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Neonatal and Adult O4⁺ Oligodendrocyte Lineage Cells Display Different Growth Factor Responses and Different Gene Expression Patterns

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Abstract

Oligodendrocytes are the myelinating cells of the central nervous system. While the CNS possesses the ability to repair demyelinating insults, in certain cases, such as the chronic lesions found in multiple sclerosis, remyelination fails. Cycling cells capable of becoming oligodendrocytes have been identified in both the developing and adult mammalian forebrain. Many studies have focused on differences in gene expression profiles as oligodendrocyte progenitors differentiate into myelinating oligodendrocytes by isolating cells at different developmental stages from animals at a single age. However, few have studied the differences that exist between the progenitors of the neonatal CNS and those of the adult CNS. This study examined the response of neonatal and adult O4⁺ cells to the growth factors PDGF-AA, bFGF, and IGF-1, and revealed marked differences. While adult cells readily differentiated *in vitro*, the majority of neonatal progenitors remained immature. Microarray analysis was used to examine differences between acutely isolated neonatal and adult progenitors further. Gene expression profiles found that the adult O4⁺ cells are more developmentally mature than neonatal cells. Neonatal cells expressed higher levels of genes involved in proliferation. Adult O4⁺ cells expressed higher levels of transcripts for genes involved in cell death and survival. Therefore, O4⁺ cells from the adult differ greatly from those of the neonate and the developmental stage of the animal models utilized must be taken into consideration when applying principles from neonatal systems to the adult.

Keywords

O4⁺ oligodendrocyte progenitors; microarray; growth factors

Introduction

Demyelinating lesions are the main pathological feature of multiple sclerosis and are also found in other illnesses such as leukodystrophies and viral infections or as the result of injury, trauma, or exposure to toxic compounds. The CNS possesses the ability to repair such damage, though remyelination occurs to varying degrees ranging from complete restoration of function to the chronic demyelinated lesions found in multiple sclerosis (Ludwin 1997). To develop new therapies for demyelinating diseases, researchers are studying the regulation of oligodendrocyte progenitor differentiation. Many studies have focused on changes in gene expression as progenitors differentiate into myelinating

oligodendrocytes (Blasi et al. 2002; Cahoy et al. 2008; Dugas et al. 2006; Nielsen et al. 2006; Scarlato et al. 2000). Most of this research utilized cells isolated from 1-3 week old animals. However, it is the oligodendrocyte progenitor cells of the adult brain that are called upon to remyelinate.

Neonatal progenitors were first identified in the developing optic nerve and were later discovered in other regions of the CNS (Raff 1989). Since then, a large body of work has accumulated detailing their properties (Baumann and Pham-Dinh 2001). Growth factors such as platelet-derived growth factor-AA (PDGF), insulin-like growth factor-1 (IGF), fibroblast growth factors, ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3) modulate their development *in vivo* and *in vitro* (McMorris and McKinnon 1996; Webster 1997). Transplantation studies in various models of demyelination have shown that transplanted neonatal cells can remyelinate demyelinated axons (Franklin and Blakemore 1995; Groves et al. 1993).

Oligodendrocyte progenitors in the adult CNS were first identified in the rat optic nerve (Ffrench-Constant and Raff 1986). Immature cycling cells capable of differentiating into oligodendrocytes have also been isolated in the adult mammalian forebrain, providing a population of cells that may be utilized in the remyelination of lesions (Gensert and Goldman 1997; Gensert and Goldman 2001). Adult progenitors isolated from the rat optic nerve exhibit significant differences in proliferation, migration, and differentiation in comparison to their neonatal counterparts (Wolswijk and Noble 1989) although these cells were identified and isolated by their binding of the A2B5 monoclonal antibody, rather than the O4 antibody. This study raised the important idea that adult and neonatal progenitors possess inherent differences and that information gleaned from neonatal or adolescent studies cannot be assumed to be applicable to their adult counterparts. Therefore, it is important that studies be done to determine whether or not cells from the neonatal and adult CNS are comparable in their responses to different stimuli and in their regulation of proliferation and differentiation.

An earlier project in the lab examined the effect of PDGF, fibroblast growth factor -2 (bFGF), and IGF on the proliferation and differentiation of O4⁺ cells from the adult forebrain (Mason and Goldman 2002). Utilizing the same methods, we have performed an identical study using O4⁺ cells isolated from the neonatal forebrain to determine whether or not these retain comparable responses to stimuli into adulthood. The growth factor response of neonatal cells differed from those of the adult cells. A far greater proportion of adult O4⁺ cells differentiated in culture. Furthermore, a BrdU labeling index was far lower in the adult cells. To examine further the differences between neonatal and adult O4⁺ cells, the gene expression profiles of acutely isolated neonatal and adult O4⁺ progenitors was analyzed using Affymetrix microarrays. Differentially regulated transcripts revealed that significant differences exist between neonatal and adult progenitors with respect to genes that are associated with developmental stage, proliferative capacity, and cell survival.

Materials and Methods

Media

Unless otherwise stated, all media reagents were purchased from Sigma (St. Louis, MO). CDMEM: DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen, Carlsbad, CA), 1mM sodium pyruvate (Invitrogen), and 100 µg/ml penicillin/streptomycin (Invitrogen). Chemically Defined Media (CDM): A modified version of the N2 media described in (Bottenstein and Sato 1979), N2B3, that is composed of DMEM/F12 (Invitrogen), 1 mg/ml BSA, 10 ng/ml d-biotin, 5 µg/ml insulin, 20 nM progesterone, 100 µM putrescine, 1.2 g/L sodium bicarbonate, 5 ng/ml selenium, 50 µg/ml transferrin, 15 mM HEPES, 30 nM

triiodothyronine (T3) and 100 µg/ml penicillin/streptomycin. Isolation Media: 0.9M sucrose in 1x MEM (Invitrogen) supplemented with 20mM HEPES, pH 7.2. O2A Media: Composed of DMEM, 10 ng/ml d-biotin, 5 µg/ml insulin, 20 nM progesterone, 100 µM putrescine, 5 ng/ml selenium, 50 µg/ml transferrin, 2 mM glutamine, 15 mM HEPES, 100 µg/ml penicillin/streptomycin.

Progenitor Isolation

O4⁺ cells were isolated from the forebrains of P2 Sprague-Dawley rat pups and the subcortical white matter of adult female rats (200-250g). The tissue was mechanically and enzymatically dissected as described elsewhere (Gensert and Goldman 2001). Briefly, tissue was shredded using forceps and digested in a solution containing 0.125% trypsin (Invitrogen), 20U/ml papain (Roche Applied Science, Indianapolis, IN), and 285U/ml DNase (Sigma, St. Louis, MO) at 37°C in a shaking water bath. Undigested tissue was triturated with a Pasteur pipet, filtered through 70 µm Nitex mesh and the trypsin neutralized with an equal volume of CDMEM. Adult tissue was subjected to additional enzymatic treatment until completely dissociated. The single cell solution was centrifuged at 1000xg for 10 minutes and the cell pellet resuspended in Isolation Media. This cellular suspension was centrifuged at 1000xg for 10 minutes to enrich for cycling progenitors. The resulting pellet was resuspended in O2A Media. O4⁺ progenitors were isolated by immunopanning to >95% purity. Immunopan plates were prepared by incubating 60mm petri dishes overnight with goat anti-mouse IgM (20µg/ml; Rockland Immunochemicals, Gilbertsville, PA) in 50mM Tris (pH 9.5) at 37°C. The plates were then washed 3X with CMF-PBS, incubated with O4 supernatant (American Type Culture Collection (ATCC), Manassas, VA) for 2 hrs, and blocked with 10% FBS/10% NGS for 30 min. Neonatal cells were pre-plated on 100cm tissue culture dishes to deplete microglia before application to immunopan plates. Dissociated cells were incubated in the immunopan plates for 1 hr at 37°C. Plates were then vigorously washed with CMF-PBS to remove immunonegative cells. O4⁺ cells were detached using trypsin-EDTA (Invitrogen) applied for 10 min at 37°C. After deactivation of the trypsin using CDMEM, progenitor cells were pelleted by centrifugation. All animal experiments were performed under the guidelines of the Columbia University Institutional Animal Care and Use Committee.

Cell Culture

O4⁺ progenitors were resuspended in CDM at 2×10^5 cells/ml and plated onto 8-well glass chamber slides (Nunc, Rochester, NY) in droplets and allowed to settle for 10 min before the addition of CDM to the appropriate volume with one or a combination of the following growth factors from Sigma: platelet-derived growth factor-AA (PDGF) (10ng/ml), basic fibroblast growth factor (bFGF) (10ng/ml), and insulin-like growth factor-1 (IGF) (10ng/ml). The final density was 2×10^4 cells/cm². Media was changed every two to three days. For proliferation studies, 10µM 5'-bromo-2'-deoxyuridine (BrdU; Sigma) was added to cell cultures for 4hr before fixation and immunodetection.

Immunofluorescence Staining

Cultured cells were fixed in a 4% paraformaldehyde (PFA) solution for 20 min at RT stored in PBS at 4°C until use. Cells to be stained with intracellular markers were permeabilized with ice-cold acetone for 7 minutes. Slides were blocked with 20% goat serum (Sigma) for 20 min and all primary antibodies were applied overnight at 4°C. Cultures to be stained with surface markers were incubated in the surface marker antibody overnight, washed 3X with PBS, and fixed with 4% PFA before permeabilization with ice-cold acetone for 8 min. Cultures were then blocked and incubated in primary antibodies to intracellular markers. Surface markers include: A2B5 hybridoma supernatant (1:5; ATCC), O4 hybridoma supernatant (1:5; ATCC), and O1 (1:10 ATCC). Intracellular markers include: mouse anti-

vimentin (1:500; DAKO Cytomation, Carpinteria, CA), rabbit anti-GFAP (1:500; DAKO Cytomation), and mouse anti-TUJ1 (1:500; Covance, Berkeley, CA). Cells were washed 3X with PBS and incubated in Alexa-Fluor conjugated secondary antibodies (1:2000, Invitrogen, Carlsbad, CA) or FITC conjugated goat-anti rabbit secondary antibody (Southern Biotech, Birmingham, AL) for one hour at room temperature and then counter-stained with Hoescht 33342 nuclear stain. BrdU was detected utilizing a 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit (Roche Applied Science). Images were captured using a Nikon Eclipse E800 microscope and Spot imaging software.

RNA purification and microarray analysis

After immunopanning, O4⁺ oligodendrocyte progenitor cell pellets were flash-frozen in liquid nitrogen and stored at -80°C. Multiple neonatal and adult samples were combined to isolate total RNA using an RNeasy kit (Qiagen, Valencia, CA). 5 µg of each of the total RNA samples were then processed as described in the Affymetrix Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, total RNA was converted into cDNA, transcribed into biotinylated cRNA using the IVT kit provided, and fragmented. Samples were hybridized to RG-U34 A (Affymetrix) gene chips and processed at the Herbert Irving Comprehensive Cancer Center Microarray facility according to Affymetrix protocols. Two neonatal microarrays and one adult microarray were processed. Microarray data was processed using Genespring GX 9.0 software (Agilent Technologies, Santa Clara, CA). The data was normalized using Genespring's per chip normalization and per gene normalization. A two-fold difference in normalized expression levels was used to identify gene transcripts that are differentially expressed between neonatal and adult progenitors. Functional pathways and components were identified using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com). The dataset consisted of fold-change data generated by GenespringGX normalization and comparison analysis. P-values were calculated using Fisher's Exact Test.

Semi-Quantitative RT-PCR

Total RNA was isolated from O4⁺ OPCs using an RNeasy kit (Qiagen) and reverse-transcribed using the Thermoscript RT-PCR system for first strand cDNA synthesis (Invitrogen). PCR using Platinum Taq (Invitrogen) was performed using the primers shown in Table IV. All primers were designed to amplify sequences that contain introns, so that spliced mRNA could be distinguished from contaminating genomic DNA and were tested for accuracy using the NCBI/Primer-BLAST tool. Primers for MAG were taken from the literature (Gupta et al. 2005), while primers for PLP, PDGFRalpha, Transferrin and 18S RNA were generated using the Lasergene software. RT-PCR for 18S RNA was used as control.

Western Blots

O4⁺ cells were lysed using the CellLytic™M Cell Lysis Reagent with Protease Inhibitor Cocktail (Sigma) and protein levels were quantified using the Bradford assay. 10 µg of total protein were separated under reducing conditions on Nupage 4-12% Bis-Tris gels (Invitrogen) at 100V for 90 min and blotted on nitrocellulose membrane. The blots were blocked using 5% dry milk in TBS-T (Tris-buffered saline with 0.05% Triton-X) for 60 min at room temperature and incubated at 4°C overnight in 3% dry milk in TBS-T with the following primary antibodies: sheep anti-Carbonic anhydrase II (1:2000, Serotec, UK), mouse anti-CNPase (1:500, Covance), rabbit anti-αB-crystallin (1:2000, Stressgen, Victoria, BC, Canada), mouse anti-MBP (1:500, Covance), rabbit anti-PLP (1:1000), mouse anti-MAG (1:1000, kind gift from Dr. M. T. Filbin, Hunter College, New York), rabbit anti-transferrin (1:1000, kind gift from Dr. J. Barasch, Columbia University, New York), rabbit anti-PDGFRalpha (1:500, kind gift from Dr. P. Canoll, Columbia University, New York).

Blots were then washed 3 times in TBS-T and incubated in 3% dry milk in TBS-T with the appropriate HRP-conjugated secondary antibody (donkey anti-goat 1:1000, Southern Biotech, goat anti-mouse and goat anti-rabbit, 1:1000, Pierce Biotechnology, Rockford, IL) for 60 minutes at room temperature. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). To ensure equal protein loading, the blots were reprobed using a mouse anti-GAPDH antibody (1:1000, EnCor Biotechnology).

Results

Comparison of the Growth Factor Responses of Neonatal and Adult O4⁺ Progenitors

O4⁺ cells were purified from P2 rat forebrains by sequential immunopanning and plated onto eight-well chamber slides at 200 cells/μl to a density of 2×10^4 cells/cm² in a serum-free medium supplemented with the growth factors, PDGF-AA (10ng/ml), bFGF (10ng/ml), and IGF (10ng/ml), individually and in combination. The initial composition of the O4⁺ panned population was examined after 2-3 hours to allow the cells time to settle. Cells were immunostained with TuJ1 to label neurons, vimentin to label immature astrocytes, GFAP to label mature astrocytes, and three stage-specific oligodendrocyte markers, A2B5, O4, and O1. Over 95% of the cells were immunopositive for O4. We found rare TuJ1⁺/O4⁺ cells (<0.5%) and no GFAP⁺ astrocytes. Approximately 5% of the cells were A2B5⁺ and 6% were O1⁺. Surprisingly, approximately 5% of the cells were vimentin⁺ and co-labeled with both A2B5 and O4. This “hybrid” cell phenotype was unique to the neonatal progenitors as no counterpart was detected in the adult population.

The O4⁺ cells were cultured for four days before fixation and immunostaining for A2B5, O4, and O1 to determine how their developmental responses to the growth factors differed from those observed in an earlier study of their adult counterparts (Mason and Goldman 2002). The most striking difference between the two populations was their ability to differentiate into O1⁺ cells. Adult O4⁺ cells readily differentiated, with 50% of the progenitors becoming O1⁺ in serum-free medium. (Mason and Goldman 2002). The addition of growth factors induced further differentiation, with as much as 80% of the progenitors acquiring O1 expression after four days. In contrast, no more than 30% of the neonatal O4⁺ progenitors acquired O1 after four days in culture (Figure 1). Unlike the adult progenitors, for which the presence of bFGF and/or IGF enhanced differentiation, no growth factor increased the differentiation of the neonatal progenitors above that of medium alone. In fact, bFGF significantly decreased the number of O1⁺ cells in the neonatal population ($p < 0.01$ in comparison to control). A similar inhibition of differentiation by bFGF was previously observed in neonatal O4⁺ progenitor cultures (Gard and Pfeiffer 1993; McKinnon et al. 1990).

A large fraction of the neonatal O4⁺ cells appeared to acquire A2B5 immunostaining, suggesting reversion to a less mature stage (Figure 1). PDGF and FGF together increased the percentage of A2B5⁺ cells in the cultures, so that a large majority were A2B5⁺. In contrast, only 10% of adult O4⁺ cells expressed the A2B5 antigens in serum-free medium and adding PDGF was only able to increase A2B5 acquisition to 25% (Mason and Goldman 2002).

Neonatal and adult O4⁺ cells also responded differentially to the growth factors with respect to proliferation, as determined by BrdU incorporation during a 4 hr pulse. Neonatal O4⁺ cells displayed a basal labeling index of about 20%, which could be increased almost 2-fold by a combination of growth factors (Figure 2). As previously described, PDGF and bFGF, alone and in combination, were capable of increasing the proliferation of neonatal progenitors (Gard and Pfeiffer 1993). In contrast, the adult O4⁺ cells showed a basal level of BrdU labeling of less than 10% (Mason and Goldman 2002). In that work, a combination of

all three growth factors was sufficient to increase the proliferation of adult progenitors to approximately 15%.

Microarray Analysis of Neonatal and Adult O4⁺ Populations

To examine differences in gene expression between neonatal and adult O4⁺ cells, we examined expression profiles of the two populations using Affymetrix RG U24A microarrays. O4⁺ cells were isolated from the P2 rat forebrain and adult subcortical white matter and immediately frozen for RNA extraction. Multiple samples were combined to harvest sufficient total RNA for processing according to Affymetrix protocols. The data from two neonatal microarrays and one adult microarray were analyzed using GeneSpringGX 9.0. A two-fold cutoff yielded 799 differentially expressed probe sets representing 584 unique transcripts, 506 of which are known genes. 214 of the 506 known genes are more highly expressed in the adult O4⁺ cells and 292 are more highly expressed in neonatal cells. Complete lists of transcripts that are differentially regulated in the neonate and adult are available as Supplemental Material (see Supplemental data as Excel spread sheet).

Verification of Microarray Data

To verify the validity of the microarray data, semi-quantitative RT-PCR was performed on several of the genes identified by the analysis. While not as sensitive as quantitative RT-PCR, this procedure is sufficient to determine if the changes in the transcript levels detected follow the same trend as that determined by the microarray data. Among the transcripts most highly upregulated in adult progenitors are those for genes encoding myelin sheath components and myelin-associated proteins (Table I). Additional genes with lower fold-changes (2-4 fold) were also selected for verification. Total RNA was extracted from acutely isolated neonatal and adult progenitors and reverse transcribed into cDNA using random oligomers. Gene-specific primers were then used to PCR amplify the following cDNAs: proteolipid protein (PLP), myelin-associated glycoprotein (MAG), myelin basic protein (MBP), transferrin (Tf) and cyclic nucleotide phosphodiesterase 1 (CNPase). In addition to the myelin-associated genes, several other oligodendrocyte-enriched genes were also examined such as: platelet derived growth factor receptor - alpha (PDGFR α), carbonic anhydrase II (CA2) and α -B crystallin (Cryab). The results show that there is a difference in the levels of six out of nine transcripts that parallels the Affymetrix array data (Figure 3). CA2 (array fold change 30.38), Cryab (24.10), Tf (78.57), and MAG (28.26) were more highly expressed in the adult O4⁺ cells, while PDGFR α (8.44) was more highly expressed in the neonatal O4⁺ cells. There were slight increases in the CNPase, PLP and MBP transcript levels in the adult O4⁺ cells. CNPase was only slightly increased in the microarray results as well (fold change 2.70). As for MBP (fold change 14.88) one possible explanation could be the presence of multiple transcripts. Five additional splice variants were amplified in the RT-PCR, which are not detected by many of the probes within the MBP probe set. The PLP microarray data shows a larger difference than does the PCR, a difference that deserves further study. However, protein levels of PLP are far higher in the adult cells (see below).

Microarray data provides a measure of the levels of transcripts present at any given time point. However, variation in the levels of transcripts does not always correspond to similar differences in protein levels. Western blot analysis was performed to determine if the changes in transcript levels of the above genes were reflected in the levels of proteins present in acutely isolated O4⁺ cells. The results confirm the observed transcript level differences in seven out of eight proteins examined. In particular, CA2, CNPase, Cryab, MAG, PLP and Tf are more highly expressed in the adult O4⁺ cells (Figure 3). However, with the exception of CA2 and Tf, the presence of transcripts does not correspond to protein expression, at least at detectable levels, in the neonatal O4⁺ cells. PDGFR α which is more

highly expressed in the neonatal cells, also shows higher protein levels in neonatal cells. Interestingly, MBP protein could not be detected in either neonatal or adult O4⁺ cells. This observation may be explained by the fact that MBP mRNA is enriched in oligodendrocyte processes, where it is translated into protein (Barbarese et al. 1999; Campagnoni et al. 1991). MBP mRNA starts to accumulate in differentiating oligodendrocytes after 3-4 days in culture and the protein can be detected 1-2 days later (Francone et al. 2007). In contrast, other myelin-specific proteins are synthesized in the oligodendrocyte cell body, like PLP and MOG (Holz et al. 1996; Sorg et al. 1986).

Functional Pathways that are Differentially Regulated in Adult O4⁺ Cells

Ingenuity Pathway Analysis (IPA) was utilized to identify and examine the components that interact or are involved in particular functions that were differentially regulated in the two progenitor populations. Analysis of the transcripts present at higher levels in the adult O4⁺ cells revealed 26 functions in which gene expression was altered (Figure 4). The four most significant functions include genes involved in cellular compromise, cell death, cell morphology, and cellular organization and assembly. Cellular compromise consists of seven genes that are involved in the degeneration of cell processes and plasma membrane projections (Supplementary Table I). Cell morphology included the genes involved in myelination that were utilized in the verification of the microarray data (Table I; $p=2.18 \times 10^{-5}$ as determined by IPA). Both cell morphology and cellular organization consisted of 19 genes that affect the growth of cell processes and plasma membrane projections with certain genes increasing outgrowth and others decreasing outgrowth. However, the genes involved in cell death comprise the largest functional group with 88 members (Table II; $p=1.98 \times 10^{-5}$). 64 of the genes affect cell death and survival in various experimental systems; 44 genes increase cell death while 43 of the genes decrease cell death instead. Several of the genes, such as *App* and *RhoB*, appear to be associated with multiple functions and are able to either promote or inhibit cell death under the appropriate conditions.

Functional Pathways that are Differentially Regulated in Neonatal O4⁺ Cells

Utilizing IPA to study the transcripts that were higher in the neonatal O4⁺ cells revealed 23 functions that are differentially regulated (Figure 5). The most significant of these functions was that of protein synthesis. Among the 30 genes associated with the synthesis of protein are 3 that are believed to increase protein synthesis: cyclin D1 (*Ccnd1*), cyclin-dependent kinase 4 (*Cdk4*), ribosomal protein S6 (*Rps6*), and one gene that is known to decrease protein synthesis, p53 (Table III). The 19 genes involved in cellular development encompass multiple processes including cellular growth, fate determination and differentiation, and the G1/S phase transition. Finally, 11 transcripts for genes that participate in post-transcriptional modifications of RNA are expressed at higher levels in neonatal O4⁺ cells. Four of the genes are involved in the processing of rRNA and seven are involved in the processing of mRNA.

Discussion

Neonatal and Adult O4⁺ Cells Respond Differently to Growth Factors

The responses of the neonatal O4⁺ cells to the growth factors varied from that of their adult counterparts. In neonatal cells bFGF increased the percentage of A2B5⁺ cells, and the combination of bFGF and PDGF increased the percentage of A2B5⁺ cells to still higher levels. This is slightly different from the results of a previous study of neonatal O4⁺ cells, in which PDGF induced the cells to revert to an A2B5⁺ state, (Gard and Pfeiffer 1993; Mason and Goldman 2002). In the current study, nearly all growth factor combinations resulted in a significant decrease in the percentage of O4⁺ cells and an increase in A2B5⁺ cells. An interesting discrepancy between neonatal and adult cell responses was the inability of IGF, a

well-known differentiation factor, to increase the number of O1⁺ cells in the neonatal cultures. The addition of IGF significantly increased the differentiation of adult O4⁺ cells, a response that is not replicated by neonatal O4⁺ cells exposed to identical culture conditions. (Mason and Goldman 2002). While IGF may be insufficient to induce the neonatal progenitors to differentiate into oligodendrocytes, it may prevent the reversion to a more immature state. Altering the composition of the culture media may enhance the differentiation of acutely isolated neonatal O4⁺ cells, as previously demonstrated in oligodendrocyte progenitor cultures generated from stem cells and mixed glial cell cultures (Espinosa-Jeffrey et al. 2002; Yang et al. 2005). While changes in the culture conditions of neonatal cells may increase differentiation, no such changes are necessary for the adult cells which readily differentiate in media alone; the addition of growth factors serves to further increase the proportion of mature oligodendrocytes. This discrepancy in the ability of neonatal and adult cells to differentiate may be the result of inherent differences in their gene expression profiles.

Adult O4+ Cells are More Mature than Neonatal O4+ Cells

Unlike the neonatal O4⁺ cells, adult cells readily differentiated into O1⁺ cells. Microarray analysis revealed that the adult progenitors higher levels of transcripts for several myelin genes, including *Mog*, *Mag*, *Mbp*, and *Plp* (Table I). Other genes that are implicated in the process of myelination and oligodendrocyte differentiation were also upregulated. *Enpp2*, which encodes ectonucleotide pyrophosphate/phosphodiesterase 2, is expressed by oligodendrocytes at the commencement of myelination, antagonizing adhesive interactions with extracellular matrix (Fox et al. 2003). The enzyme encoded by *Ugt8* is localized in myelin subfractions and is downregulated during demyelination in the twitcher mouse model for globoid cell leukodystrophy (Koul et al. 1980; Taniike et al. 1998). Zitter rats, which possess a mutation in the gene for attractin, exhibit hypomyelination (Kuramoto et al. 2001). Transferrin, whose transcript is increased in the adult, has been shown to induce oligodendrocyte differentiation both *in vitro* and *in vivo* as well as increasing myelination *in vivo* (Paez et al. 2004; Saleh et al. 2003; Sow et al. 2006). It has also been implicated in the induction of cell cycle exit, inhibition of migration, and premature differentiation of oligodendrocyte progenitors *in vitro* (Garcia et al. 2004; Paez et al. 2002). However, RT-PCR and Western blots showed less of a difference in transferrin between neonates and adults than did the microarray. We are less confident of the apparently larger difference on the microarray because only a single probe set is represented. Finally, *Egr2*, which encodes a transcription factor that controls myelination in the peripheral nervous system, is also upregulated (Topilko et al. 1994). In Schwann cells, *Egr2* inhibits proliferation and promotes cell survival and myelination; it may play a similar role in CNS oligodendrocytes (Parkinson et al. 2004). Overall, based on the expression of these genes, the adult O4⁺ cells appear to be primed for differentiation and myelination.

Myelin and myelin-associated proteins inhibit the differentiation of progenitors *in vitro* and it has been suggested that this inhibition is responsible for the maintenance of immature oligodendrocyte precursors in the adult brain (Miller 1999; Syed et al. 2008). Once removed from this restrictive *in vivo* environment, the adult O4⁺ cells promptly differentiated, a behavior that the neonatal progenitors were unable to emulate. Furthermore, adult O4⁺ cells will differentiate into myelinating oligodendrocytes after they are transplanted into a demyelinated lesion (Mason et al. 2004), pointing to the ability of these cells to complete differentiation to myelinating oligodendrocytes.

Three differentiation and myelination-associated genes were expressed at higher levels in neonatal O4⁺ cells. *Jun* encodes a transcription factor that antagonizes the effects of *Egr2* in Schwann cells, inhibiting myelination (Parkinson et al. 2004). The ability of c-Jun to induce the proliferation of Schwann cells *in vitro* and drive myelinating cells to a less mature state

in vivo may play a role in maintaining neonatal progenitors (Parkinson et al. 2008). *Hes5* overexpression in embryonic spinal cord is capable of inhibiting oligodendrocyte differentiation and maintaining the cells in an immature state when cultured in media conducive to oligodendrocyte differentiation (Wu et al. 2003). Fyn is a tyrosine kinase that is active early in the process of oligodendrocyte differentiation and is involved in myelination (Lu et al. 2005; Osterhout et al. 1999; Seiwa et al. 2000). Since the adult O4⁺ cells, which express higher levels of myelin gene transcripts, appear to be more differentiated than the neonatal progenitors, it is possible that *Fyn* has already been downregulated, resulting in higher levels of transcript in the neonatal progenitors. Again, this suggests that the transcriptional program of the neonatal progenitors maintains them in an immature state whereas that of the adult progenitors predisposes them towards differentiation when placed in a non-restrictive environment. Of course, the differential regulation of all of these genes should be eventually tested by RT-PCR and Western blots.

Another possibility is that the O4⁺ cells isolated from the adult are a mixed population consisting of oligodendrocytes at various stages of differentiation. Previous studies used the O1 monoclonal antibody to remove O4⁺ progenitors that have progressed to later stages of development. While this is possible in young animals, a similar selection becomes problematic when isolating O4⁺ cells from the mature rat brain. Similar to progenitors isolated from the adult optic nerve, the majority of adult O4⁺ cells from subcortical white matter exhibit stress-induced O1 expression, a phenomenon that is not observed in O4⁺ cells isolated from neonatal or juvenile animals (Shi et al. 1998). Since the adult O4⁺ cells readily differentiate into O1⁺ cells in culture, it is difficult to determine if O1 expression within the first day of culture is due to initial expression by mature oligodendrocytes or acquisition of O1 expression by earlier lineage progenitors that are primed to differentiate when removed from a repressive *in vivo* environment. However, after one day in culture, approximately 20% of the adult O4⁺ cells were labeled by a four hour BrdU pulse (data not shown), suggesting that a large proportion of the cells were cycling at the time of isolation, placing them at a more immature, ostensibly O1⁻, developmental stage. The use of other developmental markers to further purify the adult O4⁺ population would be very useful in further delineating genes expressed at more restricted developmental stages.

Cells are analyzed immediately after isolation. However, the isolation protocol for neonatal O4⁺ cells is different from that for adult cells. Using the shorter neonatal isolation protocol for adult white matter results in the death of a majority of the adult cells, most likely due to excessive shear forces. The dissociation of adult brain tissue requires a prolonged enzymatic digestion to enhance cell survival. We can think of no reason to suspect that the longer isolation procedure would result in the upregulation of a number of myelin genes, however. Whether the upregulation of genes involved in cell death and survival, seen in the adult cells, might result partly from a longer isolation procedure is not clear. We would argue, however, that the survival of the adult O4⁺ cells is driven by the interplay of genes involved in the control of both cell death and survival, programs that may not be present in neonatal O4⁺ cells. Examination of the gene expression profile of neonatal O4⁺ cells isolated using the more rigorous adult enzymatic dissociation process may further clarify those genes induced as a result of the isolation protocol as opposed to those that are developmentally regulated.

Differences in the Proliferative Capacity of O4⁺ Cells in Culture is reflected in the Microarray Analysis

After 4 days in culture, adult O4⁺ cells were less proliferative than the neonatal cells; fewer than 9% of the cells incorporated BrdU over 4 hrs in comparison to the 20% observed in neonatal cultures. The gene expression profiles of the neonatal and adult O4⁺ cells gives some molecular clues as to what controls a higher proliferation rate. Fifty-seven probe sets

representing 44 ribosomal genes were expressed at higher levels in neonatal progenitors (24 are listed in Table IV). Proliferating cells possess high levels of rRNA synthesis, a condition that is observed in transformed cells, which have a consequently enlarged nucleolus (Raska et al. 2004; Russell and Zomerdijk 2005). In addition to proliferation, ribosomal biogenesis is also closely linked to cell growth; continued rRNA synthesis halts cell-cycle progression until the appropriate cell size is achieved. CyclinB1/Cdk1 (Cdc2a), then inhibits rRNA synthesis to drive cells into mitosis (Hernandez-Verdun and Roussel 2003). CyclinD1 complexes with Cdk4 to promote G1/S phase progression (Lundberg and Weinberg 1998). The increased transcription of the ribosomal genes, in combination with the four cell cycle regulatory proteins, suggests that the neonatal O4⁺ progenitors are more proliferative than their adult counterparts. They could be less proliferative in the sense that more have exited cell cycle, or they could have longer cycles, or both.

Cell Death and Survival are Differentially Regulated in Neonatal and Adult O4⁺ Cells

The number of oligodendrocytes in the brain is controlled through a balance of proliferation and cell death. Approximately 50% of the developing oligodendrocytes in the optic nerve are eliminated through apoptosis (Barres et al. 1992a). In the cerebrum, 20% of premyelinating oligodendrocytes degenerate between P7 and P11 and this percentage increases to 28% by 4 weeks of age (Trapp et al. 1997). Some of this survival/death is due to competition for survival factors, such as PDGF (Barres et al. 1992b), and some of the survival is controlled through contact with axons (Burne et al. 1996). The adult O4⁺ progenitors exhibited higher expression of 44 genes associated with promoting cell death and 43 transcripts for genes involved in decreasing cell death. This group of genes includes Bcl2-like, DAPk2, Fas apoptotic inhibitory molecule 2, and *Unc5b*. How the balance of survival and death operates to maintain an O4⁺ population in the adult CNS is an important question that awaits further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The Effects of PDGF, bFGF, and IGF on the Differentiation of Neonatal O4⁺ Cells
 Neonatal O4⁺ cells were cultured in CDM supplemented with the following growth factors: PDGF (P), bFGF (F), and IGF (I) alone and in combination. After four days, cultures were fixed and stained for A2B5, O4, O1, and Hoescht 33342. Bar graph depicts the percentage of immunopositive cells out of the total number of cells present under each condition ($m \pm$ SEM $n=3$; * $P<0.05$ and ** $P<0.01$ in comparison to control as determined by paired Student's t-Test).

Figure 2. The Effects of PDGF, bFGF, and IGF on the Proliferation of Neonatal O4⁺ Cells
 Neonatal O4⁺ cells were cultured in CDM supplemented with the following growth factors: PDGF (P), bFGF (F), and IGF (I) alone and in combination. After four days, 10 μ M BrdU was added to the medium for four hours before the cultures were fixed and stained BrdU and Hoescht 33342. Bar graph depicts the percentage of BrdU⁺ cells out of the total number of cells present under each condition ($m \pm SEM$ $n=3$; * $P<0.05$ and ** $P<0.01$ in comparison to control as determined by paired Student's t-Test).

Figure 3. Expression of Myelin and Oligodendrocyte Specific Genes in Acutely Isolated O4+ Cells

A comparison of mRNA (RT-PCR, left panel) and protein (Western blots, right panel) levels of several genes expressed by neonatal (N) and adult (A) O4⁺ cells immediately after isolation. 18S RNA and GAPDH were used as controls.

Figure 4. Functional Gene Groups that are Differently Regulated in Adult O4⁺ Cells
The bar graph was generated by IPA and represents functional gene groups, comparing adult and neonatal O4⁺ OPCs. Those that are significantly more highly expressed in adult cells are represented by bars above the threshold line. Gene transcripts associated with the most significant functions are listed in Tables II and III. Threshold- p=0.05.

Figure 5. Functional Gene Groups that are Differently Regulated in Neonatal O4⁺ Cells
The bar graph was generated by IPA, and those genes that are significantly more highly expressed in neonatal cells are represented by bars above the threshold line. Gene transcripts associated with the most significant functions are listed in Table IV. Threshold- p=0.05.

Table I**Differentially Regulated Transcripts for Genes Associated with Myelination**

Entrez GeneID	Fold change	Gene Symbol	Gene Title
Upregulated in Adult O4 ⁺ Oligodendrocyte Progenitors			
25037	42.36 [*]	<i>Mobp</i>	myelin-associated oligodendrocytic basic protein
24558	67.05 [*]	<i>Mog</i>	myelin oligodendrocyte glycoprotein
29409	28.26 [*]	<i>Mag</i>	myelin-associated glycoprotein
24547	14.88	<i>Mbp</i>	myelin basic protein
24943	17.52 [*]	<i>Plp</i>	proteolipid protein
64364	9.48	<i>Pllp</i>	plasma membrane proteolipid
24660	3.51	<i>Pmp22</i>	peripheral myelin protein 22
25263	55.20	<i>Mal</i>	myelin and lymphocyte protein
24825	78.57	<i>Tf</i>	transferrin
114090	4.33	<i>Egr2</i>	early growth response 2
84050	3.66 [*]	<i>Enpp2</i>	ectonucleotide pyrophosphatase/phosphodiesterase 2
50555	3.74	<i>Ugt8</i>	UDP galactosyltransferase 8
25275	2.70	<i>Cnp1</i>	cyclic nucleotide phosphodiesterase 1
83526	2.51	<i>Atrn</i>	attractin
29584	3.89	<i>Gjb1</i>	gap junction membrane channel protein beta 1
Upregulated in Neonatal O4 ⁺ Oligodendrocyte Progenitors			
25150	2.80	<i>Fyn</i>	fyn proto-oncogene
24516	2.09 [*]	<i>Jun</i>	Jun oncogene
79225	3.07 [*]	<i>Hes5</i>	hairy and enhancer of split 5 (Drosophila)

* Average of multiple probe sets.

Table IITranscripts associated with cell death that are upregulated in adult O4⁺ oligodendrocyte progenitors

Entrez GeneID	Fold Change	Gene Symbol	Gene Title
690853	5.57	LOC690853	Similar to apoptosis-associated tyrosine kinase
25379	2.59	<i>Anp32a</i>	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
29722	4.20*	<i>Apbb1</i>	amyloid beta (A4) precursor protein-binding, family B, member 1
54226	5.55*	<i>App</i>	amyloid beta (A4) precursor protein
25389	4.51	<i>Atf3</i>	activating transcription factor 3
24211	2.07	<i>Atp1a1</i>	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide
24888	2.58*	<i>Bcl2l1</i>	Bcl2-like 1
117028	3.99*	<i>Bin1</i>	bridging integrator 1
29619	3.38*	<i>Btg2</i>	B-cell translocation gene 2, anti-proliferative
29156	4.12*	<i>Capns1</i>	calpain, small subunit 1
24936	2.13	<i>CD9</i>	CD9 antigen
25599	2.12	<i>CD74</i>	CD74 antigen, MHC, class II invariant chain
83628	15.50*	<i>Cd82</i>	CD82 antigen
25275	2.70	<i>Cnp1</i>	cyclic nucleotide phosphodiesterase 1
25420	24.10*	<i>Cryab</i>	crystallin, alpha B
171293	3.01	<i>Ctsd</i>	cathepsin D
50654	3.32	<i>Ctss</i>	cathepsin S
29467	2.34	<i>Ddit3</i>	DNA-damage inducible transcript 3
25678	8.30	<i>Ddr1</i>	discoidin domain receptor family, member 1
361384	5.31*	<i>Dnajb1</i>	DnaJ (Hsp40) homolog, subfamily B, member 1
25751	2.18	<i>Dnm2</i>	dynamamin 2
114856	7.36*	<i>Dusp1</i>	dual specificity phosphatase 1
116744	14.10	<i>Edg2</i>	lysophosphatidic acid receptor 1
94268	2.32*	<i>Efnal</i>	ephrin A1
24330	4.92*	<i>Egr1</i>	early growth response 1
114090	4.33	<i>Egr2</i>	early growth response 2
29496	2.04	<i>ErbB3</i>	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
29347	2.45	<i>Faah</i>	fatty acid amide hydrolase
246274	9.24	<i>Faim2</i>	Fas apoptotic inhibitory molecule 2
81730	2.80	<i>Fez1</i>	fasciculation and elongation protein zeta 1 (zyglin I)
79114	2.30	<i>Fgfr1</i>	Fibroblast growth factor receptor 1
25022	3.64*	<i>Fgfr2</i>	fibroblast growth factor receptor 2
288584	3.38*	<i>Fis1</i>	fission 1 (mitochondrial outer membrane) homolog (yeast)
314322	2.54*	<i>Fos</i>	FBJ murine osteosarcoma viral oncogene homolog

Entrez GeneID	Fold Change	Gene Symbol	Gene Title
25319	2.29	<i>Fth1</i>	ferritin, heavy polypeptide 1
29584	3.89	<i>Gjb1</i>	gap junction membrane channel protein beta 1
81663	3.40*	<i>Gna12</i>	guanine nucleotide binding protein, alpha 12
24416	32.68	<i>Grm3</i>	glutamate receptor, metabotropic 3
24424	6.06	<i>Gstm2</i>	glutathione S-transferase, mu 2
24426	3.07*	<i>Gstp1</i>	glutathione S-transferase pi
79239	2.80	<i>Hmox2</i>	heme oxygenase (decycling) 2
24913	10.17	<i>Hrasls3</i>	HRAS like suppressor 3
60460	2.37	<i>Hspa2</i>	heat shock 70kDa protein 2
294254	3.65*	<i>Hspa1a</i>	heat shock 70kDa protein 1A
24472	11.52*	<i>Hspa1b</i>	heat shock 70kDa protein 1B
24471	2.94*	<i>Hspb1</i>	heat shock 27kDa protein 1
290364	3.50	<i>Itm2b</i>	integral membrane protein 2B
24517	3.34*	<i>Junb</i>	Jun-B oncogene
81679	2.69	<i>Jup</i>	junction plakoglobin
25263	55.20	<i>Mal</i>	myelin and lymphocyte protein, T-cell differentiation protein
50689	3.19	<i>Mapk3</i>	mitogen activated protein kinase 3
116457	2.61	<i>Mapk8ip</i>	mitogen activated protein kinase 8 interacting protein
29477	2.75*	<i>Mapt</i>	microtubule-associated protein tau
24547	14.88	<i>Mbp</i>	myelin basic protein
24567	2.27*	<i>Mt1a</i>	metallothionein 1a
24586	1.27	<i>Ncam1</i>	neural cell adhesion molecule 1
58851	2.10	<i>Nr1h2</i>	nuclear receptor subfamily 1, group H, member 2
29431	3.16	<i>Pak1</i>	p21 (CDKN1A)-activated kinase 1
364052	2.29	<i>Pea15</i>	phosphoprotein enriched in astrocytes 15
24943	17.52*	<i>Plp</i>	proteolipid protein
24660	3.51	<i>Pmp22</i>	peripheral myelin protein 22
312754	2.88	<i>Ppp2r5a</i>	protein phosphatase 2, regulatory subunit B', alpha isoform
29411	3.67	<i>Ppt1</i>	palmitoyl-protein thioesterase 1
25522	2.13	<i>Prkcz</i>	protein kinase C, zeta
24686	3.59	<i>Prnp</i>	prion protein
25524	4.45*	<i>Psap</i>	prosaposin
81751	2.65	<i>Psen2</i>	presenilin 2
25526	120.93	<i>Ptgds</i>	prostaglandin D2 synthase
29622	2.96*	<i>Ralgds</i>	ral guanine nucleotide dissociation stimulator
117273	7.17	<i>Rhoa</i>	ras homolog gene family, member A
64373	2.42	<i>Rhob</i>	ras homolog gene family, member B

Entrez GeneID	Fold Change	Gene Symbol	Gene Title
295214	2.67*	<i>S100a1</i>	S100 calcium binding protein A1
296350	3.64	<i>Serinc3</i>	serine incorporator 3
29517	4.29	<i>Sgk</i>	serum/glucocorticoid regulated kinase
24778	3.16*	<i>Slc2a1</i>	solute carrier family 2 (facilitated glucose transporter), member 1
171565	2.05	<i>Stambp</i>	Stam binding protein
29332	3.82	<i>Stmn1</i>	stathmin 1
24807	2.08	<i>Tacr1</i>	tachykinin receptor 1
24822	3.03	<i>Tegt</i>	testis enhanced gene transcript
24825	78.57*	<i>Tf</i>	transferrin
115769	2.23	<i>Tjp2</i>	tight junction protein 2
294900	6.86	<i>Tpd52</i>	tumor protein D52
1175 14	3.68*	<i>Txnip</i>	thioredoxin interacting protein
25232	3.09	<i>Tyro3</i>	TYRO3 protein tyrosine kinase 3
50555	3.74	<i>Ugt8</i>	UDP galactosyltransferase 8
60630	2.11	<i>Unc5b</i>	unc-5 homolog B (C. elegans)
25577	2.67*	<i>Ywhaq</i>	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide
79426	4.00*	<i>Zfp36</i>	zinc finger protein 36

* Average of multiple probe sets.

Table IIITranscripts that are differentially regulated in neonatal O4⁺ oligodendrocyte progenitors organized by function

Entrez GeneID	Fold Change	Gene Symbol	Gene Title
Protein Synthesis			p=1.61E-07
58919	2.92*	<i>Ccnd1</i>	cyclin D1
94201	3.62	<i>Cdk4</i>	cyclin-dependent kinase 4
58940	4.00	<i>H2afz</i>	H2A histone family, member Z
291434	3.21	<i>LOC100042067</i>	similar to Rpl17 protein
288770	2.94	<i>Naca</i>	nascent polypeptide-associated complex alpha subunit
300079	2.45	<i>Rpl3</i>	ribosomal protein L3
64302	3.16	<i>Rpl4</i>	ribosomal protein L4
362631	2.43*	<i>Rpl11</i>	ribosomal protein L11
65043	2.28	<i>Rpl14</i>	ribosomal protein L14
245981	2.38	<i>Rpl15</i>	ribosomal protein L15
81766	2.05	<i>Rpl18</i>	ribosomal protein L18
64307	2.24	<i>Rpl24</i>	ribosomal protein L24
287417	3.17	<i>Rpl26</i>	ribosomal protein L26
29283	2.63	<i>Rpl29</i>	ribosomal protein L29
64640	2.21	<i>Rpl30</i>	ribosomal protein L30
64298	2.84	<i>Rpl31</i>	ribosomal protein L31
28298	2.43	<i>Rpl32</i>	ribosomal protein L32
296709	3.45*	<i>Rpl35</i>	ribosomal protein L35
81770	2.31	<i>Rpl37</i>	ribosomal protein L37
25347	3.03	<i>Rpl39</i>	ribosomal protein L39
290641	2.39*	<i>Rpl18a</i>	ribosomal protein L18a
83789	2.64	<i>Rps2</i>	ribosomal protein S2
25538	2.30	<i>Rps5</i>	ribosomal protein S5
29304	2.49*	<i>Rps6</i>	ribosomal protein S6
81772	2.19	<i>Rps9</i>	ribosomal protein S9
81773	2.14	<i>Rps10</i>	ribosomal protein S10
81774	2.90	<i>Rps11</i>	ribosomal protein S11
29287	2.89	<i>Rps19</i>	ribosomal protein S19
29288	2.57	<i>Rps3a</i>	ribosomal protein S3a
24842	2.11	<i>Tp53</i>	tumor protein p53
Cellular Development			p=2.07E-03
58919	2.92*	<i>Ccnd1</i>	cyclin D1
64033	3.07	<i>Ccnd2</i>	cyclin D2
94201	3.62	<i>Cdk4</i>	cyclin-dependent kinase 4
79128	4.00	<i>Dab2</i>	disabled homolog 2 (Drosophila)

Entrez GeneID	Fold Change	Gene Symbol	Gene Title
25150	2.80	<i>Fyn</i>	fyn proto-oncogene
29577	3.36	<i>Hes1</i>	hairy and enhancer of split 1 (Drosophila)
79225	3.06*	<i>Hes5</i>	hairy and enhancer of split 5 (Drosophila)
25587	3.27	<i>Id2</i>	inhibitor of DNA binding 2
24516	2.09*	<i>Jun</i>	Jun oncogene
24533	4.50	<i>Ldha</i>	lactate dehydrogenase A
116667	3.20*	<i>Map3k1</i>	mitogen activated protein kinase kinase kinase 1
117038	3.19	<i>Mt3</i>	metallothionein 3
25498	2.82*	<i>Npm1</i>	nucleophosmin 1
24679	2.36	<i>Prkar2b</i>	protein kinase, cAMP dependent regulatory, type II beta
64193	3.27	<i>Pttg1</i>	pituitary tumor-transforming 1
25353	2.04	<i>Spp1</i>	secreted phosphoprotein 1
303882	2.73*	<i>Tnk2</i>	tyrosine kinase, non-receptor, 2
24842	2.11	<i>Tp53</i>	tumor protein p53
24851	3.40*	<i>Tpm1</i>	tropomyosin 1, alpha
Post-Transcriptional RNA Modification			p=2.65E-04
114612	2.22	<i>Bat1a</i>	HLA-B-associated transcript 1A
25203	2.06	<i>Ccnb1</i>	cyclin B1
54237	2.82	<i>Cdc2a</i>	cell division cycle 2 homolog A (S. pombe)
362160	3.01	<i>Cugbp1</i>	CUG triplet repeat, RNA binding protein 1
170944	2.16	<i>Dkc1</i>	dyskeratosis congenita 1, dyskerin
432358	2.34	<i>Elavl4</i>	ELAV-like 4 (Hu antigen D)
24440	3.42*	<i>Hbb</i>	hemoglobin beta chain complex
60373	3.52	<i>Nol5</i>	nucleolar protein 5
368070	2.70	LOC368070	pinin, desmosome associated protein
252855	2.11	<i>Sfpq</i>	splicing factor proline/glutamine rich
117259	2.33	<i>Sfrs10</i>	splicing factor, arginine/serine-rich 10

P-values were generated by IPA using Fisher's Exact Test.

* Average of multiple probe sets.

Table IV

Primer Pairs

Target transcript	Primer	Sequence (5'→3')	Transcript nucleotides	RT-PCR product length
CA2	forward	AGATTGGACCTGCCTCACAAG	451-471	220 bp
	reverse	TGCTCACTGCTGACAGTAATGG	670-649	
CNPase	forward	ACGGCGTGGCGACTAGACT	530-548	516 bp
	reverse	CAGGCTCTCGGAGGATGAGG	1045-1026	
Cryab	forward	CAAGCCGCCTCTTTGACCAGT	79-99	375 bp
	reverse	CAGTGAGGACTCCATCCGATG	453-433	
MAG	forward	CCCCACCCGCGTCATTTGT	1607-1626	542 bp
	reverse	CCGCCCCACCCCTACCACT	2148-2129	
MBP	forward	TCACAGAAGAGACCCTCACAG	84-104	362 bp (var. 1,3)* 284 bp (var. 2,4,5)
	reverse	GGTGTACGAGGTGTCACAATG	445-425 / 367-347	
PDGFRa	forward	GGCTTCAACGGAACCTTCAG	659-678	640 bp
	reverse	CGCTGTCTTCTTCCTTAGCC	1298-1279	
PLP	forward	TCATTCTTTGGAGCGGGTGTG	518-538	456 bp
	reverse	TAAGGACGGCAAAGTTGTAAGTGG	927-904	
Tf	forward	TATGCCGTGGCTGTGGTGAAGG	1373-1394	453 bp
	reverse	CAGGTGGCAGGTGGCGAACTC	1825-1805	
18S	forward	TTGACGGAAGGGCACCACCAG	1205-1225	131 bp
	reverse	GCACCACCACCCACGGAATCG	1335-1315	

* amplification of all primer pairs generates one RT-PCR product, with the exception of MBP, where 5 out of 6 known transcript variants (var.) can be amplified.