The gene expression profile of PDGF-treated neural stem cells corresponds to partially differentiated neurons and glia

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Abstract

We have previously shown that platelet-derived growth factor AA (PDGF-AA) stimulates the expansion of neuronal progenitors from neural stem cells, but is unable to replace fibroblast-growth factor 2 (FGF-2) as a stem cell mitogen. In the present study, we compared gene expression in neural stem cells that were grown in the presence of FGF-2 and in cells cultured with PDGF-AA or in the absence of growth factor, which induces differentiation. The genetic program elicited by PDGF-AA (156 significantly regulated genes) was not unique, but an intermediate between the ones of FGF-2-cultured stem cells and differentiated cells. These observations are compatible with the hypothesis that PDGF-AA induces a partial differentiation of neural stem cells, which retain the ability to proliferate, rather than acting solely as an instructing agent for neuronal differentiation. Finally, the transcriptional signature of stem cells grown with FGF-2 included a large number of genes over-expressed in gliomas and a core set of conserved genes periodically expressed during the eukaryote cell cycle.
Platelet-derived growth factors (PDGF) constitute a family of soluble dimeric proteins, which includes PDGF-AA, -AB, -BB, -CC and -DD. They bind with different affinities to specific receptor tyrosine kinases, namely PDGF receptors α and/or β (Heldin and Westermark, 1999). Ligand-induced dimerization of the receptors leads to the activation of the tyrosine kinase domain, which initiates signaling via SH2-domain containing proteins (including Grb2, phosphotyidinositol-3 kinase, phospholipase Cγ, SHP-2, c-Src), and other types of adaptor proteins such as Gab1 and NHERF (Demoulin et al., 2003; Heldin and Westermark, 1999; Kallin et al., 2004).

PDGFs and their receptors are widely expressed in both embryonic and adult central nervous system (CNS), where PDGF was first reported to cause proliferation and differentiation of oligodendrocyte progenitor cells (Heldin et al., 1981; Noble et al., 1988; Raff et al., 1988). Additional studies show a neurotrophic effect of PDGF (Smits et al., 1991), involvement in neuroprotection (Pietz et al., 1996), and promotion of neuronal differentiation (Johe et al., 1996; Williams et al., 1997).

PDGFs are also involved in brain cancer. Amplification of the PDGF α-receptor gene occurs in a subset of high-grade gliomas. A mutation causing partial deletion of the α-receptor gene, leading to constitutive activation of the receptor, was also found in one patient (Clarke and Dirks, 2003). In addition, all PDGF isoforms, namely PDGF-AA, -AB, -BB, -CC, and -DD, have been implicated in the growth of gliomas which express both PDGF α− and β-receptors (Lokker et al., 2002). Furthermore, PDGF over-expression using retrovirus in mouse models results in gliomagenesis (Dai et al., 2001; Uhrbom et al., 1998).

Neural stem cells are the origins of neurons and glia (Davis and Temple, 1994). Evidence from biopsy specimens and studies of cell lines derived from pediatric brain tumors suggests that neural stem cells or intermediate precursors may be the origin of primitive neuroectodermal tumors and possibly also gliomas (Fung et al., 1995). Recent studies indicate that brain tumor cells resemble neural stem cells (Oliver and
Wechsler-Reya, 2004) and the isolation of cells with neural stem cell properties from both adult (Ignatova et al., 2002) and pediatric (Hemmati et al., 2003; Singh et al., 2003) brain tumors supports this notion.

We have previously reported that PDGF has a unique effect on neural stem/progenitor cells (NSPCs) cultured in vitro. In contrast to fibroblast growth factor-2 (FGF-2), which promotes neural stem cells renewal, PDGF induces a partial differentiation of NSPCs, which acquire a morphology clearly distinct from fully differentiated cells (in the absence of growth factors) (Erlandsson et al., 2001). PDGF-treated neurons retain an immature phenotype, expressing markers both for the neuronal lineage and cell proliferation.

Global gene expression analysis using microarrays has helped to better characterize neural stem cells, by comparing them to differentiated cells (Geschwind et al., 2001; Karsten et al., 2003; Luo et al., 2002). To our knowledge, global gene regulation in neural stem cells in response to a single growth factor has not been characterized yet. This study therefore aimed to compare the genetic profile of cells treated with FGF-2 or PDGF-AA with differentiated cells. We here report the identification of both novel and known genes that are regulated at early stages of NSPC differentiation. We found that the PDGF-treated NSPC gene expression profile constitutes an intermediate between proliferating cells and differentiated neurons and glia.
Methods

Stem cell culture

Neural stem/progenitor cells (NSPCs) were isolated from embryonic neocortex of timed-pregnant Sprague-Dawley rats (B&K, Sollentuna, Sweden) on embryonic day 14.5 (E14.5; E0.5 was defined as the day of copulatory plug) and cultured as described previously (Erlandsson et al., 2001). Briefly, monolayer NSPC cultures were maintained in N2-medium with 10 ng/ml fibroblast growth factor 2 (FGF-2; Pepro Technologies, London, England) on tissue culture plastic, pre-coated with poly-L-ornithine (Sigma Chemical, St Louis, MO) and fibronectin (Invitrogen, Carlsbad, CA). Fresh FGF-2 was added daily and the medium changed every other day. When subconfluent, the cells were passaged using a cell scraper, re-plated and used for experiments 2-4 days thereafter. For differentiation experiments platelet-derived growth factor AA (PDGF-AA, Pepro Technologies, London, England) was added daily at a concentration of 10 ng/ml to stem cell cultures.

Immunocytochemistry

Cells were grown on glass cover slips pre-coated as described above, and fixed in ice-cold acid ethanol (90% ethanol, 5% acetic acid). Prior to staining, the cells were treated with 0.2% Triton X-100 in PBS for permeabilization, washed in PBS, and incubated in blocking solution (20% normal goat serum (Dako A/S, Glostrup, Denmark), 0.1% saponin (Sigma) and 4 mg/ml bovine serum albumin (BSA, Sigma) in PBS) for 30 min at room temperature. For O4 staining, the cells were fixed in 3% paraformaldehyde in PBS and the permeabilization step was omitted. The cell cultures were incubated with primary antibody for 1 h at 37°C, washed 5-6 times in PBS, and incubated with fluorochrome-coupled secondary antibody for 45 min at 37°C. The antibodies were diluted in blocking solution. After the last washing step, cover slips were mounted with Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA). The primary antibodies were monoclonal anti-MAP2 (dilution 1:200, clone HM2, Sigma, St. Louis, MO), polyclonal anti-GFAP (dilution 1:200, Dako A/S, Glostrup, Denmark), monoclonal anti-O4 (dilution 1:30, Chemicon, Hampshire, UK), polyclonal anti-nestin (dilution 1:200, kind gift from Dr. R. McKay, NIH, Bethesda, MD), and monoclonal anti-PLP (dilution 1:200, clone plpc1, Serotec, Oxford, UK). The secondary antibodies were Cy3-goat-anti-mouse-IgG, FITC-goat-
anti-rabbit-IgG, Cy3-goat-anti-rabbit-IgG, and FITC-goat-anti-mouse-IgM (Jackson ImmunoResearch Laboratories, West Grove, PA).

**cDNA microarray analysis**

Microarray hybridization and analysis were performed essentially as described (Demoulin et al., 2004). Total RNA was isolated using the RNeasy kit (Qiagen, Germany). Total RNA (40 µg) from cells treated for a given period of time with PDGF-AA or control medium were labeled in reverse transcription reactions (Superscript II kit, Invitrogen) with dCTP-Cy5 (Amersham). RNA isolated from FGF-treated cells was used as a reference and labeled with dCTP-Cy3. In every second replicate experiment the fluorescent deoxynucleotides were swapped. Purified cDNA probes labeled with Cy3 and Cy5 were mixed per pair, and hybridized to cDNA microarray chips (Mver1.1.1, containing 15000 single stranded cDNA spots) from the Sanger Institute/LICR/CRUK Consortium (see the Sanger Institute web site for details and hybridization protocols). For each time point, we performed at least four independent hybridizations, and used at least two different batches of RNA. Chips were scanned in a Perkin Elmer/GSI Lumonics ScanArray 4000 scanner and spot intensities were measured using the QuantArray software (histogram method with background subtraction). Normalization and statistical analysis of the quadruplicate data sets were performed using GeneSpring 5.0 analysis software (Silicon Genetics). A Lowess non-linear normalization was applied and the median of the ratio distribution for each array was set to 1. Regulated spots were selected based on the average ratio values ≥ 2 for up-regulated genes and ≤ 0.5 for down-regulated genes. In addition, we considered only genes that were significantly regulated (t-test, p<0.05) based on replicate hybridizations (global error model, GeneSpring). For all features selected using this protocol, the signal was significantly above the background, indicating that the expression of these genes was detectable. Finally, Mver1.1.1 microarrays contain replicate spots corresponding to the same gene. Genes represented by spots that were not regulated in a similar manner were discarded. We show the average ratio of one representative spot for each regulated gene, with standard error calculated from multiple hybridizations and with the annotation provided by the microarray facility (Mver1.1.1_NCBI30).
Quantitative RT-PCR

These experiments were performed essentially as described (Demoulin et al., 2004). Briefly, cDNA was prepared from 5 µg of DNAse-treated total RNA using the Superscript II kit (Invitrogen), and diluted with water to 100 µl. Quantitative RT-PCR experiments were performed with 2 µl of cDNA, SYBR green master mix (Eurogentec, Belgium), and oligonucleotides designed using the PrimerExpress software (Applied Biosystems), according to the manufacturer’s instructions. PCR reactions were run in triplicate on an ABI7000 sequence detection system. Assays were validated as recommended by Applied Biosystems (Foster City, CA). Quantitative data were calculated using the threshold cycle (Ct) method. Transcript levels were normalized against acidic ribosomal protein P0 (arp0) levels and changes in transcript levels were expressed as relative values.
Results

Differentiation of neural stem/progenitor cells

The embryonic rat cerebral cortex is a common source of neural stem/progenitor cells (NSPCs) that can be grown as monolayers in serum-free medium containing FGF-2 (McKay, 1997). These cells (named NSPC-FGF in this study) feature a rosette-like colony growth pattern, uniform nestin expression, and low expression of differentiated cell markers (Fig. 1A). FGF-2 withdrawal initiates differentiation into neurons and glial cells. The process of differentiation takes several days and no change in nestin expression, cellular morphology (Fig. 1B), or expression of cell lineage markers (data not shown) could be detected at 12 hours after FGF-2 withdrawal.

The later stage of differentiation, when both morphology and cell lineage markers display a manifest phenotype, was represented by cell cultures kept for 6 days without FGF-2 (NSPC- noF, noF = no factor added). We have previously shown that neural stem/progenitor cells treated with PDGF-AA (NSPC-PDGF) express neuronal markers, but have an immature morphology compared to untreated cells (Erlandsson et al., 2001). After six days in the presence of PDGF-AA, cells stained with antibodies to neurons (MAP2), astrocytes (GFAP), and oligodendrocytes (O4) (Fig. 1C-E). The presence of markers for all three cell lineages and their immature appearance indicated that besides its effects on immature neurons, PDGF also allowed the differentiation of glial cells. To further study this process, we analyzed gene expression in NSPC treated with FGF-2, PDGF-AA or in the absence of growth factors for 12 hours and 6 days.

Neural stem/progenitor cell transcriptome

Given the very high level of similarity between rat and mouse transcripts (often close to 99%), we used mouse Mver1.1.1 cDNA microarrays to monitor gene expression in rat NSPCs. The Mver1.1.1 chips contain 15000 single-stranded DNA elements derived from the NIA Mouse 15K clone set, which is mainly derived from embryo cDNA libraries (Kargul et al., 2001). In two-color hybridizations, RNA extracted from NSPC-FGF was used to produce reference cDNA and hybridized together with labeled NSPC-PDGF cDNA or NSPC-noF cDNA. Therefore, transcripts enriched in
NSPC-FGF appeared down-regulated in result tables (Fig. 2). We considered only genes that were regulated at least two-fold in a significant manner based on replicate hybridizations ($p<0.05$). Using these criteria, we identified 309 differentially regulated genes, including 62 that had no known function (Fig. 2). Nestin, which was present on the Mver1.1.1 array, was decreased 1.85 fold upon differentiation (not shown). To validate our data, the expression of 15 genes was checked by quantitative PCR. For 14 genes, the microarray data was consistent with quantitative PCR performed with oligonucleotides designed from the rat sequences (see below, and data not shown). Although we cannot rule out the presence of artefacts due to the hybridization of rat cDNA onto mouse chips, this validated our experimental design. Overall, the microarray analysis underestimated the amplitude of each specific gene response, in line with previous observations using similar arrays (Demoulin et al., 2004).

A detailed kinetics for a limited number of genes was obtained by quantitative PCR experiments specific for each rat transcript. We chose a set of regulated genes that are associated with NSPC proliferation or differentiation. Cystatin C acts as a necessary cofactor for FGF-2 in neural stem cells (Taupin et al., 2000). The expression of cystatin C is reduced about fourfold by 6 days of differentiation (Fig. 3A), in agreement with the microarray data. Synaptotagmin XI belong to a family of proteins involved in synaptic vesicle trafficking. The increase in expression of synaptotagmin XI as differentiation proceeded was confirmed (Fig. 3B).

The gene showing the highest level of regulation on the microarray was myelin proteolipid protein, PLP, which is an integral membrane protein that accounts for 50% of the protein mass in the myelin sheath (Griffiths et al., 1998). The expression of PLP was stimulated to an even larger extent than was suggested from the microarray analysis (Fig. 3C). Typically, NSPC-noF yield between 5 and 10% oligodendrocytes (Johe et al., 1996). In addition, it has been reported that the PLP transcript is expressed in an unidentified population of neural progenitors in the ventricular zone abutting the floor plate (Yu et al., 1994), thus an area not associated with myelination. Immunocytochemistry of NSPC-noF (6 days) using PLP and O4 antibodies, revealed PLP staining only in cells positive for O4 (Fig. 3D). PLP is expressed in fewer cells than those staining with O4, which is expected because O4 expression precedes myelin formation.
Gene expression profile of PDGF-treated NSPCs

Strikingly, although PDGF-exposed cells presented a morphology distinct from cells differentiated without growth factors, the gene expression profiles were closely related (Fig. 2 and 4). When analyzing each time point separately, 57% of the regulated genes were common between NSPC-PDGF and NSPC-noF, 12 h after FGF removal. That percentage dropped to 29% after 6 days (Fig. 4A). Overall, expression of 146 genes was regulated more than two-fold in both conditions (Fig. 2B). Only ten genes were expressed at a higher level in NSPC-PDGF compared to NSPC-noF. No transcript was regulated in an opposite manner by the two treatments, i.e. no genes were up-regulated in NSPC-noF and down-regulated in NSPC-PDGF and vice versa. In addition, the expression of 153 genes passed the two-fold regulation threshold (up-regulated by a factor of 2 or down-regulated to 0.5 times the value for cells in FGF-2) only in NSPC-noF. Most of these genes were also regulated to some extent by PDGF as shown in Fig. 4C. When we performed a direct statistical comparison of the two conditions, the expression of only 17 genes was significantly higher in NSPC-noF compared to NSPC-PDGF. Among them was insulin-like growth factor 2 (IGF-2), which may play a role in the nervous system development (Sullivan and Feldman, 1994). IGF-2 regulation was confirmed by quantitative PCR (not shown). We found no genes specifically regulated by PDGF. In summary, the transcriptional profile of NSPC-PDGF was an intermediate between the ones of NSPC-FGF and NSPC-noF.

FGF-2-supported NSPC are mainly enriched in cell cycle-related transcripts

The majority of the transcripts enriched in NSPC-FGF play a role in cell division (70% of the down-regulated genes that have a known function, Fig. 2). After 6 days, genes related to cell division were still strongly down-regulated in differentiated cells, but not in PDGF-treated cells. This is consistent with our published observation that cells treated with PDGF-AA, such as neuronal precursors, are still cycling (Erlandsson et al., 2001).

The cell division gene category included many genes whose expression is known to be regulated during the human cell cycle (Whitfield et al., 2002). Surprisingly, that comparison was not only valid for mammalian cells but also for the recently published core set of periodically expressed genes in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Cho et al., 1998; Rustici et al., 2004; Spellman et al.,
Our results confirmed the existence of a conserved core set of genes that are highly regulated during the cell cycle of all eukaryotic mitotic cells. In conclusion, FGF-2-supported neural stem/progenitor cells are highly enriched in transcripts connected to cell division, a feature that has been underestimated in the definition of a transcriptional stem cell signature.

**Differentiated neural cell transcriptome**

Twelve hours after FGF-2 removal, the expression of a large number of genes decreased (Fig. 4A). At that stage, NSPC-noF and NSPC-PDGF behave in a very similar manner, although gene expression was changed to a somewhat larger extent in the absence of growth factor (Fig. 4C). Genes up-regulated by differentiation fell into several categories, such as structural proteins, growth factors and their receptors, and signal transduction molecules. Genes with specialized functions in the nervous system were also identified among the up-regulated transcripts.

An intermediate wave of transcription encoded genes involved in cell cycle exit, including p57kip2 and the growth arrest and DNA damage inducible protein Gadd45γ. Gadd45γ is a Cdk1/cyclin B1 kinase inhibitor that has been implicated in terminal differentiation and growth suppression (Liebermann and Hoffman, 1998; Vairapandi et al., 1996). Gadd45γ has also been suggested to play a role in neuronal cell death (Kojima et al., 1999).

The Pink 1 gene (PTEN-induced kinase 1) was identified in a screen for genes transactivated by PTEN (Unoki and Nakamura, 2001) and carries a putative serine/threonine protein kinase catalytic domain. We find that Pink 1 expression peaks at 7 to 8-fold by 2 days, levels off at 4 days and starts to decline at 6 days of differentiation. The expression pattern is similar for NSPC-PDGF but at a lower level of regulation (Fig 5A). Up-regulation of Pink 1 was also found in differentiating postnatal neurospheres (Gurok et al., 2004) and mutated Pink 1 has recently been associated with hereditary Parkinson’s disease (Valente et al., 2004).

Two ESTs were chosen for further analysis based on their high level of early regulation in the microarray experiments. The first, T2DL [GenBank: BC031720 and NM_172702], is a member of the TMS membrane protein/Tumor Differentially
Expressed protein family expressed in the brain (Grossman et al., 2000). The expression of T2DL is lower in proliferating cells and reaches a peak of 8 times the reference level at 4 days of differentiation (Fig. 5B). T2DL was expressed also in response to PDGF-AA, but to a lower extent, with a maximum level of 4-fold up-regulation. The second EST, with the GenBank accession number BC011468 (Fig. 5C), is an unknown gene with 60% similarity to NRBP/MADM, a multidomain putative adapter protein (Hooper et al., 2000) and 15-20% similarity to WNK kinases (Kahle et al., 2004). BC011468, which appears to contain a truncated kinase domain, reaches its maximum expression levels at 6 days, both in NSPC-noF (11-fold) and NSPC-PDGF (7-fold) cultures.

In addition to IGF-2, the gene for insulin-like growth factor binding protein 5 (Igfbp-5) was highly up-regulated both at 12 hours and 6 days (Fig. 2). In line with this result, several IGF binding proteins have previously been detected as regulated in neural stem cell differentiation irrespective of the neural stem cell origin and culture system (Easterday et al., 2003; Gurok et al., 2004). The genes for PDGF-A and the PDGF receptors were also found to be regulated. Transcripts for the PDGF α-receptor and the PDGF-A chain were strongly increased at 6 days of differentiation in NSPC-noF and NSPC-PDGF. The PDGF β-receptor gene was regulated to a lesser extent. These results were confirmed by quantitative PCR (data not shown).

Comparison with glioma transcriptional profile

In a recent study, Johansson and colleagues analyzed gene expression in mouse gliomas induced by injecting intracerebrally a recombinant retrovirus encoding PDGF-B. They used microarrays based on the same NIH clone set as ours, which allowed us to perform a detailed comparison of the expression data. They first compared gene expression in tumors and in normal brain. The expression changes observed during tumorigenesis strikingly contrast with neural stem cell differentiation (Fig. 6): genes that were up-regulated in NSPC-noF (compared to NSPC-FGF) are down-regulated in tumors compared to normal brain tissue, and vice-versa. This difference is particularly convincing for cell cycle genes, but it is not restricted to that cluster. Cystatin C, IGF-2, PLP and PINK1 are down-regulated in tumors. Johansson also compared early onset (glioblastoma-like) tumors with late-onset tumors (lower grade). Again, genes over-expressed in aggressive tumors are down-regulated in
differentiated NSPC (Fig. 6). Although one should be cautious in comparing relative data generated on different microarray platforms, this suggests that PDGF-induced aggressive tumors present a transcriptional profile closer to neural stem/progenitor cells. In the minority of genes that were regulated in a similar manner during tumorigenesis and differentiation, we found PDGF receptors, cathepsins and cystatin C.

We also found a limited overlap of our gene list with results published in two human glioma microarray studies (Markert et al., 2001; Rickman et al., 2001). In the first one, human primary glioblastomas, which showed no sign of PDGF receptor amplification, were compared to normal brain tissue. Eighteen differentially expressed genes were present in our analysis. Fourteen genes (78%) are regulated in an opposite manner in human tumors and differentiated rat NSPC, as observed with Johansson's data (Fig. 7A). In a third study, grade IV glioblastomas were compared with lower grade gliomas (Rickman et al., 2001). Among the 12 hits that are common with our list of regulated genes, 10 have an increased expression in high grade tumors and in FGF-2-treated NSPC, compared to their respective controls, in line with the two other reports (Fig. 7B). In conclusion, these results suggest that high grade glioma cells and neural stem cells grown with FGF-2 may share a common transcriptional signature.
Discussion

We previously investigated the role of PDGF for neural stem/progenitor cells (NSPC) (Erlandsson et al., 2001; Forsberg-Nilsson et al., 1998) and have now used expression profiling to identify genes associated with neural stem cell differentiation in the absence or presence of PDGF. We characterized the genetic profile of PDGF-AA-treated NSPC and found it to be an intermediate between the ones of proliferating NSPC and differentiated neurons and glia.

The addition of PDGF-AA at the time of FGF-2 withdrawal resulted in the formation of cells with neuronal and glial lineage markers, but these continued to proliferate and retain an immature morphology. The idea that PDGF-AA causes expansion of all progenitors, rather than acts as an instructive differentiation agent for neurons, is supported by the data presented here, where we did not find genes specifically regulated by PDGF. Instead, most of the transcripts that were enriched in NSPC-noF cells also showed an increased expression in NSPC-PDGF, but to a lesser extent. The same was true for genes whose expression is reduced. Furthermore, several genes associated with cell division, which were reduced early during differentiation and continued to be repressed in the absence of FGF-2, were initially down-regulated by PDGF-AA and then expressed again. Examples of these are CDK1, cyclin A2, cyclin B2, cyclin D1, proliferating cell nuclear antigen (PCNA), and many ESTs. This is compatible with the observation that PDGF stimulate the proliferation of partially differentiated cells.

A member of the PDGF family, PDGF-A, as well as the PDGF α-receptor and the PDGF β-receptor were up-regulated upon differentiation of NSPCs, both in the presence and absence of PDGF-AA. Altogether, these data suggested that endogenous PDGF may stimulate NSPC-noF cells in an autocrine manner. Interestingly, we also found increased IGF-2 expression in differentiated cells. In the developing brain, IGF-2 expression has been attributed to the choroid plexus and the leptomeninges. However, some reports show a transient IGF-2 mRNA expression also in neurons and glia of the developing CNS (Sullivan and Feldman, 1994). Further studies will have to determine if the autocrine production of PDGF and IGF-2 plays a role in the differentiation of NSPC cultures in the absence of exogenously added growth factors.
Endogenous PDGF production might also explain the similarity of the transcriptional profiles of NSPC-noF and NSPC-PDGF.

Our study overlapped to some extent with previously published genetic profiling of neural stem cells (41 out of 253 annotated genes had been mentioned earlier, see supplementary table). Different microarrays have been used for hybridizations and the differences in tissues used for stem cell isolation, donor age, as well as culture differences may account for the variation in the resulting genetic profiles. The use of a defined cell media containing a single growth factor instead of a mix of several growth factors should help to understand the regulation of gene expression in stem cells. The purity of the stem cell preparations used is also a critical parameter that most likely varies between different reports. Although the high expression of cell cycle genes in stem cells has been reported previously, they represented only a minor cluster of genes preferentially expressed in stem cells (Karsten et al., 2003). The importance of this functional group of genes was therefore further emphasized by this study. An extensive expression of the cell cycle machinery genes in stem cells seems likely, given the high cycling rate of these cells. Many of the 28 uncharacterized genes down-regulated upon FGF-2 removal are probably also involved in the cell cycle progression. This list may therefore serve as a basis for the discovery of new players in cell division.

In a comparison of genes expressed during the human cell cycle and in tumors, Whitfield et al suggested that several of the periodically expressed genes contribute to the tumor phenotype (Whitfield et al., 2002). Several of these genes are shared with genes whose expression changes during the yeast cell cycle (Cho et al., 1998; Rustici et al., 2004; Spellman et al., 1998) and with genes associated with the neural stem/progenitor cell state (Table 1, this study). For instance, PTTG1/securin, a proto-oncogene involved in chromosomal stability and cell division, was recently associated with malignant glioma growth (Tfelt-Hansen et al., 2004). The level of PLK-1 (polo-like kinase), which belongs to the family of serine/threonine kinases and is involved in spindle formation, centrosome cycles and chromosome segregation, has been correlated with grade of glioma malignancy (Dietzmann et al., 2001). Furthermore, the serine/threonine kinase STK15 (aurora kinase) has been associated with unfavorable prognosis of medulloblastomas (Neben et al., 2004). These observations
were confirmed and extended by performing a global comparison of our data with published microarray analysis of gliomas, suggesting that aggressive brain tumors and neural stem cells may share a common transcriptional signature. In that respect, it has long been suggested that primitive neuroectodermal tumors and malignant glioma may arise from neural stem cells (Fung et al., 1995), and recent reports confirm the existence of neural stem cells in brain tumor specimens (Oliver and Wechsler-Reya, 2004). Our results may therefore serve as a source for identification of genes for improved diagnosis of these highly malignant tumors for which very little treatment options exist at present.

Our findings indicate that PDGF-treated neural stem cells, according to their transcriptional profile, are an intermediate between proliferating stem cells and their differentiated progeny. These results support the notion that PDGF causes expansion of progenitor cells rather than acting solely as an instructing agent for neuronal differentiation. Furthermore, we identified a number of uncharacterized genes that are active during stem/progenitor cell division. In addition to an increased understanding of how normal stem cell division is regulated, further knowledge about these genes may contribute to the improvement of brain tumor diagnosis and therapy.
Acknowledgements

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References

The Sanger Institute web site [http://www.sanger.ac.uk/Projects/Microarrays].


Table I

Core set of genes expressed in stem cells and regulated during eukaryote cell cycle.

The list of genes that were expressed preferentially in NSPC-FGF was compared with genes regulated during the cell cycle of *Shizosaccharomyces pombe* (Rustici et al., 2004), and *Saccharomyces Cerevisiae* (Cho et al., 1998; Spellman et al., 1998).

<table>
<thead>
<tr>
<th>Rat stem cells</th>
<th>S. pombe</th>
<th>S. cerevisiae</th>
<th>Function</th>
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</thead>
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<tr>
<td>Polδ2, Polε</td>
<td>pol1, cdc20</td>
<td>POL1, POL2</td>
<td>DNA polymerase</td>
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<td>cdc22</td>
<td>RNR1</td>
<td>Ribonucleotide reductase</td>
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<td>hta1, hta2</td>
<td>hta1, hta2</td>
<td>Histone H2A</td>
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<td>plo1</td>
<td>CDC5</td>
<td>Polo kinase</td>
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<td>ark1</td>
<td>IPL1</td>
<td>Aurora kinase</td>
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<td>KAR3, KIP1, CIN8</td>
<td>Kinesin microtubule motor</td>
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Figures

Figure 1 - Expression of cell lineage markers during NSPC differentiation

A. NSPC (neural stem/progenitor cell) monolayer cultures from the embryonic rat cortex were grown in the presence of FGF-2 and stained with antibodies to nestin (red, left panel), the neuronal marker MAP2 (red, right panel), the astrocyte marker GFAP (green, right panel), and DAPI nuclear stain (blue).
B. NSPC cultures in which differentiation was induced by withdrawal of FGF-2 for 12 hours in the absence (NSPC-noF) or presence of PDGF-AA (NSPC-PDGF).
C-E. NSPC-noF and NSPC-PDGF were stained after 6 days of differentiation with anti-MAP2 antibodies (neurones, red) (C), anti-GFAP antibodies (astrocytes, green) (D) or anti-O4 antibodies (oligodendrocytes, green) (E). DAPI nuclear stain, blue. Magnification 20X. This confirmed our previous report (Erlandsson et al., 2001).

Figure 2 - Functional classification of regulated genes

The relative gene expression for each condition was shown using a color code ranging from blue (for down-regulated genes, i.e. genes that are expressed at a higher level in NSPC-FGF) to red (for genes up-regulated during the differentiation treatment). A detailed table containing all data and annotations is available as supplementary materials on the Experimental Medicine web site (http://www.icp.ucl.ac.be/mexp/pdgf). Raw microarray results were also submitted to the databases GEO (accession #GSE3251) and ArrayExpress (accession #E-TABM-39).

Figure 3 - Validation of microarray results

A-C) RNA was prepared from rat NSPC cultures treated with or without PDGF-AA for periods of time, ranging from 4 hours to 6 days. Values for proliferating neural stem/progenitor cells (NSPC-FGF) = 1. All values are normalized to the expression of the acidic ribosomal protein arbp. Error bars represent standard deviations of triplicate samples. Circles = NSPC-FGF; Squares = NSPC-noF; Triangles = NSPC-PDGF. A) Expression of Cystatin C, a cofactor for FGF-2. B) Synaptotagmin XI, Syt11, a neuronal marker. C) Myelin proteolipid protein, PLP, a component of myelin. D)
Immunostaining of NSPC-noF, 6 days differentiation, with antibodies to O4 (green) and PLP (red). Nuclei are visualized by DAPI (blue). Arrows indicate double positive cells.

**Figure 4 - Overview of gene expression in NSPC treated with FGF-2, PDGF-AA or differentiated in the absence of growth factor**

Normalized microarray results for each gene were expressed as a ratio between expression levels in differentiated cells (NSPC-noF or NSPC-PDGF) and FGF-2, used as a common reference. A) For each condition and time point, genes that were regulated significantly (p<0.05) at least two-fold were selected. B) Comparison of the list of significantly regulated genes for both conditions. C) Each gene regulated in at least one of the two conditions for one particular time point is represented by a line showing the difference in expression between NSPC-PDGF and NSPC-noF. The color code is the same as in Fig. 2.

**Figure 5 - Quantitative PCR expression of novel differentiation-regulated genes**

RNA from NSPC-noF and NSPC-PDGF (4 hours to 6 days) was prepared. Circles = NSPC-FGF (= 1); Squares = NSPC-noF; Triangles = NSPC-PDGF. All values are normalized to the expression of arbp. Error bars represent standard deviations of triplicates. A) PTEN-inducible kinase, Pink1; B) TD2L; C) EST BC011468.

**Figure 6 – Gene expression in neural stem cells compared to mouse gliomas**

The data obtained from rat NSPC was compared with published gene expression in murine gliomas induced by injection of recombinant retrovirus encoding PDGF-B (Johansson et al., 2005). In lane 5, a pool of early and late tumors was compared to normal brain tissue. In lane 6, early glioblastoma-like tumors were compared to late lower grade tumors. Data are presented as base-two logarithms of the expression ratios. The lines on the right of the colour-coded expression blocks indicate genes that are similarly regulated during brain cell differentiation and tumorigenesis.
Figure 7 – Gene expression in neural stem cells compared to human brain tumors

Our data was compared with published gene expression data obtained in human brain tumors compared to normal brain tissue (A) (Markert et al., 2001). In a second study, high grade human gliomas were compared to low grade gliomas (B) (Rickman et al., 2001). Data are presented as base-two logarithms of the expression ratios. The lines on the left of the colour-coded expression blocks indicate genes that are similarly regulated during brain cell differentiation and tumorigenesis.

Additional file

Additional file 1 - File name: Table_full array results.xls; File format: excel

Title of data: Microarray data and annotations.
Description of data: A detailed table containing all microarray data and annotations.
Figure 1
Figure 3

A. Cystatin C

B. Synaptotagmin 11

C. PLP

D. Merge PLP/DAPI

NSPC-FGF, NSPC-PDGF, NSPC-noF
A

<table>
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B

C

Figure 4
Figure 5

A. PINK1

B. TD2L

C. BC011468

Legend:
- NSPC-FGF
- NSPC-PDGF
- NSPC-noF

Rel. expression (fold change)

Time points: FGF-2, 4 h, 12 h, 2 d, 4 d, 6 d
Cell cycle

Other functions

EST

Expression (log2)

Demoulin, et al.  Fig. 6
Expression ratio

Demoulin, et al. Fig. 7