pattern of neuritic arborization. In the ML, as described by Ramon y Cajal (1911), the small, stellate interneurons can be seen. There are two subtypes of stellate cells, horizontally oriented stellate cells in the most superficial aspect of the ML and spiny stellate cells within the core and deeper aspect of the ML. In the PCL, the medium-sized basket cells radiate long, slender dendrites up into the ML and project horizontal axons across the PCL, forming dense pericellular baskets around Purkinje cell bodies. In the IGL, two types of interneurons can be seen. One, the Lugaro cell, is situated just beneath the Purkinje cells, extending long, horizontal processes that contact the descending Purkinje cell axons. The other, the Golgi cell, projects thin, ascending dendrites into the ML, and a thick skirt of axons down into the...
IGL. In addition to these six types of cerebellar neurons, two classes of astroglial cells can be seen - the Bergmann glia, located just above the PCL with numerous, radial processes coursing to the pial surface, and astrocytes of the IGL. Although cerebellar glial cells (Hatten and Liem, 1981) and Purkinje cells (Ross et al., 1989) can be readily identified by cellular antigen markers, other cerebellar cells are classically identified by their size, laminar position and pattern of neuritic arborization.

Among cerebellar neurons, the granule cell presents an opportune model for studying CNS neuronal specification. Unlike the other five cerebellar neurons, the granule cell arises in a displaced proliferation zone, the external germinal layer (EGL) (Ramon y Cajal, 1911). Experimental chick/quail chimeras provide evidence that, whereas the other cerebellar neurons originate from the caudal aspect of the menencephalon, the EGL arises from the rostral portion of the metencephalon via a complex series of transverse migrations. The distinct origin of the EGL (Hallonet et al., 1990; Hallonet and Le Douarin, 1992; Martinez and Alvarado-Mallart, 1989) raises the question as to whether granule cell identity is specified by regulatory factors, localized to the superficial germinal zone. In vitro studies with purified EGL precursors support the conclusion that the close apposition of granule cell precursors within the EGL restricts the fate of these progenitor cells to a granule cell identity (Gao et al., 1991). In the present study, we have further examined whether the EGL provides local signals that restrict precursor cells to a granule cell identity, by re-implanting labeled EGL precursors into the EGL and examining their laminar positioning and neuritic arborization after short survival times (1-7 days).

Another general approach to understanding the control of neural fate is to study progenitor cell immortalized by oncogene transfer. Although experiments in non-neuronal systems, including the hemopoietic system (Klinken et al., 1988), have cautioned against the use of immortalized cells in lineage analyses, the demonstration that immortalized CNS neuronal precursors would incorporate into developing brain, and differentiate into identifiable cell classes has suggested that immortalized CNS cells provide a convenient means of studying developmental processes in brain (for reviews see Cepko, 1988; Lendahl and McKay, 1990). However, as previous CNS cell lines have been generated by immortalizing a mixture of progenitor cells (e.g. Renfranz et al., 1992), it has not been possible to assess the effects of oncogene expression on the specification of the primary cell of a given CNS sub-lineage.

In the current study, we transferred the tsA58 allele of the SV40 large T antigen oncogene into purified early postnatal EGL precursor cells and compared the development of primary EGL cells with infected cells both in vitro and in vivo. To compare the fate of the cells, we implanted primary EGL cells and immortalized EGL cells into cerebellar EGL on postnatal day 6. After implantation, whereas primary EGL cells gave rise exclusively to granule neurons, immortalized cells gave rise to granule neurons, stellate interneurons, neurons and astroglia.

In the same transplantation assay, precursor cells taken from the cerebellar primordium on embryonic day 13 (E13) generated Purkinje neurons, interneurons, granule neurons and astroglia after implantation into the early postnatal EGL. These results indicate that whereas E13 cells show multiple fates, early postnatal EGL cells have a restricted potential, giving rise only to granule neurons. The control of precursor cell potential is apparently subverted by the expression of the SV40 large T-antigen oncogene as immortalized cells generate multiple programs of cell differentiation.

## MATERIALS AND METHODS

### Preparation of early postnatal EGL precursor cells and astroglia and of embryonic day 13 cerebellar cells

The present experiments were carried out with primary cells from C57Bl/6j mouse cerebella harvested on the 5-6 postnatal days (P5-P6). EGL cells were purified as described previously (Gao et al., 1991; Hatten, 1985). Astroglial cells were purified from the same preparations used to harvest EGL cells, as described by Hatten, (1985). Cells from the E13 cerebellar anlage were prepared as described by Hatten and Sidman, (1978). Briefly, cerebella were incubated sequentially in 0.08% and 0.25% trypsin in CMF-PBS containing 0.02% EDTA for 15 minutes at 37°C each., after which soybean trypsin inhibitor (0.05 mg/ml in CMF-PBS containing 0.05 mg/ml DNase) was added. The tissue was then triturated and dissociated into single cells.

### Implantation of EGL cells, astroglia, E13 cerebellar cells and immortalized EGL cells into P6 cerebellum

Prior to implantation, purified EGL cells, astroglial cells, E13 cerebellar cells or GC-B6 cells (see below) were labeled with green fluorescent latex microbeads (Lumafluor, Inc., NJ) in vitro for 1 hour at a dilution of 1:300 in the culture medium. The cells were then washed with medium or CMF-PBS for several times, collected in a test tube and stained with PKH-26 at a concentration of 4 µM for 5 minutes (Zynaxis, Inc.; see Gao et al., (1992)). The labeled cells were then washed several times and suspended in DMEM + 9 mg/ml glucose in the presence of 20 µM Hepes buffer (pH 7.4). Approximately 25,000 cells were implanted into the EGL of P5-6 C57Bl/6j mice (Gao and Hatten, 1993). Prior to the cell implantation procedure, P6 mouse pups were rendered unconscious by chilling the animals at 4°C for 1-2 minutes and placed in a Stoelting stereotoxic device fitted with a neonatal rat adapter and a vertical holder for a Hamilton syringe (Wood Dale, IL). The skin overlying the midbrain and hindbrain was rinsed with alcohol, a small incision was made in the skin and the Hamilton syringe needle was lowered gently through the incision to a position just beneath the meninges (just above the EGL). The animals insensitivity to the surgical procedure was judged by their lack of movement. Approximately 1 µl of the cell suspension (2.5x10^7 cell/ml) was injected slowly on each side of the cerebellum, after which the syringe was removed, the skull was rinsed with a solution of penicillin-streptomycin (0.25%), and the skin was replaced and sealed with Vetbond (Henry Schein Inc). The animal was then warmed to 35.5°C and returned to the litter.

After survival of 1-7 days, animals were anaesthetized with ketamine prior to perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The cerebella were removed by dissection and post fixed in the same fixative prior to being washed in PBS and embedded in 3% agar gel. Serial sections (90-100 µm) were cut with a vibratome and labeled cells were visualized with epifluorescence microscopy using a Zeiss Axioshot microscope fitted with phase contrast, Nomarski and epifluorescent illumination, Plan-neofluor 20x and 40x objectives and an Axioshot camera module. To examine the extent of PHK-26 dye leakage and re-uptake, we double labeled cells with PKH-26 and microbeads and followed the incorporation of labeled cells. In a cell sample of several thousand cells, we did not observe a cell that was not double labeled, suggesting that the dye was not transferred from implanted, double-labeled cells to endogenous, unlabeled cells. As a further control, we labeled primary EGL cells with PKH-26, killed them by freezing and thawing.
four times at −80°C, and implanted the dead, labeled cells into the P6 EGL. In those experiments, no PKH-26 labeled cells were seen distal to the site of injection, confirming previous findings (Gao et al., 1992; Gao and Hatten, 1993; Horan and Slezak, 1989) that the PKH-26 dye does not transfer to other cells in intact tissue.

In some experiments, cells were visualized with an Axiovert 135 microscope with DIC and epifluorescent illumination, Plan-Neofluor 20× objective, and a computer-driven (z-axis, Ludl system) stage. Images were acquired with a high-sensitivity Biorad MRC-600 scanning, imaging head controlled with an 80386 host computer with scan control and imaging acquisition and analysis system, using two detectors for acquisition of simultaneous Nomarksi and fluorescence imaging. A 15 mW argon/krypton mixed gas multi-line mode laser with lines at 488, 568 and 647 was used for imaging of blue line excitation (FITC, Rhodamine 1,2,3). Images were stored on an optical disk, Ethernet-linked to a Silicon Graphics Iris computer system and printed on a Sony U-811 Video Printer System.

Immortalization of EGL cells using retroviral constructs

Purified EGL cells from P5-6 mouse cerebella were infected with conditioned medium from psi 2 cells packaging the retrovirus encoding tsa58/C197 antigen and neomycin, at a density of 10^7/ml in the presence of 8 µg/ml polybrene (Sigma) in a untreated culture dish. The virus-producing cells were kindly provided by R. McKay (NIH). After being infected twice (6 hours each time), the virus-containing medium was replaced with BME plus 10% horse serum, 5% fetal bovine serum, 9 mg/ml glucose, and cells were placed at 35°C for 2 days then transferred to a polylysine coated dish (500 µg/ml). Several days later, cultures were selected in the above medium containing G418 (200 µg/ml). The selection medium was replaced every 2 days, and G418-resistant colonies were observed in 2 weeks and picked with cloning rings. The clones were then expanded, frozen, subcloned, and cultured in DMEM plus 10% newborn calf serum. In the present experiments, although similar results were obtained from one of the other clones, only the data from subclone GC-B6 were described and used for transplantation.

Immunocytochemistry

Immunocytochemical labeling of primary EGL cells was as described previously (Gao et al., 1991). In the present experiments, GC-B6 cells were plated in 16-well lab-tek slides coated with poly-D-lysine (0.1 mg/ml) (35°C, overnight) in either DMEM plus 10% newborn calf serum or serum-free medium (Redu-Ser II from Upstate Biotech Inc.) at 39°C for 16-24 hours before being fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) for 30 minutes, and mounted in gel/mount (Biomedical Co.). The implanted cells were visualized with a Zeiss Axiophot microscope fitted with phase contrast, Nomarksi and epifluorescent illumination and Plan-neofluor 40× objectives and an Axiosphot camera module.

RESULTS

Previous in vitro analyses of primary EGL cells in reaggregate cultures (Gao et al., 1991; Gao et al., 1992) demonstrated that local interactions among EGL precursor cells promote granule cell neurogenesis and differentiation. A striking aspect of these studies was the finding that proliferating cells in homotypic reaggregate cultures had a restricted cell fate, expressing cellular antigen markers and morphological features of granule cells, but not other classes of cerebellar neurons (Gao et al., 1991). To examine the fate of purified EGL cells in vivo, we double-labeled purified EGL cells with fluorescent microbeads and PKH-26, injected them just beneath the pia of P6 animals, allowed the animals to survive for short periods of time (1-7 days), and examined the morphology and location of labeled cells in the cerebellar cortex. Using fluorescent and confocal microscopy it was found that labeled cells remained at the injection site during the first 1-24 hours in vivo, with some cells undergoing mitosis in the superficial aspect of the EGL, as evidenced by labeled, mitotic figures (not shown). 24-48 hours after implantation, primary EGL cells, characterized by their small (4-6 µm) size and globular shape, descended into the deeper aspect of the EGL, where they extended long fibers, 100-200 µm in length, parallel to the surface of the anlage (Fig. 1A). This sequence of developmental events confirmed the hypothesis of Ramon y Cajal, who suggested that granule cell axon extension precedes the inward migration of the cell soma (Ramon y Cajal, 1911).

A second stage of development commenced 48 hours after implantation, the polarization of the cell soma to extend a descending process perpendicular to the plane of parallel fiber extension (Fig. 1B). Following extension of this descending or ‘leading process’ (Edmondson and Hatten, 1987; Rakic, 1971; Ramon y Cajal, 1911), many of the implanted cells started to migrate along Bergmann glial fibers, requiring approximately 10-12 hours to reach the internal granule cell layer (IGL). During migration, the trailing process of labeled cells displayed an ascending ‘T-shape’ (Fig. 1C), characteristic of the granule cell axon. At low magnification, in a coronal plane of section, several dozen labeled cells could be seen migrating through the molecular layer (ML) and settling in the IGL (Fig. 1D). All of these cells expressed the morphology described in classical studies of Golgi-impregnated tissue (Ramon y Cajal, 1911).

After settling within the IGL, labeled cells extended four to six, short radiating dendrites, with branched claw-like endings, characteristic of mature granule cells. Among the more than 80 animals injected with several thousand labeled EGL cells each,
and analyzed by serial sectioning of the brain, more than 99% of the cells that underwent differentiation expressed a granule neuron identity, positioning their soma in the IGL, forming 4-6 short dendrites, and extending an ascending 'T-shaped' parallel fiber into the molecular layer. Less than 0.5% of the differentiated cells we counted assumed the glial phenotype, consistent with the level of contamination of the cell preparation with glial cells. No other classes of cerebellar neurons were observed after injection of labeled EGL cells (Table 1), suggesting that EGL precursor cells were normally fated to a granule cell identity.

By confocal microscopy, approximately 10-20% of the cells we injected into the host tissue differentiated as described. The same general distributions of cell types, were obtained in each of the 80 animals we injected with labeled cells. As a control, we implanted labeled, primary astroglial cells purified from...
Table 1. Differentiation of implanted cells in developing cerebellum

<table>
<thead>
<tr>
<th>Cells implanted</th>
<th>Granule neuron</th>
<th>Purkinje neuron</th>
<th>Interneurons</th>
<th>Bergmann glia</th>
<th>Astroglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary E13 cells</td>
<td>99</td>
<td>0</td>
<td>0</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Primary astroglial</td>
<td>0</td>
<td>0</td>
<td>20-30</td>
<td>40-60</td>
<td></td>
</tr>
<tr>
<td>GC-B6 cells</td>
<td>5-10</td>
<td>10-15</td>
<td>20-30</td>
<td>10-20</td>
<td>20-40</td>
</tr>
<tr>
<td>E13 cerebellar cells</td>
<td>10-15</td>
<td>30-40</td>
<td>10-20</td>
<td>20-40</td>
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</tr>
</tbody>
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Primary P6 E13 cells, P6 astroglia, GC-B6 or E13 cerebellar cells were implanted into the EGL on postnatal day 6 as described in the text. After 1-7 days in vivo, the morphology and position of labeled cells was used to classify them as granule neurons, Purkinje neurons, interneurons, Bergmann glia or astrocytes. The percentage of dye-labeled cells with the morphology and laminar position of specific cerebellar cell types is indicated.

*Approximately 30% of the implanted cells could not be identified.

P4-6 cerebellum, identified by their expression of GFAP, into the developing cerebellum. Purified glial cells differentiated into Bergmann glial cells (20-30%) and astrocytes (40-60%) after implantation into P6 cerebellar cortex. None of the labeled glial cells we injected differentiated into granule neurons or other types of cerebellar neurons (Table 1), suggesting that glial cell fate was specified prior to P6.

Implantation of primary E13 cerebellar cells generates all major classes of cerebellar cells

To test whether the early postnatal EGL contains local factors that inhibit the development of other classes of cerebellar neurons, including the Purkinje cell, we injected embryonic precursors of other types of cerebellar cells into the EGL and followed their development. As all other classes of cerebellar cells are thought to arise from the ventricular zone lining the IVth ventricle, between embryonic days 11 and 13 (Altman and Bayer, 1985; Miale and Sidman, 1961), we used the E13 cerebellar anlage as a source of cells. At this age, the anlage contains a mixture of postmitotic and proliferating Purkinje cell precursors, as well as proliferating precursors of other neurons, the emerging EGL, and glial precursor cells. To test whether this complex population of cells would generate all classes of cerebellar cells if placed in the EGL, we labeled the dissociated E13 cells with PKH-26, and injected them just beneath the pia of the cerebellar cortex of P6 animals. Implantation of E13 cerebellar precursor cells resulted in the appearance of all cerebellar cell types, including granule neurons with ascending, T-shaped axons (Fig. 2A), Bergmann glial cells with multiple, slender, ascending processes (Fig. 2B), Purkinje neurons with several thick ascending processes and one descending axon (Fig. 2C,D), and multiple classes of interneurons and astrocytes of the IGL and white matter (Table 1). Moreover, most of these differentiated implanted, primary precursor cells were positioned in the correct layer (Fig. 2).

Immature Purkinje cells seen after implantation of E13 precursor cells resembled both young Purkinje cells in the process of migration (Fig. 2C) and cells that were settling into the Purkinje cell zone (Fig. 2D).

Among the E13 cells that differentiated after incorporation into the early postnatal EGL, approximately 10-15% of the cells expressed the features of granule neurons, 30-40% resembled Purkinje neurons, 10-20% formed interneurons and 20-40% astroglia. The granule cells we observed most likely originated in the lateral aspect of the E13 anlage, as the EGL is just beginning to emerge across the rhomic lip at this age. The observation of Purkinje neurons, interneurons and glial cells, after implantation of the cells harvested from the E13 cerebellar anlage suggests that local signals within the early postnatal EGL can support the differentiation of precursors of all of the principal classes of cerebellar neurons.

Infection of primary EGL cells with a replication-deficient retrovirus containing the tsA58 allele of SV40 large T antigen oncogene

In order to examine the role of oncogene expression in the specification of granule cells, we infected purified, granule cell progenitors with a retrovirus carrying the tsA58 allele of SV40 large T antigen oncogene and the neomycin gene (Renfranz et al., 1992). Retroviral transfer was carried out in reaggregate cultures under conditions where EGL cell proliferation occurs (Gao et al., 1991). As a first step, G418-resistant colonies were picked, expanded, and subcloned by standard limited dilution methods. Among several dozen clonal cell lines established, most generated cells that expressed neuronal antigen markers at the non-permissive temperature. We next tested the cell lines for their ability to differentiate in co-culture with primary neurons (see below) and their ability to rescue weaver granule neuron differentiation (not shown). As one of the clonal lines, GC-B6, expressed both of these properties of ‘normal’ granule neurons, this line was chosen for further study. As shown by immunostaining with a monoclonal antibody against large T antigen, all GC-B6 cells showed nuclear labeling of the large T antigen, demonstrating expression of the tsA58 allele of SV40 large T antigen oncogene in EGL precursor cells (Fig. 3A,B).

At the permissive temperature, GC-B6 cells had either a flat or bipolar morphology when cultured on a surface treated with polylysine, laminin or Matrigel, and expressed low levels of neuronal or glial markers (see below). These cells underwent rapid proliferation with a cell generation time of approximately 12-14 hours. Shifting the cells to the non-permissive temperature (to allow differentiation) slowed proliferation to generation times of 48-72 hours. Although a large number of the cells became elongated at 39°C, neurite formation was not evident. Addition of growth factors (basic fibroblast growth factor, epithelial growth factor, insulin-like growth factor-1, nerve growth factor, brain-derived neurotrophic factor and neurotrophin 3) or medium conditioned by either granule cells or astroglia, did not induce neurite formation (not shown). Maximal differentiation, including the extension of slender, bipolar neurites, was seen when the cells were plated on a monolayer of purified, primary EGL cells (Fig. 4A,B), suggesting that EGL cells induce differentiation of the GC-B6 cell line by a mechanism that involves close cell apposition. This is consistent with previous findings on primary EGL cells (Gao et al., 1991).

Immunocytochemical characterization of the expression of cellular antigen markers by GC-B6 cells, cultured for 16-24 hours at the non-permissive temperature to allow differentiation of the cells, revealed heterogeneity within the population, with a subpopulation of cells expressing the germinal zone antigen GD3 (Goldman et al., 1984) (Fig. 3C,D). While all of the cells expressed the neuronal cell adhesion molecule...
(NCAM) (Thiery et al., 1977) (Fig. 3E,F), fewer than 5% expressed markers for differentiated granule cells, including the axonal glycoproteins L1 (Rathjen and Schachner, 1984) and TAG-1 (Dodd et al., 1988), the neuron-glia ligand astrotactin (Edmondson et al., 1988) (Fig. 3G,H), or the neurofilament protein (see Table 2). Instead, 20-30% of the cells within a given culture expressed the glial filament protein (GFAP), while a subpopulation of GFAP+ cells co-expressed the neu-
rofilament protein. Cells that expressed both neurofilament protein and GFAP were process-bearing cells, resembling astroglia. Thus, within clonal populations of immortalized EGL cells, a range of differentiated marker proteins were expressed by the cells in culture. The results with GC-B6 cells contrast with previous results on primary EGL precursor cells in which cells undergo neuronal differentiation, including neurite extension, cell migration in a number of different culture systems (Fishell and Hatten, 1991; Gao et al., 1991, 1992), and express neuronal but not glial cell markers in vitro (Gao et al., 1991).

Twenty-eight cell lines established by immortalizing purified EGL cells with retroviruses containing the temperature-sensitive large T antigen showed similar immunocytochemical heterogeneity. Although this was not investigated in detail, in the several cases where subcloning of clonal lines was carried out, heterogeneous populations of cells resulted. In addition, immortalization with E1A constructs containing the v-myce oncogene gave rise to clones of cells that generated both neuronal and glial progeny, as judged by cellular antigen marker expression (not shown).

Imortalized EGL cells have multiple fates after implantation into the EGL

We next examined the fate of GC-B6 cells in vivo, under environmental influences where primary EGL fate was restricted to a granule cell identity. As seen for primary cells, 24 hours after implantation many of the GC-B6 cells appeared to undergo proliferation at the superficial aspect of the EGL. Thereafter, GC-B6 cells descended into the deeper layers of the EGL and integrated into the host cerebellar cortex. In contrast to the primary EGL cells, which generated granule neurons exclusively, GC-B6 cells differentiated into several types of cells (see Table 2). A minority of the cells (5-10%) were identified as granule cells by their globular morphology, extension of parallel fibers, migratory profiles, and/or formation of an ascending T-shaped axon (Fig. 5A,B). In addition to granule neurons, interneurons, including stellate cells in the upper aspect of the ML (Fig. 5C) and Golgi neurons (Fig. 5D), could be identified by their laminar position and morphology (see Introduction).

A larger number of the immortalized cells, 20-30% of the differentiated population, differentiated into Bergmann glia, as evidenced by a somata 8-10 \( \mu \)m in diameter, positioned just above the Purkinje cell layer, and the extension of multiple discrete fibers ascending vertically to the pial surface (Fig. 5F), giving the typical ‘candelabrum’ appearance of Bergmann cells. In addition, approximately 10-20% of the implanted cells were identified as astroglia within the IGL and the white matter, based on morphology and position (Fig. 5E and Table 2).

Although the GC-B6 cells differentiated into multiple types of cells after implantation, they did not generate all of the classes of neurons seen in P6 cerebellar cortex. Most notable was the absence of Purkinje neurons, small stellate cells in the deeper layers of the EGL, basket and Lugaro cells. The same subset of cells – granule cells, stellate cells of the upper aspect of the ML, Bergmann glial cells and astrocytes in the IGL – were obtained in each of the injection experiments we carried out, totalling more than 25 animals. Approximately 0.5-1% of the immortalized cells we implanted incorporated into the host tissue. As stated above, 10-20 times more primary cells incorporated into developing cerebellar cortex than did immortalized GC-B6 cells. The levels of GC-B6 cell incorporation are consistent with the results of Renfranz et al. (1992) and Snyder et al. (1992). The incorporation rates of both primary and immortalized cells probably underestimated the capacity of the cells to integrate into host brain, as we used short survival times to chronicle the developmental steps followed by implanted cells.

**DISCUSSION**

**Granule neurons arise from precursors with a restricted potential**

The re-implantation of purified EGL precursors into early postnatal EGL provided a test of the role of local signals in the EGL to the development of cerebellar granule neurons. In an in vivo transplantation assay, cells taken from the cerebellar primordium on embryonic day 13 generated multiple fates, with each of the major classes of cerebellar cells evident. E13 was chosen because it is the time point when precursors of all of the principle neuron classes are present (Altman and Bayer, 1978, 1985) with EGL precursors just emerging across the rhombic lip to establish the external germinal layer. The demonstration that EGL cells purified in the early postnatal period give rise to cells with the laminar position and neuritic profile of granule neurons, and not other cerebellar cell types, after implantation into the EGL suggests that the fate of EGL cells is normally restricted (Fig. 6). Thus granule cells appear to arise from precursor cells with a restricted potential.

The homotypic transplantation experiments support the conclusion that the EGL is normally fated to produce granule neurons. This result is consistent with our finding that EGL cells have a restricted fate when cultured in homotypic cellular reaggregates (Gao et al., 1991) and with studies on chick-quail chimeras (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Hallonet and Le Douarin, 1992) showing that EGL precursors give rise to granule neurons and not other cerebellar cell types. The present study extends our previous findings by demonstrating that the entire program of granule cell development, including axon extension, laminar position, and

| Table 2. Immunocytochemical characterization of GC-B6 cells |
|------------------|------------------|
| Markers          | Immunopositive cells (%) |
| Germinal zone antigens | 20-30            |
| R24a (GD3)       |                  |
| Neuronal antigens |                  |
| N-CAM            | 100              |
| L1               | 0                |
| TAG-1            | 0                |
| Astroactinin     | 5-10             |
| Neurofilament protein | 2-5              |
| Glial antigens   |                  |
| Glial filament protein | 20-30          |

Immortalized cerebellar granule cells were plated in 500 µg/ml polylsine-coated 8-well Lab-Tek culture slides (1x10^6 cells/well) in serum-supplemented medium (Gao et al., 1991) for 24-48 hours and were then immunostained with the antibodies listed above.
to appear in such extreme abundance, accounting for nearly 20% of the total neuronal population of human brain (Kandel et al., 1991). The vast numbers of EGL cells suggest that these cells represent a sublineage of CNS cells that undergo amplification after commitment, in a hemopoietic model. In this model, granule cell identity would be specified, either at the site of origin in the primordium, or during the segregation of these precursor cells into an external zone (embryonic days 13 and 17). Evidence for partial commitment of the cells comes from studies on the neurotransmitter uptake systems of EGL cells. As early as E13, EGL cells do not show any ability for uptake of GABA, the neurotransmitter system used by other cerebellar neurons (Hatten et al., 1983). In the mouse, the amplified proliferation of EGL cells occurs between P0 and P10 (Miale and Sidman, 1961), when the EGL thickens from a thin, unicellular layer to a layer 8-10 cells deep. In vitro studies suggest that local signals promote the continued proliferation of EGL precursor cells (Gao et al., 1991).

**Immortalized EGL cells do not show a restricted cell potential**

In contrast to the results obtained with primary EGL cells, EGL cells infected with retroviral constructs containing the tsA58 allele of SV40 large T antigen oncogene did not retain a granule neuron specification. Our observation that immortalized cells gave rise to multiple types of cells after implantation into the cerebellum (Fig. 6) is consistent with the findings of Renfranz et al. (1992), who showed that immortalized progenitor cells differentiated into a variety of cerebellar cell classes after implantation into early postnatal cerebellar cortex. This result is also consistent with the results of Snyder et al. (1992), who showed that immortalized cerebellar cells can participate in formation of the cerebellum. A major difference between the previous studies and the present analysis is that we have directly compared the fate of a single class of purified CNS primary precursor cells with immortalized precursor cells. Our finding that immortalized EGL cells did not express the fate seen for their primary counterparts suggests that introduction of the tsA58 allele of the SV40 large T antigen oncogene into EGL precursor cells altered the response of the cells to regulatory components needed to specify a granule cell identity.

In spite of the failure of immortalized EGL cells to retain a granule cell commitment after implantation into developing cerebellar cortex, EGL cell lines should prove useful for a number of other experiments, as they express selected features of granule cells. These include the ability to differentiate upon close apposition with primary EGL cells, shown here, and the ability to rescue weaver granule cell differentiation (Gao et al., 1992). Comparison of these and other EGL cell lines might also provide insights to the molecular determinants of pluripo-

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**Fig. 3.** Immunocytochemical characterization of GC-B6 cells. Immunocytochemical staining of GC-B6 cells, grown in the permissive temperature in vitro for 16-24 hours, with a monoclonal antibody against large T-antigen (A,B) reveals nuclear expression of the large T antigen by all cells in the population. Immunocytochemical labeling of cells, grown at the non-permissive temperature in vitro for 16-24 hours, with antibodies against the neural cell adhesion molecule N-CAM (E,F) labeled all cells, and antibodies against the neuron-glial adhesion system astrotactin (G,H) labeled a small number of cells. Thus, although all cells expressed the large T antigen and NCAM, more restricted neuronal markers were expressed by a subset of cells within the cell population. (A,C,E,G) Fluorescence microscopy. (B,D,F,H) Phase contrast microscopy of paired fields. Bar, 50 µm.

**Fig. 4.** Co-culture of GC-B6 cells with primary EGL cells induces neurite formation. GC-B6 cells were labeled with PKH-26 and cultured on a monolayer of primary EGL cells for 24 hours in vitro. Two cells were seen extending long, slender, bipolar neurites characteristic of granule neurons, suggesting differentiation of GC-B6 cells on a monolayer of primary EGL cells. (A) Fluorescence microscopy and (B) phase contrast microscopy of the same field. Bar, 50 µm.

dendritic development, is realized after re-implantation of the cells. These findings do not exclude the possibility that EGL precursors would express a different potential after transplantation to heterotypic brain regions.

The finding that EGL cells give rise only to granule neurons suggests that segregation of this population of precursor cells, from the time of their origin in the primordium (Hallonet et al., 1990; Hallonet and Le Douarin, 1992), and continued proliferation in displaced germinal zone, sets forth the pattern of cell fate restriction leading to a granule cell identity. Interestingly, the granule neuron is the only class of CNS neurons to arise from a displaced germinal zone, and also the only CNS neurons...
tency versus a restricted fate; to primary EGL cells or other CNS cells.

Effects of SV40 large T oncogene on neuronal fate specification

In previous studies, immortalized CNS cell lines have been proposed to represent multipotential stem cells, owing to the diversity of cells seen after their implantation into developing brain (Renfranz et al., 1992; Snyder et al., 1992). In this model, immortalization of the cells is thought to capture cells that have entered a neural sublineage, but remain multipotential. As these cell lines were generated from a mixture of uncharacterized progenitor cells, it was difficult to assess the effect of the transfected oncogene to cell specification. The present experiments suggest that, although the cells retain a neural character, immortalization subverts the specification of granule cells. The most striking feature of the GC-B6 cell line was the odd variety of types of cerebellar cells generated after implantation of GC-B6 cells. For example, although we observed one subclass of inhibitory stellate cells, those located in the upper aspect of the molecular layer, we did not observe the more common stellate cells seen in deeper aspects of the molecular layer. As the par-
EGL cells (hatched circles) give rise to a variety of cell types, granule cell development seen in vivo. By contrast, immortalized to a granule neuron fate, proceeding through all of the steps of cortex, primary EGL precursor cells (stippled circles) are committed developing cerebellar cortex support cell differentiation (represented Fig. 6. Oncogene expression subverts granule cell lineage together with our current findings, point to the potential for Stein, 1977). These general features of CNS neural tumors, medulloblastoma, give rise to multiple cell types in situ (Russell and Rubenstein, 1977). These general features of CNS neural tumors, together with our current findings, point to the potential for oncogenes to subvert normal cell fate specification.

The model that emerges from our studies is one where a particular variety of cell types generated by GC-B6 cells have not been suggested to arise from a common precursor or even the same germinal zone, it seems unlikely that the GC-B6 cell line represents a restricted progenitor found in vivo.

Although the EGL is not inhibitory for Purkinje cell differentiation, as evidenced by the ability of E13 cells to differentiate into Purkinje cells forming transplantation into the EGL, it may not contain local signals required for the specification of Purkinje cells (or the classes of interneurons not seen). The failure of the GC-B6 cell line to form Purkinje cells after implantation into the EGL may therefore be due to the absence of Purkinje cell determinants in the EGL zone. It will be interesting to examine, in future experiments, if transplantation of GC-B6 progenitors to the site of origin of the Purkinje neuron, the embryonic VZ lining the fourth ventricle, results in the generation of a Purkinje cell. This would be in agreement with the cortical transplantation studies of McConnell and Kaznowski (1991), showing that environmental cues in the cortical VZ influence the laminar fate of neural progenitor cell.

The interpretation that immortalizing oncogenes subvert normal mechanisms of cell fate commitment is supported by studies on non-neural cells. In the hemopoietic system, overexpression of the v-raf oncogene leads to lineage switching, converting B lineage cells, from either lymphomas or preleukemic bone marrow cells of Eμ myc transgenic mice, to macrophages. As the B cell and macrophage lineages are not closely related, v-raf expression appears to subvert normal mechanisms of lineage specification (Klinken et al., 1988). More generally, lineage instability is common among transformed cell populations. Many tumors, including a tumor which is thought to arise from EGL progenitor cells, the medulloblastoma, give rise to multiple cell types in situ (Russell and Rubenstein, 1977). These general features of CNS neural tumors, together with our current findings, point to the potential for oncogenes to subvert normal cell fate specification.

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Fig. 6. Oncogene expression subverts granule cell lineage specification. On postnatal day 6, a variety of factors present in the developing cerebellar cortex support cell differentiation (represented by squares). When implanted into the EGL of the P6 cerebellar cortex, primary EGL precursor cells (stippled circles) are committed to a granule neuron fate, proceeding through all of the steps of granule cell development seen in vivo. By contrast, immortalized EGL cells (hatched circles) give rise to a variety of cell types, suggesting they are not committed to a granule cell fate.
implantation of weaver granule cell precursors into wild-type cerebellar cortex. Science 260, 367-369.


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