Analysis of Gene Expression in the Normal and Malignant Cerebellum

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ABSTRACT

The developing nervous system consists of a small number of multipotent precursors that undergo extensive proliferation to generate the neurons and glia that make up the adult brain. Elucidating the mechanisms that control the growth and differentiation of these cells is important not only for understanding normal neural development but also for understanding the etiology of central nervous system tumors. A particularly striking example of this is in the cerebellum. Recent studies have suggested that the Sonic hedgehog-Patched signaling pathway plays a critical role in regulating the proliferation of cerebellar granule cell precursors and is also a major target of mutation in the cerebellar tumor medulloblastoma. In light of these observations, identification of additional genes that control cerebellar growth and differentiation is likely to provide important insight into the basis of cerebellar tumors. Similarly, analysis of gene expression in medulloblastoma will no doubt shed light on previously unknown signaling pathways that regulate normal cerebellar development. The advent of high-throughput gene expression analysis techniques — such as adapter-tagged competitive polymerase chain reaction (ATAC-PCR), serial analysis of gene expression (SAGE), and DNA microarrays — makes identification of such genes faster and easier than ever before. This review summarizes recent studies of gene expression in the cerebellum and discusses the value of such approaches for understanding development and tumorigenesis in this tissue.

I. Development of Cerebellar Granule Cells

The cerebellum is required for motor coordination and has been implicated in a variety of cognitive and affective functions as well (Leiner *et al.,* 1993; Altman and Bayer, 1997). These functions depend on precise interactions among at least five types of neurons (Altman and Bayer, 1997). The most abundant of these are the Purkinje cells, which carry signals from the cerebellum to other parts of the brain, and the granule cells, which regulate the activity of Purkinje cells. The murine cerebellum contains about 10^8 granule cells, more than the total number of neurons in the rest of the brain. The critical importance of these cells is evident from mutant mice, in which loss of granule cells leads to severe ataxia (Mullen *et al.,* 1997), and from patients with congenital granule cell degenera-

tion, who have severe deficits in motor coordination, language use, and cognitive function (Pascual-Castroviejo *et al.,* 1994).

Granule cell development has a number of unique features that distinguish it from other kinds of neurogenesis (Hatten and Heintz, 1995; Altman and Bayer, 1997). Whereas most neurons are born around the ventricles and then migrate outward toward the surface of the brain, granule cells are generated on the outside of the cerebellum and migrate inward (Figure 1). Granule cell precursors (GCPs) initially arise from a dorsal hindbrain structure called the rhombic lip (Alder *et al.,* 1996; Alcantara *et al.,* 2000; Wingate, 2001). During embryonic life, these cells leave the rhombic lip and stream across the outer surface of the cerebellum to form a region called the external germinal layer (EGL). After birth, cells in the EGL undergo extensive proliferation to generate a large pool of GCPs (Fujita *et al.,* 1966; Mares *et al.,* 1970). As new GCPs are generated, older cells move inward, then exit the cell cycle and differentiate (Fishell and Hatten, 1991; Komuro and Rakic, 1998). The differentiating cells extend axons that form synapses with Purkinje cells, then continue to migrate inward past the Purkinje cell bodies to their final destination, the internal granule layer (IGL). The waves of GCP proliferation and differentiation continue until about 3 weeks of age, at which time the EGL disappears and all GCPs complete their migration and differentiation into mature granule cells (Nicholson and Altman, 1972).

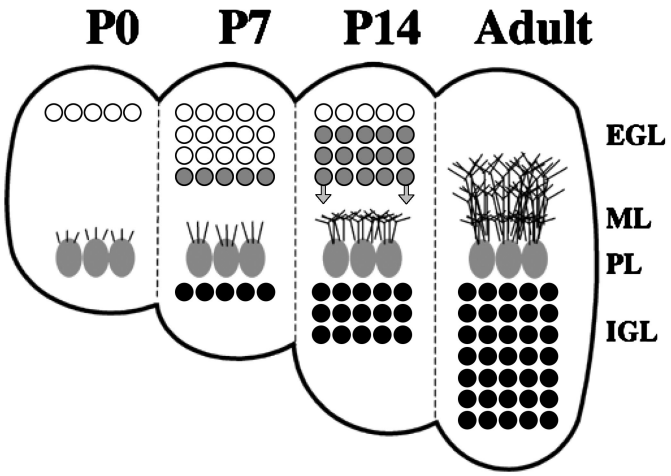

FIG. 1. Granule cell development. Granule cells are generated in the external germinal layer (EGL). After birth (P0), they proliferate to generate a large pool of precursors (white circles, P0–P14). These cells move inward, exit the cell cycle, and differentiate (gray circles). They extend axons that synapse with Purkinje cells (gray ovals). Finally, cells migrate through the molecular layer (ML) and Purkinje cell layer (PL) to the internal granule layer (IGL). By adulthood, all precursors have become mature granule cells (black circles) and no EGL remains.

II. Control of Granule Cell Precursor Proliferation

The observation that mutations in the Sonic hedgehog (Shh) signaling pathway result in cerebellar tumors in both mice and humans (Johnson *et al.*, 1996; Goodrich *et al.*, 1997; Raffel *et al.*, 1997) suggested that this pathway might play a role in normal cerebellar growth and differentiation. Our own studies (Wechsler-Reya and Scott, 1999) and others (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999) demonstrated that Purkinje cells make Shh and that developing GCPs express all of the elements of the signaling pathway necessary to respond to it. Addition of recombinant Shh protein to GCPs in culture induces a 75- to 100-fold increase in thymidine incorporation within 3 days. Treatment of cerebellar slice cultures with Shh also causes a dramatic proliferative response and inhibits granule cell differentiation and migration. Finally, intracranial injection of Shh-blocking antibodies during early postnatal development leads to decreased proliferation of GCPs and a significant reduction in the thickness of the EGL. Together, these studies suggest that Shh is a critical regulator of GCP proliferation.

A number of factors present in the outer EGL enhance Shh-induced proliferation of GCPs. One of these is the extracellular matrix molecule, laminin (Wechsler-Reya and Scott, 1999; Pons *et al.*, 2001). GCPs cultured on laminin show a 1.5- to 2-fold increase in Shh-induced proliferation, compared to cells cultured on other substrates. The chemokine stromal cell-derived factor-1 alpha (SDF-1α), produced by the pia mater that surrounds the EGL, induces GCP chemotaxis and also can synergize with Shh to enhance GCP proliferation in culture (Klein *et al.*, 2001). Finally, heparan sulfate proteoglycans (HSPGs), which have been shown to be required for maximal hedgehog signaling in *Drosophila*, are present in the postnatal EGL and can increase Shh-induced proliferation of GCPs (Rubin *et al.*, 2002). While none of these factors induces significant proliferation on its own, all may contribute to the proliferation of cells in the EGL.

Growth factors that act through receptor tyrosine kinases (RTKs) – epidermal growth factor (EGF), insulin-like growth factor (IGF-1), and basic fibroblast growth factor (bFGF) – have been reported to induce GCP proliferation (Gao *et al.*, 1991; Tao *et al.*, 1996; Ye *et al.*, 1996; Lin and Bulleit, 1997). On their own, these factors cause a 2- to 4-fold increase in thymidine incorporation in cultured GCPs. Interestingly, however, they show no evidence of synergy with Shh; rather, EGF and IGF-1 cause a modest suppression of Shh-induced proliferation and bFGF reduces the Shh response by 90% (Wechsler-Reya and Scott, 1999). This suggests that RTK-binding growth factors control proliferation through mechanisms distinct from the one used by Shh or that they act on distinct subsets of cerebellar cells. The fact that EGF, IGF-1, and bFGF also have potent effects on granule cell survival and differentiation (Hatten *et al.*, 1988; Dudek *et al.*,

1997; Lin and Bulleit, 1997; Saffell *et al.,* 1997; Gunn-Moore and Tavare, 1998; Liu and Kaczmarek, 1998) raises the possibility that these factors may control aspects of cerebellar development besides proliferation.

III. Granule Cell-cycle Exit and Differentiation

GCPs undergo extensive proliferation during the first 2–3 weeks after birth. But even as proliferation in the EGL reaches its peak, some GCPs are beginning to exit the cell cycle and differentiate into neurons. One possible explanation for this cell-cycle exit could be movement of cells away from Shh protein. For example, Shh might be present only in the outer EGL and, as cells move inward and away from the mitogenic signal, they might exit the cell cycle. Although this model has not been completely ruled out, the available evidence argues against it. Shh is made by Purkinje cells (Wechsler-Reya and Scott, 1999) and the protein has been detected throughout the EGL (Gritli-Linde *et al.,* 2001; Pons *et al.,* 2001). Moreover, Shh expression begins during embryonic life and persists into adulthood (Traiffort *et al.,* 1999). Thus, termination of the proliferative response probably is not due to reduced exposure to Shh. Rather, it is likely to result from reduced responsiveness to Shh or conversion of the proliferative response into a differentiative one.

To date, three signals have been shown to overcome Shh-induced proliferation: the extracellular matrix molecule vitronectin (VN), the protein kinase A (PKA) activator forskolin, and bFGF. In contrast to laminin, VN is found primarily in the *inner* EGL and granule cells in this region express alpha-v integrins, which can function as VN receptors (Pons *et al.,* 2001; Wechsler-Reya, 2001). GCPs grown on VN show a 20% reduction in the proliferative response to Shh and increased β-tubulin expression and neurite outgrowth, compared to cells grown on other substrates. Growth on VN also causes increased phosphorylation of the transcription factor cAMP response element binding protein (CREB). CREB function appears to be required for VN's effects on neurite outgrowth (Pons *et al.,* 2001). These observations suggest that VN may contribute to granule cell-cycle exit and differentiation.

Forskolin also can inhibit Shh-induced proliferation of GCPs (Wechsler-Reya and Scott, 1999; Kenney and Rowitch, 2000). This is not surprising, since PKA activators can inhibit Shh responses in most tissues and in most species (Li *et al.,* 1995; Hammerschmidt *et al.,* 1996; Ungar and Moon, 1996). Because forskolin is not a physiologic signal, there must be an endogenous factor that activates PKA for this to play a role in granule cell development. One good candidate for a PKA activator is pituitary adenylate cyclase activating polypeptide (PACAP) (Sherwood *et al.,* 2000). PACAP is made by Purkinje cells (Nielsen *et al.,* 1998; Skoglosa *et al.,* 1999) and its receptors are expressed by GCPs in the outer EGL (Basille *et al.,* 1993). To date, most studies of PACAP

in the cerebellum have indicated that it increases proliferation of GCPs and promotes survival in the presence of various death-inducing stimuli (Vaudry *et al.,* 1999,2002; Tabuchi *et al.,* 2001). On the other hand, recent studies of neurons in the embryonic cortex and hindbrain indicate that PACAP can inhibit proliferation of those cells (Suh *et al.,* 2001; Lelievre *et al.,* 2002). Thus, PACAP might contribute to cell-cycle exit in the cerebellum as well.

Finally, bFGF is a potent inhibitor of proliferation, reducing the response to Shh by 90% (Wechsler-Reya and Scott, 1999). bFGF is made by cerebellar astrocytes and Purkinje cells (Hatten *et al.,* 1988; Matsuda *et al.,* 1994). At least two receptors capable of binding it (i.e., fibroblast growth factor (FGF)-1 and -4) are expressed in GCPs in the EGL (el-Husseini *et al.,* 1994; Miyake *et al.,* 1995; Ozawa *et al.,* 1996; Meiri *et al.,* 1998; Sleptsova-Friedrich *et al.,* 2001). The observation that bFGF can prevent proliferation is consistent with a variety of studies demonstrating that it promotes granule cell differentiation and neurite outgrowth *in vitro* and *in vivo* (Williams *et al.,* 1994; Hatten and Heintz, 1995; Saffell *et al.,* 1997; Liu and Kaczmarek, 1998). However, as mentioned earlier, bFGF has been reported to induce proliferation of cerebellar cells *in vitro* and following subcutaneous injection (Tao *et al.,* 1996; Cheng *et al.,* 2001). In fact, we have observed that bFGF induces a small increase in thymidine incorporation in our cultures as well (Wechsler-Reya and Scott, 1999). One possible explanation for these observations is that the proliferative response to bFGF occurs in a subset of GCPs or in a distinct class of progenitor cells that are present in the postnatal cerebellum.

As granule cells differentiate, they extend axons and migrate inward, past the Purkinje cells, to the IGL. Bergmann glia are thought to provide a substrate for the migration (Edmondson and Hatten, 1987; Hatten, 1990; Komuro and Rakic, 1998) and therefore are a likely source of signals and guidance cues. Autocrine signaling by brain-derived neurotrophic factor (BDNF) is critical for initiation of migration from the EGL (Borghesani *et al.,* 2002). Astrotactin, a granule cell surface molecule with EGF and fibronectin repeats, has been shown to be important for guiding granule cell migration (Edmondson *et al.,* 1988; Zheng *et al.,* 1996). Migration also has been shown to be regulated by N-methyl-D-aspartate (NMDA) receptor activity and intracellular calcium concentration (Komuro and Rakic, 1993,1996).

IV. Gene Expression Analysis of the Developing Cerebellum

The studies described herein have revealed a number of important factors that control proliferation and differentiation of granule cells. However, many important questions remain. For example, the molecular mechanisms by which Shh and other factors regulate growth of GCPs and the identity of the signals that control granule cell differentiation and migration *in vivo* are poorly understood.

Moreover, the molecules that regulate growth and differentiation of other cell types in the developing cerebellum have not been identified. One important tool for identifying such molecules is gene expression analysis. By analyzing changes in gene expression during the course of cerebellar development, or by isolating specific cell types and comparing their gene expression under conditions of growth and differentiation, it may be possible to identify molecules that are important in normal growth and differentiation.

One of the first efforts at broad-based gene expression analysis in the cerebellum was carried out by Kuhar *et al.* (1993). These investigators raised polyclonal antisera against immature GCP and adsorbed them against PC12 cells (a cell line resembling peripheral neurons) and adult cerebellum to deplete non-GCP-specific antibodies. They then used the antisera to screen a cDNA expression library derived from GCPs, to identify GCP-specific genes. Using this approach, they cloned 39 unique cDNAs, 28 of which represented novel genes and showed tissue- and stage-specific expression. The majority were expressed at high levels early in development (i.e., postnatal days 0–10), then downregulated by adulthood. Among these genes were at least four distinct expression patterns: 1) those that were localized in the outermost part of the EGL, where GCPs are proliferating; 2) those expressed in the inner EGL, where cells have exited the cell cycle and begun to differentiate; 3) genes expressed in the upper IGL, in postmitotic cells that had not yet completed migration; and 4) genes expressed in fully differentiated, postmigratory granule cells. These genes (some of which have now been cloned) (Miwa *et al.*, 1999) not only provide valuable molecular markers for the stages of granule cell development but also represent a set of molecules that may play important functional roles in this process.

More-recent studies of gene expression in the cerebellum have taken advantage of high-throughput approaches using polymerase chain reaction (PCR) or microarrays. For example, Matoba and colleagues (2000a,b,c) generated cDNA libraries from cerebella of 4-day-old (P4), 12-day-old (P12), and 6-week-old (6W) mice and sequenced several thousand clones from each library. They then chose approximately 400 of these genes (i.e., those with the highest expression levels) and quantitated their expression levels using a technique known as adapter-tagged competitive PCR (ATAC-PCR) (Figure 2A). This involves isolating RNA from each sample (i.e., stage of development), converting it to cDNA, and then "tagging" one end of that cDNA with an adapter. For each sample, the cDNA is tagged with a different-sized adapter. The samples from each stage are mixed into a single tube and amplified by PCR using an adapter-specific primer and a gene-specific primer. After separation by gel electrophoresis, products from each sample can be discriminated based on their size (determined by the unique adapter). The amount of each fragment reflects the amount of original template. Relative expression levels in each sample can be deduced from their signal intensities.

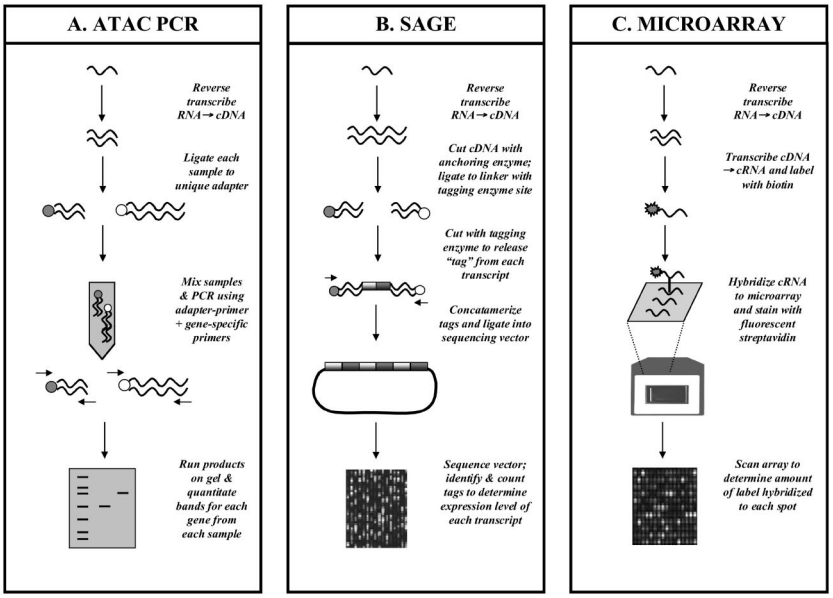

FIG. 2. Methods of genomic analysis. Procedures for adapter-tagged competitive polymerase chain reaction (ATAC-PCR), serial analysis of gene expression (SAGE), and Affymetrix GeneChip (Microarray) analysis are illustrated. [See text for details.]

Using this type of analysis, Matoba *et al.* (2000a,b) determined the expression of genes in the P4, P12, and 6W samples and divided genes into groups based on expression profiles. Although a number of different cell types were represented, the differences in gene expression profiles correlated best with the development of granule cells, which are by far the most abundant cell type. Early in development (P4), when GCPs are undergoing rapid proliferation, the most abundant genes are those encoding ribosomal proteins, cytoskeletal proteins (e.g., tubulin, actin, thymosin β4), and genes associated with proliferation (e.g., cdc2 kinase). Later in development (P12), when granule cells are engaged in axon extension and synapse formation, genes for mitochondrial activities (cytochrome c oxidase, mitochondrial proton/phosphate symporter), ion channels (Na/K-ATPase), and markers of differentiated neurons (NeuroD, glutamate transporter, trkC) are elevated. Expression of many of these genes continues to increase during development. Finally, at 6 weeks, when differentiation of cerebellar neurons and glia is complete, elevated expression of synaptic proteins (e.g., soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein SNAP25), neurotransmitter receptors (gamma aminobutyric acid receptors, GABA-Rs), and components of myelin (myelin basic protein and myelin proteolipid protein) are detected.

In a subsequent study, Matoba *et al.* (2000c) expanded their analysis to include 1800 genes expressed over six different time points in cerebellar development: 2, 4, 8, and 12 days and 3 and 6 weeks. Their results again were clustered into 1) genes elevated early in development (e.g., 2, 4, and 8 days); 2) genes elevated later in development (e.g., 12 days, 3 weeks, 6 weeks); and 3) genes with complex expression patterns. About 80% of these could be classified based on function. The investigators looked for correlations between temporal expression profiles and functional categories and found that genes expressed early in development were enriched for those encoding cancer-related proteins (e.g., adenomatous polyposis coli (APC), c-erbA, abl, Krox24/Egr1) and ribosomal proteins. Other genes in this group were involved in RNA processing, cell adhesion/migration (e.g., VN, tenascin, neural cell adhesion molecule (NCAM), reelin), signaling, and transcription. In contrast, the genes expressed primarily in late postnatal development included those involved in protein transport, carbohydrate metabolism, "brain-specific functions," ion channels and transporters (ryanodine receptor 2, potassium channels, sodium channels, Na/K ATPases), lipid metabolism and neurotransmitter receptors (GABA-A receptors, NMDA receptors, AMPA (alpha-amino-3-hydroxy-5-methylisoxasole proprionic acid) receptors) and synapse components, and genes associated with mature oligodendrocytes (myelin basic protein, myelin proteolipid protein). Having found a number of known genes with documented functions in cerebellar development, the authors suggest that this approach may be used to identify novel genes that regulate granule cell growth and differentiation.

Gene expression in the postnatal cerebellum has also been studied by Kaltschmidt and Kaltschmidt (2001). These investigators initially were interested in the role of the transcription factor nuclear factor kappa B (NF-κB) in neuronal survival. They found that NF-κB is expressed at high levels in the EGL before P7 and declines between P7 and P12. To identify signals that might account for this change in expression, the researchers used cDNA arrays to compare gene expression in P4 vs. P12 cerebellum. The arrays they used were commercial nylon membranes (macroarrays) spotted with 588 known cDNAs. mRNA from each developmental stage was labeled with ^{32}P and hybridized to a filter. Then, the level of hybridization was quantitated and compared using a phosphorimager. While most of the genes did not change, a small group ($\approx$ 40/588) was developmentally regulated; most of these increased between P4 and P12 but some decreased. Changes in expression were found among genes associated with proliferation and differentiation (c-myc, cyclin D3, cdc-like kinase 2, Id3), apoptosis and DNA repair (growth arrest and DNA damage (GADD45), topoisomerase II, TNF receptor-associated death domain (TRADD)), cell-signaling molecules (extracellular signal-regulated kinase (ERK)1, ERK3, jun kinase (JNK)3, mitogen-activated protein kinase (MAPK)2), cell adhesion (contactin 1, integrin β4, N-cadherin, lymphocyte function-associated antigen 1 (LFA-1)), and

growth factors and their receptors (insulin receptor, fibroblast growth factor receptor (FGFR)1, transforming growth factor (TGF)-β2, macrophage colony-stimulating factor (MCSF), fms-like tyrosine kinase (FLT-3) ligand).

The most highly induced gene during this period of development was the cytokine, TGF-β2. The authors confirmed expression of TGF-β2 in the EGL by *in situ* hybridization (ISH) and antibody staining and showed that expression levels increased significantly from P4 to P12. To test the functional effects of TGF-β2, they added the factor to granule cells in culture and showed that it causes a rapid decrease in expression of the transcription factor, NF-κB. Since TGF-β2 can promote granule cell apoptosis (de Luca *et al.*, 1996) and NF-κB can promote survival (Koulich *et al.*, 2001; Piccioli *et al.*, 2001), the authors suggest that increased expression of TGF-β2 may play a role in the apoptosis that occurs during normal granule cell development *in vivo* (Wood *et al.*, 1993).

Among the other genes whose expression increased from P4 to P12 were a number of elements of the FGF signaling pathway, including FGF receptor 1, MAPK2, ERK1, ERK3, and CREB2. As previously discussed, FGF signaling can promote granule cell-cycle exit and differentiation *in vitro.* The fact that multiple components of the FGF signaling pathway are coordinately regulated during a period of granule cell differentiation is consistent with the possibility that it plays this role *in vivo* as well. However, further studies of the effects of FGF signaling on granule cell development are necessary to validate this notion.

A more-comprehensive study of gene expression in the developing cerebellum was carried out by Zhao *et al.* (2002). These investigators initially examined gene induction in GCPs following Shh treatment, to identify target genes induced by this stimulus. They isolated cells from P4–5 cerebellum and treated them with Shh protein for 3 or 24 hours, then harvested RNA for microarray analysis using Affymetrix GeneChips (Figure 2B). These arrays consist of thousands of short oligonucleotides (25-mers) conjugated to a solid matrix. Each gene is represented by 10–15 oligonucleotides that match the coding sequence and a corresponding set of oligonucleotides that contain a one-base mismatch; the latter serve as controls for the specificity of hybridization. To analyze gene expression in a sample, RNA is isolated, converted to cDNA, and transcribed to generate biotin-labeled cRNA. This cRNA is hybridized to the Chip, incubated with a streptavidin-conjugated fluorescent dye, and scanned to detect the amount of fluorescence for each oligonucleotide. Analytical software is used to determine the expression levels of each gene and to compare expression between samples.

In their experiments, Zhao and colleagues found that $\approx$ 4% of the 13,000 genes on their microarrays were upregulated by Shh. Among these genes, $\approx$ 90% were involved in cell-cycle regulation (e.g., cyclins D1, B1, B2, A2, proliferating cell nuclear antigen (PCNA), E2F1, Ki67, cdc20). Other genes overexpressed in Shh-treated cells included transcription factors (Pax2, Math1, Gli2, N-myc) and genes involved in DNA replication and ribosome assembly. For comparison, the

researchers also examined cells that were growth arrested and then treated with Shh, to identify genes that are induced by Shh in postmitotic granule cells. Although Shh did induce gene expression in these cells, the targets were largely distinct from those induced in GCPs.

To test whether the targets of Shh in proliferating GCPs were expressed in these cells *in vivo*, Zhao *et al.* examined expression of a number of genes by ISH. These studies revealed that 10/12 of the genes analyzed were expressed in EGL at postnatal day 1–7 and were downregulated by postnatal day 15. Based on these findings, the authors proposed the concept of a temporal gene regulation profile (TRP) for the EGL: a set of genes that is temporally regulated in a pattern consistent with expression and function in proliferating GCPs in the EGL. In other words, genes whose expression was high from P1–P7 and low after P15 were likely to represent genes expressed in the EGL. If this concept could be validated, EGL-specific genes could be identified based solely on their expression profile during postnatal cerebellar development.

To test this hypothesis, the authors performed microarray analysis on whole cerebellum from P1–P30. In an initial test, they found that 10/12 genes that were known to be expressed in the EGL were expressed at high levels in P3–7 and downregulated from P15–30. To further validate this, they used microarray data to generate a list of genes whose developmental expression pattern fit that of the TRP-EGL, then examined their localization by ISH. The TRP-EGL profile correctly predicted EGL expression for more than 80% of the genes. These studies suggest that one can prospectively identify genes expressed in GCPs based on temporal changes in expression within the whole cerebellum.

These studies demonstrate that gene expression analysis can be a powerful tool for studying development and can provide valuable information about the genes that regulate cell growth and differentiation in a tissue. But when studying changes in gene expression during development – especially within an intact tissue – it is important to keep several issues in mind. First, changes in gene expression in the tissue may reflect changes in expression levels within a given cell type or changes in abundance of that cell type within the tissue. In fact, for much of cerebellar development, the abundance of granule cells tends to swamp out gene expression from most other cell types, including Purkinje cells, astrocytes, oligodendrocytes, and other interneurons. In order to learn about the genes that control development of these cells, it may be necessary to isolate them using laser capture microdissection (LCM) (Vincent *et al.,* 2002) or antibody-based purification methods such as panning or fluorescence-activated cell sorting (Trotter and Schachner, 1989; Baptista *et al.,* 1994; Catapano *et al.,* 2001; Sawamoto *et al.,* 2001). Alternatively, studies of mutant mice that have defects in particular cell types (Mullen *et al.,* 1997) may shed light on the gene expression patterns important for particular aspects of cerebellar development.

Regardless of the cell type or stage being studied, it is clear that gene expression analysis is only a first step towards understanding the molecular mechanisms of development. Once a profile of gene expression is identified, additional criteria must be used to narrow down the list of genes to a manageable number for further study. For example, one might focus on transcription factors or secreted proteins or genes that are likely to control cell adhesion or movement. Alternatively, it might be useful to perform additional screens to determine which of the expressed genes is dysregulated in a particular mutant mouse. Given a short list of genes, functional analysis — both *in vitro* and *in vivo* — will be necessary to understand how these genes contribute to a particular developmental process.

In addition to their importance in understanding cerebellar development, studies of gene expression in the normal cerebellum may have important implications for understanding the etiology of cerebellar tumors. For example, a number of genes expressed in the EGL and in GCPs stimulated with Shh (e.g., cyclin D1, N-myc, gli2) also have been shown to be expressed at high levels in some types of medulloblastoma (Pomeroy *et al.*, 2002). This raises the possibility that other genes involved in regulating cerebellar growth, differentiation, and apoptosis might play a role in tumorigenesis.

V. Medulloblastoma

Medulloblastoma is a highly malignant tumor of the cerebellum. It occurs most commonly in children between 5 and 10 years of age and accounts for 20–30% of all pediatric brain tumors (Schiffer, 1997; Zakhary *et al.*, 2001). The disease usually is treated with a combination of surgery, radiation, and chemotherapy. While these approaches often are effective at shrinking the primary tumor, recurrence and metastasis are common and only 50% of patients survive for 5 years after diagnosis. Moreover, aggressive treatment of children with radiation and chemotherapy has been found to impair intellectual and physical development (Zakhary *et al.*, 2001). New approaches to the diagnosis and treatment of medulloblastoma clearly are necessary and are most likely to come from a deeper understanding of the cellular and molecular basis of this disease.

Histologically, medulloblastoma often is divided into two major subtypes: classic and desmoplastic (Zakhary *et al.*, 2001; Pomeroy *et al.*, 2002). Classic medulloblastoma consists of small, round, densely packed cells that show little evidence of morphologic differentiation. Desmoplastic (or nodular) medulloblastomas contain regions of densely packed cells with extensive reticulin fibers surrounding “pale islands” of cells that are much less dense. The majority of medulloblastomas have the classic morphology; only 20–25% of tumors are considered desmoplastic. The cell of origin is not clear for either subtype but some studies suggest that desmoplastic tumors may arise from GCPs in the EGL,

whereas classic tumors may derive from multipotent precursors that surround the ventricles and normally give rise to Purkinje cells, cerebellar interneurons, and glia (Katsetos and Burger, 1994; Buhren *et al.,* 2000).

An important step in understanding the molecular basis of medulloblastoma came from studies of the Sonic hedgehog-Patched signaling pathway (Ingham and McMahon, 2001). As discussed, Shh is a secreted molecule that plays a critical role in embryonic development and is a major regulator of proliferation in the developing cerebellum (Dahmane and Ruiz-i-Altaba, 1999; Wechsler-Reya and Scott, 1999). Patched (Ptc) is a transmembrane protein that functions as both a Shh receptor and an antagonist of Shh signaling. Several lines of evidence indicate that Shh-Ptc signaling is involved in medulloblastoma. First, the human *patched* gene has been identified as the locus mutated in Gorlin's syndrome, a disease characterized by skin tumors, craniofacial defects, and increased incidence of medulloblastoma (Hahn *et al.,* 1996; Johnson *et al.,* 1996). Second, many sporadic medulloblastomas (especially those of the desmoplastic type) have been found to harbor mutations in *patched* and other elements of the Shh pathway (Pietsch *et al.,* 1997; Raffel *et al.,* 1997; Lam *et al.,* 1999; Taylor *et al.,* 2002). Finally, mice in which the *patched* gene has been disrupted develop tumors that resemble medulloblastoma (Goodrich *et al.,* 1997; Hahn *et al.,* 2000).

Among the other genes associated with human medulloblastoma are components of the Wnt signaling pathway. Patients with Turcot's syndrome – which results from mutations in the APC gene – have a high incidence of colorectal cancers and brain tumors, especially medulloblastoma (Hamilton *et al.,* 1995). About 4% of sporadic medulloblastomas have been shown to contain APC mutations (Huang *et al.,* 2000). In addition, 8–15% have been reported to harbor activating mutations in β-catenin and 12% have mutations in Axin, a negative regulator of Wnt signaling (Zurawel *et al.,* 1998; Eberhart *et al.,* 2000; Huang *et al.,* 2000; Dahmen *et al.,* 2001). These findings suggest that a subset of medulloblastomas may result from activation of the Wnt pathway. Medulloblastomas also have been found to exhibit overexpression of the transcription factors N-myc, c-myc, pax5, and zic and of the receptor tyrosine kinase ErbB2 (Garson *et al.,* 1989; Bigner *et al.,* 1990; Gilbertson *et al.,* 1995; Kozmik *et al.,* 1995; Yokota *et al.,* 1996). Whether these genes contribute to the development or progression of medulloblastoma, or whether they simply represent markers of the transformed cell type, remains to be determined. Animals in which these genes are misexpressed will provide valuable insight into their role in the etiology of medulloblastoma.

One of the most important genes involved in medulloblastoma has yet to be identified. Of human medulloblastomas, 30–50% have a deletion or rearrangement of part of chromosome 17 (Cogen and McDonald, 1996; Bigner *et al.,* 1997; Burnett *et al.,* 1997). In most cases, the short arm (17p) is lost and head-to-head apposition of the long arms (17q) occurs, which is referred to as

isochromosome 17q [i(17q)]. This rearrangement frequently is detected in leukemias, lymphomas, and cancers of the stomach, colon, and cervix. The loss of 17p in a number of types of cancer suggests that at least one potent tumor suppressor gene is located there. Fine mapping of deletions from different tumors has narrowed the region of interest considerably. Most investigators now believe that the putative tumor suppressor is located at 17p13.3, a region of $\approx$ 20 known genes, including those encoding the lissencephaly-associated protein Lis1, the breakpoint cluster region (BCR)-related gene *ABR*, the Max-binding protein Mnt, and the transcription factor hypermethylated in cancer-1 (Hic1) (Koch *et al.*, 1996; Steichen-Gersdorf *et al.*, 1997; Sommer *et al.*, 1999; Hoff *et al.*, 2000; Rood *et al.*, 2002). Although none of these genes has been linked definitively to the etiology of medulloblastoma, there is no question that when the chromosome 17 tumor suppressor is identified, it will provide important insight into the basis of cerebellar tumors as well as into normal cerebellar development.

VI. Gene Expression Profiling of Medulloblastoma

In an effort to identify important genes involved in medulloblastoma, a number of investigators have begun to carry out gene expression analysis on primary tumor samples. One of the first efforts was performed by Michiels and colleagues (1999), who analyzed gene expression in a human medulloblastoma sample using serial analysis of gene expression (SAGE) (Figure 2C). SAGE is based on the principle that a sequence of 9–10 nucleotides can be used to identify a transcript, if the position of these nucleotides within the transcript is known. A biotinylated oligo(dT) primer is used to synthesize cDNA from mRNA and, after digestion with a restriction enzyme, fragments are isolated from the 3′ end of each transcript. Fragments are ligated to linkers, then cleaved with a restriction enzyme to release a short sequence (i.e., 9–10 bp) from a defined region of the original cDNA (a "tag"). Tags are ligated together to form long concatamers, which are cloned and sequenced. Using this approach, one sequencing reaction can yield information about the number and distribution of many different tags. With the appropriate software, the sequence and position of the tag can be used to identify the gene from which each tag was derived. Moreover, the number of times each tag appears in the sequencing reaction is proportional to the abundance of that gene in the original sample. In this manner, SAGE gives a quantitative representation of gene expression in a sample.

For their study, Michiels and colleagues used SAGE to compare genes expressed in medulloblastoma to those expressed in 24.5-week fetal brain. They sequenced 10,000 tags from each sample and found about 6000 unique genes in each case. Among the most highly expressed genes in both samples were those encoding ribosomal proteins, consistent with the idea that both of these tissues contain highly proliferative (and protein-synthesizing) cells. Comparing the

medulloblastoma and fetal brain samples, the investigators found 138 genes whose expression differed significantly. About half of these were known genes, including the transcription factors Zic1, Otx2, and Sox4; the secretory protein secretogranin; vascular endothelial growth factor (VEGF); and PCNA. Expression of Zic1 and Otx2 was examined by Northern blotting and found to be elevated in a number of independent medulloblastoma samples. The fact that both of these genes are expressed in the EGL lends support to the notion that at least some cases of medulloblastoma arise from GCPs.

A particularly elegant study of medulloblastoma gene expression was carried out by Pomeroy and colleagues (2002), who used Affymetrix GeneChips to compare medulloblastoma to other types of brain tumors. Since pathologists commonly have grouped medulloblastoma with primitive neuroectodermal tumors (PNETs) from other parts of the brain, the investigators were particularly interested in determining how similar these tumor types were. Studying the gene expression profiles of 42 brain tumors — including medulloblastoma, malignant glioma, atypical teratoid/rhabdoid tumors, and PNETs — they found that each of these tumor types was molecularly distinct. For example, gliomas expressed markers of astrocytic and oligodendrocytic cells (phosphoprotein-enriched in astrocytes-15 kDa (PEA15), SRY-related box2 (SOX2), peripheral myelin protein 2 (PMP2), Olig-2, S100, glial fibrillary acidic protein (GFAP)), whereas medulloblastomas expressed genes characteristic of cerebellar granule cells (e.g., Zic, neurological stem cell leukemia transcription factor (NSCL1)). Interestingly, although medulloblastoma and PNET look similar morphologically, they are molecularly distinct, with PNETs lacking Zic and NSCL1 and expressing high levels of nicotinic cholinergic receptor subunits and DNA polymerase delta 1 (POLD1).

Pomeroy *et al.* next addressed whether the two major subtypes of medulloblastoma described by pathologists – classic and desmoplastic – had distinct gene expression profiles. Consistent with the observation that desmoplastic tumors have a high incidence of Shh pathway mutations (Pietsch *et al.*, 1997; Taylor *et al.*, 2002), the researchers showed that desmoplastic tumors express high levels of Patched, Gli1, IGF-2, and N-myc, which have been identified as transcriptional targets of the Shh pathway (Goodrich *et al.*, 1996; Hahn *et al.*, 2000; Wetmore *et al.*, 2000; Zhao *et al.*, 2002). In addition, desmoplastic tumors express high levels of the antiapoptotic protein Bcl2 and ribosomal proteins, similar to those seen in normal EGL GCPs. In contrast, classic tumors show elevated levels of TGFβ3 and elements of its signaling pathway (Smad2, Smad5) and increased expression of the transcription factors distal-less homeobox gene 7 (DLX7), LIM-homeobox domain protein-2 (LH-2), and NeuroD3. These data clearly indicate that desmoplastic and classic medulloblastoma are not only histologically distinct but also have different gene expression profiles.

In interpreting these differences, it is important to consider several possibilities. First, they may reflect differences in the cell type of origin or cellular composition of the tumors. For example, desmoplastic tumors may arise from GCPs, while classic tumors may arise from multipotent precursors in the ventricular zone. On the other hand, these tumors could arise from the same cell type. Differentially expressed genes might reflect activation of distinct signaling pathways that contribute to the etiology of tumor formation. Determining the functional significance of each of these genes is an important task for future study.

Independent of the role of these genes in the etiology of tumor formation, they may be significant as indicators of tumor prognosis and responsiveness to therapy. This is particularly important in medulloblastoma, since a significant percentage of patients may be cured by chemotherapy and radiation, while others are resistant and succumb to the disease. To address this possibility, Pomeroy *et al.* analyzed tumors from patients who had been treated and followed to determine the outcome of treatment. The data then were analyzed using a "supervised learning" scheme to determine which genes were best correlated with long-term survival. Among the best predictors of survival were genes associated with cerebellar differentiation (the vesicle coat protein β-NAP, the transcription factor NSCL1, the neurotrophin receptor TrkC, and sodium channels), and genes encoding extracellular matrix proteins (procollagen lysyl hydroxylase (PLOD), collagen type Vα1, elastin). On the other hand, poor prognosis was associated with increased expression of ribosomal proteins, the proliferation-associated genes B-myb and E2F5, and metabolic genes such as lactate dehydrogenase and cytochrome C oxidase. The multidrug resistance gene sorcin also was associated with poor outcome. Exactly how these genes might contribute to tumor progression (or resistance to therapy) remains to be determined. However, as a diagnostic tool, the ability to predict tumor outcome based on gene expression profile is likely to be extremely valuable.

A study of gene expression in metastatic medulloblastoma by MacDonald *et al.* (2001) may offer similar advantages. About one third of patients with medulloblastoma have metastatic disease at time of diagnosis and these patients are known to have a particularly poor outcome. In fact, disseminated disease is among the most powerful predictors of poor survival in medulloblastoma patients. To find molecular markers of metastatic medulloblastoma, these researchers used Affymetrix GeneChips to analyze gene expression in 10 metastatic (M+) and 13 nonmetastatic (M0) tumors. Of the 1992 genes on these arrays, 59 (3%) showed significantly increased expression in M+ tumors and 26 (1%) showed significantly decreased expression. One prominent category of regulated genes included cell-surface and secreted proteins involved in adhesion and angiogenesis: α-catenin; α- and β-integrins; secreted protein, acidic and rich in cysteine (SPARC); tissue inhibitor of matrix metalloproteinases (TIMP1); and

TIE (a receptor for angiopoietin). Genes involved in growth factor receptor-ras-MAP kinase signaling (platelet-derived growth factor receptor alpha (PDGFRA), FGF receptor 2, the adapter protein Src homology/collagen-related-1 (SHC1), the guanine nucleotide exchange factor (GEF2), rac-kinase β, protein kinase C-IIβ, JNK1) also were differentially expressed in metastatic vs. nonmetastatic tumors. Finally, metastatic tumors showed altered expression of transcription factors, including Hox A4 and A7 (which were overexpressed) and Nur77 and c-myb (which were expressed at lower levels). No differences were seen in expression of N-myc, patched, or ErbB2, which had previously been shown to be associated with medulloblastoma.

MacDonald and colleagues went on to develop an algorithm to predict whether a tumor was metastatic or nonmetastatic, based on its gene expression profile. This algorithm was able to predict tumor class with 72% accuracy and correctly categorized four new tumors. It predicted nonmetastatic tumors more accurately than metastatic ones, possibly because metastatic tumors are more heterogeneous with respect to cell type or gene expression profile. In addition to primary tumors, the authors analyzed the gene expression profile of several medulloblastoma cell lines. Although some of these lines could not be categorized, Daoy cells, a commonly used medulloblastoma cell line, clearly were categorized as metastatic.

The authors were particularly interested in elevated expression of PDGFRA and elements of the Ras-MAP kinase pathway in metastatic tumors, since PDGF has been shown to regulate angiogenesis, adhesion, and metastasis in other systems. They confirmed expression of PDGFRA protein in a panel of independent metastatic tumors as well as in Daoy cells. They then showed that soluble PDGFA causes activation of the Ras-MAPK pathway (including phosphorylation of MEK1, MEK2, and p42/p44 MAPK) in Daoy cells and enhances migration of these cells in culture. These effects could be prevented by PDGFRA-blocking antibodies and by MEK inhibitors. These findings suggested that inhibitors of the PDGFR or the Ras pathway could be used to treat metastatic medulloblastoma.

Together, these studies clearly demonstrate that gene expression profiles can be used to categorize tumors and to learn about their etiology. They also can be employed as diagnostic tools to predict prognosis and to choose appropriate treatment strategies. Finally, by highlighting signaling pathways that are dysregulated in tumors, gene expression information may yield new molecular targets or new approaches to treating tumors.

As with studies of normal development, there are a number of caveats to studying gene expression in intact tumors. First, tumors are heterogeneous and may contain a variety of cell types, including proliferating tumor cells, tumor cells that have undergone differentiation or apoptosis, blood and endothelial cells, and reactive astrocytes. Again, isolation of these cell types may yield important information about the molecular mechanisms of tumorigenesis. In

addition, it is important to note that differences in gene expression between normal and tumor cells (or between different types of tumors) may reflect differences in the cell types represented, in genes that are important for the etiology of the tumor, or genes that are expressed as a consequence of tumor growth. Distinguishing between these possibilities may not be important if the goal is to identify prognostic or diagnostic markers but will be critical for understanding the underlying mechanisms of tumorigenesis.

ACKNOWLEDGMENTS

The authors thank Audra Carroll for designing the figures and Trudy Oliver for critical review of the manuscript.

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