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Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation

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Abstract

Multipotent neural stem/progenitor cells (NSPCs) can be isolated from many regions of the adult central nervous system (CNS), yet neurogenesis is restricted to the hippocampus and subventricular zone in vivo. Identification of the molecular cues that modulate NSPC fate choice is a prerequisite for their therapeutic applications. We previously demonstrated that primary astrocytes isolated from regions with higher neuroplasticity, such as newborn and adult hippocampus and newborn spinal cord, promoted neuronal differentiation of adult NSPCs, whereas astrocytes isolated from non-neurogenic of the adult spinal cord inhibited neural differentiation. To identify the factors expressed by these astrocytes that could modulate NSPC differentiation, we performed gene expression profiling analysis using Affymetrix rat genome arrays. Our results demonstrated that these astrocytes had distinct gene expression profiles. We further tested the functional effects of candidate factors that were differentially expressed in neurogenesis-promoting and -inhibiting astrocytes using in vitro NSPC differentiation assays. Our results indicated that two interleukins, IL-1 β and IL-6, and a combination of factors that included these two interleukins could promote NSPC neuronal differentiation, whereas insulin-like growth factor binding protein 6 (IGFBP6) and decorin inhibited neuronal differentiation of adult NSPCs. Our results have provided further evidence to support the ongoing hypothesis that, in adult mammalian brains, astrocytes play critical roles in modulating NSPC differentiation. The finding that cytokines and chemokines expressed by astrocytes could promote NSPC neuronal differentiation may help us to understand how injuries induce neurogenesis in adult brains.

Keywords

neurogenesis; neural stem cells; astrocytes; differentiation; Microarray; cytokines

Introduction

During mammalian embryonic development, neurogenesis precedes gliogenesis, and neurons are generated from neural stem cells (NSCs) with minimal influence from glia (1). Upon birth,

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neurogenesis ceases in most brain regions; however, the dentate gyrus (DG) of the hippocampus and the subventricular zone of the lateral ventricles maintain neurogenic abilities throughout life (2). Unlike embryonic neurogenesis, NSCs in adult CNS are in intimate contact with surrounding glia, which form a so-called “stem cell niche” that can influence NSC proliferation and differentiation (3-5). Astrocytes may play critical roles in regulating neurogenesis in both intact adult brains and after injuries. Primary evidence of this is seen following neural injuries when reactive astrocytes secrete both growth factors and inflammatory proteins that can affect the survival of neurons and the regeneration of the neural network (3,6-9). Furthermore, astrocyte-expressed factors have been shown to promote NSCs and embryonic stem cells to differentiate into neurons (10-13). Therefore, determining how astrocytes regulate adult neurogenesis at the molecular level is an essential step towards understanding the regulation of adult neurogenesis.

Our previous work indicated that both membrane-associated and secreted factors expressed by newborn hippocampal (NBH), adult hippocampal (ADH), and newborn spinal cord (NBS) astrocytes could significantly promote neuronal differentiation of co-cultured adult NSPCs (13), whereas astrocytes isolated from non-neurogenic adult spinal cord (ADS) astrocytes and control adult skin fibroblasts (ASF) exhibited inhibitory effects on NSPC neuronal differentiation (13). Identification of the genes that are responsible for such differential effects among these astrocytes will provide further understanding of the molecular cues that regulate adult neurogenesis. Here, we demonstrated that astrocytes isolated from different CNS regions had distinct gene expression profiles. By comparing neurogenesis-promoting astrocytes (NBH, ADH, NBS) and neurogenesis-inhibiting cells (ADS, ASF), we have identified candidate genes that might modulate NSPC neuronal differentiation. Of the genes with significant differential expression, we have confirmed that IGFBP6 and decorin, inhibitors of IGF and TGF β 2 respectively, and enkephalin, an opioid receptor agonist, inhibited neuronal differentiation of NSPCs that were cultured with NBH astrocytes. Unexpectedly, we found that two inflammatory cytokines, IL-1 β and IL-6, could promote NSPC neuronal differentiation at relatively low concentrations, a finding that was contrary to the common assumption that inflammatory cytokines only inhibit neuronal differentiation of NSPCs (14, 15). Our work further supports the concept that, in mammalian brains, astrocytes can affect NSPC differentiation by expressing unique factors in different CNS regions and at different developmental stages, where astrocyte-derived factors such as pro-inflammatory cytokines may have contrasting effects depending on concentrations and components. Identification of these influences will facilitate our understanding of adult neurogenesis and the development of NSC-based therapies.

Materials and Methods

Cell Culture

Isolation of astrocytes from different regions at different developmental stages was performed using established methods as described (13). For NBH astrocytes, hippocampus was dissected from brains of 3-6 rat pups (postnatal day 0), diced into small pieces in HBSS and 10 mM HEPES solution (Invitrogen), followed by enzymatic digestion by papain (Worthington #LS0003126, 100 μ l of 40 μ gP/ml) and DNase 1 (1mg/ml) in HBSS/HEPES at 37°C for 20-30 min with occasional shaking. Digested tissues and cells were then allowed to settle to the bottom of the tube. Most of the supernatant was removed, and enzymatic digestion was stopped by adding 5 ml of DMEM (Invitrogen) supplemented with 10% FBS (Omega Scientific). Single cells were collected by passing the digested tissues and cells through a 70- μ m cell strainer (BD Falcon), centrifuged, and resuspended in astrocyte medium [MEM (Invitrogen) supplemented with 10% FBS, 20 mM Glucose, and N2 supplement (GIBCO)]. The isolated cells were plated in T25 flasks and cultured in a 37°C, 5% CO₂ incubator until cells reached confluence. The

flasks were then shaken at 100 rotations/min for 3 days at room temperature to shake off proliferating cells and neurons. After changing to fresh astrocyte medium, the flake was incubated in 37°C incubator with 5% CO₂ until the remaining astrocytes reached confluence. The astrocytes were then trypsinized and plated either for co-culture experiments or for ELISA assays. Nearly 100% of cells in these cultures were GFAP⁺ astrocytes.

Affymetrix genome array

RNA isolation and gene expression profiling were performed as described (16,17). Briefly, confluent primary astrocytes grown on 10 cm dishes were harvested by using 1 ml Trizol reagent (Gibco BRL, Gaithersburg, MD); then the total RNA was isolated according to manufacturer's protocol (GIBCO). The synthesis of cRNA targets and hybridization to Affymetrix U34A rat genome arrays were described in the Affymetrix manual (Affymetrix, Santa Clara, CA). Astrocytes isolated from individual animals were treated separately as independent replicates. Independent triplicates were used for each experimental condition. To assess the reproducibility of our data, the Affymetrix .CEL files were analyzed using MAS 4.1 (Affymetrix), and the target intensity was set at 200. The reproducibility of duplicate samples was analyzed by correlation coefficient of the Average Difference value and the number of falsely changed genes. The signal intensity representing expression levels of genes was used to determine correlation coefficients between replicates and experimental conditions to generate Figure 1a. To determine gene expression differences among these astrocytes, and between astrocytes and ASF, we used the "Present Calls" of MAS 4.1 software and obtained the lists of all the genes that have been identified as "Present" (P) in each astrocyte and ASF. We then compared these gene lists using the Venn Diagram function of GeneSpring 4.0.1 (Silicon Genetics, Redwood City, CA) to generate Figure 1 b & c.

Determination of candidate genes that affect NSPC differentiation

Affymetrix .CEL files from each of the five groups were analyzed using four different analytical tools: dChip1.2 (18), MAS4.1 (Affymetrix), Felix Naef algorithms (19), and the Drop Method (20). The use of multiple methods allowed us to minimize false negatives caused by analytical assumptions while providing a secondary measure of confidence when probe sets were returned by multiple methods (21). The neurogenic astrocyte chips (NBH, ADH, and NBS) were compared to the non-neurogenic chips (ADS and ASF). Pair-wise fold changes were used in dChip, Felix Naef, and MAS, whereas Drop determined a statistical confidence (c-value) between the two groups. The criteria used are as follows: dChip 1.2 – Pair-wise fold change >1.3 and group p-value < 0.05; Felix Naef – Pair-wise fold change > 1.3 and pair-wise p-value < 0.05; Affymetrix MAS4.1 – Pair-wise fold change > 1.3; Drop Method – c-value > 90%. We took all probe sets returned by multiple methods and/or by multiple probe sets as our candidate genes. These 86 probe sets representing 50 unique genes and 4 unannotated EST sequences are listed in Tables 1 and 2. Reference fold changes are from dChip and confidence values were from Drop. Ranges in fold changes and confidence values are the maximum and minimum if multiple probe sets were returned for the same gene.

Real time quantitative PCR

Analyses were performed using our previously established methods (17). Briefly, total RNA was isolated from cells using Trizol (Gibco BRL, Gaithersburg, MD). The cDNA was synthesized using SuperscriptII reverse transcriptase (Invitrogen, CA). Each RNA sample and corresponding cDNA sample was generated from a single animal, and 3 or 4 independent samples were used for each experimental condition. PCR primers were designed using Primer Express software (Applied Biosystems) ordered from Integrated DNA Technology (Coralville, IA). The primers and subsequent PCR products were first evaluated by gel electrophoresis to determine that a single PCR product of the predicted size was generated. The real time PCR

reactions were performed in an ABI 7700 Detection System (Applied Biosystems). Each cDNA sample was acquired in at least triplicate. Data analyses were performed according to the protocol provided by Applied Biosystems. Standard curves were generated using a pre-made pool of rat CNS (a mixture of brain and spinal cord) total RNA. The amount of each mRNA for tested genes was calculated according to the standard curve for that particular primer set. Finally, the relative amount of the tested message was normalized to the level of an internal control message, either hypoxanthine phosphoribosyl transferase (HPRT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results were statistically analyzed using 2-tailed, unpaired student's t-test.

ELISA analysis

Confluent astrocyte cultures were changed from astrocyte medium (see above) into co-culture medium [MEM with 25mM HEPES, 0.1% chicken egg albumin (Sigma A-5503), 0.2 M Glucose, 1mM sodium pyruvate, and N2 supplement (GIBCO)] (2 ml for 6-cm dish, and 6 ml for 10-cm dish). The medium was then collected after a 1 day or 4 day incubation period. The amounts of IL-1 β and IL-6 protein secreted by astrocytes into the medium were determined by ELISA kits (R&D system; Rat IL-1 β /IL-1F2 kits, Cat. #RLB00; Rat IL-6 ELISA kit: Cat. #R6000B). The medium was diluted to 1:2, 1:5 and 1:10 and assayed in duplicates for each concentration. The co-culture medium without astrocyte incubation was used as baseline control. The amounts of IL-1 β and IL-6 in each sample were calculated with standard curves generated by using the positive controls provided by the kits, according to manufacturer's protocols.

Recombinant proteins and other reagents

All recombinant proteins were purchased from R&D Systems, unless stated otherwise. The following proteins were used at 20 ng/ml for 4 days: rat IL-1 β (Cat. #501-RL), rat IL-6 (Cat. #506-RL), human TGF- β 2 (Cat. #302-B2), human VCAM-1 (Cat. #ADP5), rat IP-10 (Cat. #1117-IP), and human Cathepsin S (Cat. #1183-CY). The following proteins were used at 50 ng/ml for 4 days: human IGFBP-6 (Cat. #876-B6), human decorin (Cat. #143-DE), neuregulin/EGF domain peptide (NRG-ED, Cat. #395-HB), neuregulin/extracellular domain peptide (NRG-ECD, Cat. #377-HB), and, Camarillo, CA). Blocking antibodies to IL-1 β (R&D Systems Cat. #AF-501-NA) and IL-6 (R&D Systems, Cat. #AF-506) were used at 1 μ g/mL.

NSPC culture and in vitro differentiation analysis

Multipotent adult NSPCs and GFP-expressing adult NSPCs have been characterized previously using clonal analyses (22,23). NSPCs were plated in polyornithine and laminin (P/L)-coated dishes using N2 media [DMEM/F12 (1:1) media with N2 supplement (Life Technologies, Gaithersburg, MD)] containing 20 ng/ml FGF2 (PeproTech, Inc., Rocky Hill NJ) and cultured in a 5% CO₂ 37°C incubator. For in vitro differentiation, cells were changed into N2 medium alone (negative control), N2 media containing factors to be tested, or N2 medium containing 1 μ M *all-trans* retinoic acid (RA, positive control) for 4 days. Cells were then fixed by 4% paraformaldehyde, followed by immunocytochemical staining and stereological quantification as previously described (24). Primary antibodies used were: rabbit anti-type III β -tubulin (1:4000, Babco, Richmond, CA); and guinea pig anti-GFAP (1:500; Advanced Immunochemical, Inc, Long Beach, CA). Secondary antibodies were all used at 1:250 dilution: donkey anti-rabbit Cy3 and donkey anti-guinea pig Cy5 (Jackson ImmunoResearch, West Grove, PA). Cell phenotypes were captured using an Olympus BX51 microscope-equipped motorized stage. Cell counting was performed using a fractionator sampling design and formula (25) with the assistance of StereoInvestigator (MicroBrightField) using a 40 $\times$ oil-submersion objective. Between 20 and 30 frames were sampled from each chamber slide well (~2 cm² area) and approximately 1000 DAPI-positive cells were counted.

The results were statistically analyzed using 2-tailed, unpaired student's t-test. The image in Figure 5c was obtained using a Zeiss LSM510 laser scanning confocal microscope (Thornwood, New York) using split channel and z-stacking.

Co-Culture Assay

Co-culture of GFP-expressing NSPCs and astrocytes was performed as previously described (13). Briefly, confluent primary NBH astrocytes grown in T25 flasks (see above for isolation method) were trypsinized and plated onto coated [10% Collagen (Becton Dickinson) 20ug/ml poly-D-lysine (Becton Dickinson)] glass coverslips in 24-well plates (10,000 cells/coverslip). The cells were then incubated in astrocyte medium (see above) in a 5% CO₂ incubator until confluence. One day before plating GFP-NSPCs onto the astrocyte feeder layer, the astrocyte medium was changed into co-culture medium (see above). GFP-expressing NSPCs were then plated at 7,500 cells/well onto an astrocyte feeder layer in co-culture media. Four hours after plating, recombinant factors or antibodies were added into the co-culture and incubated for 4 days in a 5% CO₂ incubator. Cells were then fixed by 4% paraformaldehyde, followed by immunocytochemical analysis as described above.

Analysis of NeuroD1 promoter activity in NSPCs using luciferase assay

Murine NeuroD1 promoter (2 kb) was cloned by PCR from genomic DNA and inserted into lentiviral vector, pCSCPW-Luci at the site of CMV promoter by using the restriction enzyme sites of Cla I and Bam H1. The detailed method in constructing this plasmid has been submitted for a separate publication (Kuwabara and Gage, unpublished results). The production of lentivirus has been described elsewhere, and nearly 100% of the cells were infected [viral titers were $>1.5 \times 10^4$ Tu/ng defined by the P24 assay (26)]. NeuroD1 promoter-driving luciferase was introduced into cultured NSPCs using recombinant lentivirus [pCSCPW lentivector system (27,28)]. Because lentivirus is integrated into the cellular genome, all progenies of infected cells also contain the transgenes. NeuroD1-luciferase lentivirus-infected NSPCs were then plated onto 24 well plates (7.5×10^4 cells/well), and factors were added at 2 hours post-plating. Cells were harvested 4 days later, using 100 µl/well 1× Lysis buffer (Promega); 20 µl of each sample were used to analyze the luciferase activity using the Luciferase Assay System (Cat. # E1500, Promega) and a luminometer (Bioscan, Washington, DC). The OD 280 reading of each sample, representing the amount of protein and number of harvested cells, was measured after 1:100 dilution of the samples. The final NeuroD1 promoter activity of each sample was calculated as luciferase activity normalized by the OD 280 reading. The results were statistically analyzed using 2-tailed, unpaired student's t-test.

Analysis of GFAP promoter activity in NSPCs using luciferase reporter system

NSPCs were plated in P/L-coated 24 well plates (0.5×10^5 /well) as described above. The DNA plasmids carrying 2.5 kb glial fibrillary acidic protein (GFAP) promoter- firefly luciferase reporter gene (GF1L-pGL3) or its mutant version with STAT3 binding site mutated (GF1L-S-pGL3) and an internal control plasmid containing sea pansy luciferase driven by human elongation factor 1α promoter (EF1 α-Luc) (29) were transfected into each well using FuGENE 6 Transfection Reagent (Roche Cat. # 1815443) according to the manufacturer's instructions. Immediately after transfection, NSPCs were treated with different factors and harvested 4 days later using 100 µl/well 1× Lysis buffer (Promega); 20 µl of each sample was used to analyze the luciferase activity using the Dual Luciferase Assay System (Promega, # E1910 a) in a luminometer (Bioscan). The final results of promoter activity were presented as firefly luciferase values normalized by EF1 α-Luc values. The results were statistically analyzed using 2-tailed, unpaired student's t-test.

Cell proliferation assay

Analysis was performed as described (30) with modifications. Briefly, at 24 hours post-plating (also factor treatment), BrdU was added to the NSPC culture at 2.5 μ M final concentration for 8 hours, followed by fixation using 4% PFA. Cells were then stained using rat anti-BrdU ascites (1:500; Accurate Chemicals, Westbury, NY), mouse Ki67 (1:1000, NovoCastra Laboratories), and 1 μ g/ml DAPI according to a published protocol (30). DAPI staining was weaker but still visible. The percentage of BrdU+ cells or Ki67+ cells over the total DAPI+ cells was obtained using unbiased stereology (as described above). The results were statistically analyzed using 2-tailed, unpaired student's t-test.

Results

Neurogenesis-promoting and -inhibiting astrocytes exhibited different gene expression patterns

We have previously shown that primary type I astrocytes isolated from the spinal cord and the hippocampus of both newborn and adult rats had distinct effects on the neurogenesis of co-cultured adult NSPCs (13). To identify the genes that were responsible for such different effects, we performed gene expression profiling on astrocytes isolated from NBH, NBS, ADS, and ADH. We used ASF samples as negative controls. For each condition, astrocytes were isolated from at least 3 rats, cultured and then processed independently as replicates. The total RNA isolated from these astrocytes and from ASF was labeled and hybridized to Affymetrix rat genome U34A high density oligonucleotide microarrays using established methods (see Methods).

We first assessed the reproducibility of our data by analyzing the signal intensity of each gene (generated by Affymetrix MAS4.1 software) between replicates and between experimental conditions. Our data sets had 96-99% correlation coefficients (CCs) between replicates whereas the CCs between experimental conditions were 83-96% (examples of correlation coefficient plots are shown in Fig 1a). To determine whether these astrocytes indeed expressed different sets of genes, we used MAS4.1 software to identify genes that were defined as "Present" (the expression levels of these genes were above the threshold in at least 2 of 3 replicates) in each type of astrocyte. Using the Venn Diagram function of GeneSpring software (Silicon Genetics, CA), we compared genes that were common or unique to each type of astrocytes. We found that among 5,190 genes that were "Present" in at least one astrocyte sample, 3,394 (65.4%) genes were shared by all four types of astrocytes, 914 (17.6%) genes were shared by 3 types of astrocytes, 537 (10.3%) genes were shared by two types of astrocytes, and 527 (10.2%) genes were only "Present" in one type of astrocyte (Fig 1b). In addition, our data indicated that the newborn (NBH and NBS) astrocytes shared more common genes with each other (4271 genes, a sum of genes shared by NBH and NBS as displayed in Fig. 1b) than the adult samples (ADH and ADS: 3706 genes), indicating that more molecular divergence has developed in the adult CNS. Furthermore, the hippocampal samples (NBH and ADH) shared more common genes (4161) than spinal cord astrocytes did (NBS and ADS: 3631), indicating that the adult hippocampal astrocytes might be more similar to their newborn counterparts at gene expression levels than the adult spinal cord astrocytes to their newborn counterparts. Among the 3,394 common genes shared by astrocytes, 3,231 genes (95.2%) were also shared with ASF, whereas 163 genes (4.8%) were astrocyte-specific (Fig 1c).

To obtain genes that were differentially expressed by neurogenesis-promoting and -inhibiting astrocytes, we grouped NBH, NBS, and ADH together as the neurogenesis-promoting group, and ADS and ASF as the neurogenesis-inhibiting group. Our initial data analysis using MAS4.1 software indicated that even though these astrocytes expressed different sets of genes, many of these genes were expressed at low levels and/or did not exhibit large fold changes among

astrocytes (see online data set). We thought that many important functional genes might be expressed at low levels and their mild fold changes could have significant impact on cellular functions. To include genes with low expression levels and genes with mild fold changes, while minimizing the number of false positives, we performed data analysis of Affymetrix .CEL files using 4 different analytical software: dChip1.2 (18), MAS4.1 (Affymetrix), Felix Naef algorithms (19), and the Drop Method (20) using relaxed data analysis criteria (see Methods for details). The use of multiple methods allowed us to minimize false negatives caused by analytical assumptions while providing a secondary measure of confidence when probe sets were returned by multiple methods. Such a data analysis approach has previously been used to successfully analyze Affymetrix results (21). A total of 178 probe sets were found by at least one method using these criteria, and 48 of them were returned by multiple methods. Interestingly, of the 130 single-method probe sets, 38 were identified by at least one other probe set for the same gene. We included 50 unique genes and 4 un-annotated EST sequences identified either by multiple methods and/or multiple probe sets in our candidate lists (Tables 1 and 2). We obtained the reference fold changes and confidence values using dChip and Drop analyses, respectively. The complete data file will be published online as supplemental data and the original data set (.CEL files) will be available for further data mining.

To identify the genes expressed by astrocytes that affect NSPC fate, we focused on genes encoding secreted and cell surface proteins and confirmed expression patterns of these genes of interest using real time quantitative PCR (examples are shown in Fig. 2). For all genes analyzed, the PCR results strongly correlated with Affymetrix gene array data.

Proteins expressed at higher levels in neurogenesis-inhibiting astrocytes inhibited neuronal differentiation of adult NSPCs

Our goal was to identify the factors that affected NSPC differentiation; therefore, we compared results from this study with genes that we have previously found to be differentially expressed between neurogenic regions (DG of hippocampus tissue) and non-neurogenic regions (CA1 of hippocampus and spinal cord tissue) of adult CNS (17). Because both membrane and secreted factors expressed by astrocytes were shown to affect NSPC differentiation (13), we focused on these groups of factors, with emphasis on the factors known to share the same pathways, for further functional tests. We selected insulin-like growth factor-6, IGFBP6, a negative regulator of IGF-II signaling (31) that was expressed at higher levels by ADS astrocytes, because we previously found that IGFBP5, a positive regulator of IGF signaling, had a higher expression level in neurogenic tissue (17,32). Furthermore we found that TGF β 2 was expressed at higher levels in neurogenic tissue (17), whereas decorin, a small proteoglycan that can inhibit TGF β 2 (33), was expressed at higher levels in ADS astrocytes. We also selected enkephalin, an agonist for delta opioid receptor, in our study since we found it expressed higher levels in non-neurogenic regions in our previous study (17). Activation of the delta opioid system has been shown to inhibit adult hippocampal neurogenesis (34,35), and we hypothesized that enkephalin may directly inhibit NSPC neuronal differentiation.

To analyze the functional effect of neurogenesis-inhibiting proteins, we used GFP-expressing NSPCs co-cultured with NBH astrocytes. NBH astrocytes promoted neuronal differentiation of co-cultured NSPCs [(13), Fig. 3a,b, and d Control]. We treated co-cultured NSPCs and astrocytes with recombinant IGFBP6, decorin, or enkephalin at 50 ng/ml for 4 days and analyzed differentiation of NSPCs using immunofluorescent staining for an early neuronal marker, β III-tubulin (TuJ1). The results showed that IGFBP6 (41.3% inhibition, $p < 0.05$, $n = 4$), decorin (Dcn, 44.1% inhibition, $p < 0.01$, $n = 4$), and enkephalin (enk, 53.5% inhibition, $p < 0.05$, $n = 4$) all significantly reduced neuronal differentiation of NSPCs co-cultured with NBH astrocytes, compared to untreated co-cultures (Fig 3c and d), whereas neurogenesis-promoting candidate factors, such as IL-1 β , had no inhibitory effect on NSPC neuronal differentiation in

this co-culture assay (data not shown). We further performed the dose-response analysis of IGFBP6 and found that IGFBP6 could significantly inhibit neuronal differentiation of NSPCs at concentrations as low as 10 ng/ml (40.0% inhibition, $p < 0.05$ $n = 4$); an increased concentration of IGFBP6 had a more profound inhibitory effect (Fig. 3e). Therefore, IGFBP6, decorin and enkephalin, which were expressed at higher levels by astrocytes in non-neurogenic regions, indeed had inhibitory effects on NSPC neuronal differentiation.

Proteins expressed at higher levels by neurogenesis-promoting astrocytes enhanced NSPC neuronal differentiation

To determine the functional effect of neurogenesis-promoting factors, we used adult NSPCs cultured on P/L-coated plates that exhibited a very limited effect on neuronal differentiation of NSPCs even in the absence of the mitogen, FGF-2 (13). To confirm the neurogenesis-promoting effect of the large number of candidate factors, we used luciferase reporter systems that allowed us to analyze the functional effects of many factors in a timely manner. To detect neuronal differentiation of NSPCs, we used promoter activity analysis of NeuroD1, a basic Helix-loop-Helix transcriptional factor that is expressed when NSCs initiate their neuronal differentiation (36,37)(T. Kuwabara and F.H. Gage, unpublished observation). NSPCs were infected with a recombinant lentivirus carrying NeuroD1 promoter-luciferase reporter, and nearly 100% of the cells were infected as described previously (26). To analyze the effect of these factors on astrocyte differentiation of NSPCs, we transfected NSPCs cultured on P/L-coated plates with GFAP promoter-luciferase construct and a control EF1 α - sea pansy luciferase construct, and about 20-30% of the cells were transfected (29). NSPCs infected or transfected with luciferase constructs were treated with individual factors at 20 ng/ml, in the absence of FGF-2, for 4 days, followed by luciferase activity assays. To simplify our initial analysis, we used 20 ng/ml for all candidate factors, because most growth factors for adult NSCs, such as FGF-2, were used at this concentration (24,38). Examples of promoter activity results are shown in Figure 4a and b. Both the EGF domain of neuregulin (NRG/ED) and IL-6 increased the NeuroD1 promoter activity in NSPCs (NRG/ED: 63% increase, $p < 0.05$, $n = 8$, t-test; IL-6: 19.3% increase, $p < 0.05$, $n = 14$), but the effects of both were less than the positive control, retinoic acid (RA) treatment (Fig 4a, RA: 112.5% increase, $p < 0.001$, $n = 14$). Using BrdU labeling, we subsequently found that NRG/ED also significantly increased NSPC proliferation (data not shown), which may account for the proliferation effect of NBH astrocytes on NSPCs (13). We decided to focus on the factors that mainly modulated NSPC differentiation in this study; the functional analyses of NRG will be published elsewhere. Among tested factors, only IL-6 significantly increased astrocyte differentiation of NSPCs, shown by increased GFAP promoter activity (Fig. 4b, 6.7-fold increase, $p < 0.05$, $n = 6$), which was consistent with published literature (39). Its effect was also smaller than that of leukemia inhibitory factor (LIF) (Fig 4b, 49.8-fold increase, $p < 0.05$, $n = 6$). Neither IL-6, nor LIF increased activity of mutant GFAP promoter with mutation in the STAT3 binding site, indicating that both IL-6 and LIF act through the JAK/STAT pathway to instruct adult NSPCs into the astrocyte lineage, similar to what has been shown in embryonic NSPCs (39).

Because both the NeuroD1 and GFAP promoters that we used were partial promoters and displayed relatively high basal activities in undifferentiated NSPCs, the functional effects of certain factors on NSPC differentiation might not be detected by this method. Therefore, we further tested the neuronal differentiation effects of a subset of candidate factors using more accurate immunofluorescent staining with antibodies against early neuronal marker (TuJ1) and astrocyte marker (GFAP). The phenotypes of treated cells were quantified using unbiased stereology methods. We found that most factors tested did not significantly increase neuronal differentiation of NSPCs, but two cytokines, IL-1 β and IL-6, could promote neuronal differentiation of treated NSPCs when used at 20 ng/ml (Fig 4 c and Fig. 5b and c, IL-6: 6.3-fold increase, $p < 0.01$, $n = 7$; IL-1 β : 5.0-fold increase, $p < 0.01$, $n = 7$). To confirm that the

concentrations we used were within physiological ranges, we analyzed the amounts of IL-1 β and IL-6 proteins released into the culture media by NBH astrocytes using ELISA assays and found that NBH astrocytes secreted 7.9 ng/ml of IL-6 and 1.5ng/ml IL-1 β into their culture medium (total of 6-ml medium collected from one 10-cm plate of confluent NBH astrocytes). We collected medium after either a 1-day or 4-day incubation periods and found no significant difference in the amounts of IL-1 β and IL-6 in the culture medium secreted by astrocytes, indicating that the amounts of IL-1 β and IL-6 proteins expressed by astrocytes reached their stable levels after 1 day of incubation. Because the local concentration of IL-1 β and IL-6 secreted by astrocytes adjacent to co-cultured NSPCs was likely higher, we decided to use IL-1 β and IL-6 at the 20 ng/ml concentration for the rest of this study. To further confirm the effects of IL-1 β and IL-6 on the neuronal differentiation of NSPCs, we used specific blocking antibodies to these two cytokines and found that these antibodies specifically blocked the neuronal differentiation effects of the IL-1 β and IL-6 (Fig 4c, $p < 0.05$, $n = 3$ for both, independent experiments from Fig. 4e). To investigate whether IL-1 β and IL-6 indeed mediated the neurogenesis-promoting effect of NBH astrocytes, we further added the specific blocking antibodies to the NSPCs and primary astrocyte co-culture, and the results showed that the blocking antibodies against IL-6 and IL-1 β partially blocked the neural differentiation of NSPCs promoted by NBH astrocytes (Fig. 4d, $p < 0.5$, $n = 3$ for all conditions). Consistent with the literature and our luciferase assay results, IL-6 also increased astrocyte differentiation of NSPCs (Fig 4e, GFAP+%, 2.8-fold increase, $p < 0.01$, $n = 4$), whereas IL-1 β had no such effect ($p > 0.05$, $n = 3$). To determine whether IL-1 β and IL-6 also affected NSPC proliferation, we incubated cytokines-treated NSPCs with BrdU for 8 hours to label proliferating cells, followed by immunofluorescent staining and quantification. We found that in the absence of FGF-2, neither IL-1 β nor IL-6 significantly altered the cell proliferation (indicated by percentage BrdU + cells) and cell survival (indicated by number of DAPI+, non-apoptotic cells/well) of treated NSPCs compared to untreated NSPCs (data not shown), indicating that, at the concentration we used in our assays, both IL-1 β and IL-6 promoted NSPC differentiation without significantly affecting cell proliferation and cell death. Using standard RT-PCR, we also confirmed that adult NSPCs expressed mRNA of the receptors for IL-1 β (IL-1R1 and IL-1RII) and IL-6 (IL-6R and gp130) (data not shown).

IL-6 has been shown to inhibit the neuronal differentiation of cultured NSPCs that were treated with RA (15). To understand the difference between our results and the published literature, we treated cultured NSPCs with both 50 ng/ml [published concentration (15)] and 20 ng/ml (our condition) IL-6 in the absence and presence of RA for 60 hours [published condition (15)] and 4 days (our condition). Consistent with published work, we found that 50 ng/ml IL-6 inhibited NSPC neuronal differentiation that was initiated by RA (data not shown). However, in the absence of RA, 20 ng/ml IL-6 increased the neuronal differentiation of NSPCs (Fig. 4c) and did not inhibit RA-triggered neuronal differentiation (Fig. 5c, RA vs. IL-6+RA: $p = 0.4$, $n = 4$; RA vs IL-1 β +RA: $p = 0.2$, $n = 4$), suggesting that IL-6 could have distinct effects on NSPC neuronal differentiation at different concentrations and in different context.

Combination of candidate factors had more profound effects on neuronal differentiation of adult NSPCs

Since the majority of the candidate factors that we tested showed no effect on NSPC neuronal differentiation in either luciferase assays or immunofluorescent staining, we considered two explanations: first, even though some of the factors were expressed at higher levels by neurogenesis-promoting astrocytes, they might not have any functional effect on neuronal differentiation of NSPCs; second, since neurogenesis-promoting astrocytes expressed these factors together, a combination of these factors might be necessary to have a significant effect on NSPC differentiation. Therefore, we treated cultured NSPCs with different combinations of the neurogenic candidate factors and analyzed their effect on NeuroD1 promoter activity in

NSPCs using the luciferase assay. After a series of experiments with different combinations, we found that a combination of IL-1 β , IL-6, vascular cell adhesion molecule-1 (VCAM-1), interferon-induced protein 10 (IP-10, also known as CXCL10), Cathepsin S, and TGF β 2 had the strongest effect on NSPC neuronal differentiation (Fig. 5a, Combo, 1.6-fold increase $p < 0.01$, $n = 14$). In addition, the effect of this combination was significantly higher than IL-1 β or IL-6 alone (Fig 5a, d). We further confirmed the effect of these combined factors on NSPC neuronal differentiation using immunofluorescent staining and quantification (Fig 5b, d, Combo, 12.0-fold increase, $p < 0.001$, $n = 4$; Combo vs. LI-6, 1.1-fold higher, $p < 0.05$, $n = 4$; Combo vs. IL-1 β , 1.4-fold higher, $p < 0.01$, $n = 4$). Using BrdU labeling, we confirmed that the combination of these factors also did not alter the proliferation of treated NSPCs, compared to untreated NSPCs.

It has been shown that RA can initiate NSPCs to differentiation down the neuronal lineage (26,40). However, the downstream signaling mechanism of RA is not fully understood. To understand whether IL-1 β , IL-6 or the combination of factors was acting through the same pathways as RA, we treated NSPCs with RA and these factors at the same time. We found that neither IL-1 β nor LI-6 had an additional effect on the neuronal differentiation of RA-treated NSCs (Fig. 5d, IL-1 β , $p = 0.2$; IL-6: $p = 0.4$, $n = 4$). The combination of IL-1 β , IL-6, VCAM1, IP-10, Cathepsin S, and TGF β 2 (Combo) also did not significantly increase the neuronal differentiation of RA-treated NSPCs (Combo vs Combo+RA: $p = 0.1$, $n = 4$). These results suggested that these factors did not have a synergistic effect with RA and therefore, these factors might activate through the same pathway as RA to induce NSPC neuronal differentiation.

Discussion

In this study, we performed gene expression profiling of primary astrocytes that exhibited differential effects on the neuronal differentiation of adult NSPCs (13). In conjunction with our previous study analyzing genes differentially expressed in neurogenic and non-neurogenic regions (17), we identified candidate genes that potentially affect NSPC lineage determination. Using luciferase reporter assays, we tested the functional effects of a large number of the candidate genes encoding secreted and membrane proteins on adult NSPC differentiation, followed by confirmation using more precise immunofluorescent staining and quantification. We demonstrated that two interleukins, IL-1 β and IL-6, and a combination of a group of factors that included these two interleukins could promote NSPC neuronal differentiation, whereas IGFBP6, decorin, and enkephalin inhibited neuronal differentiation of cultured adult NSPCs. These results provide molecular evidence that astrocytes play critical roles in modulating adult NSPC fate choices. The finding that cytokines and chemokines could promote adult NSPC neuronal differentiation might help us to understand how injuries induce neurogenesis in adult brains.

The lack of neurogenesis in adult spinal cord could be due to both the lack of positive regulators and the presence of inhibitory factors. Our findings indicate that at least 3 molecules that were expressed at higher levels in ADS astrocytes or spinal cord tissue had negative effects on the neuronal differentiation of NSPCs: IGFBP-6, decorin and enkephalin. IGF-1 has been shown to promote neurogenesis both in vivo and in cultured NSCs (41-43). IGFBPs are a family of proteins that can regulate IGF function both positively and negatively (31). While IGFBP5, a positive regulator of IGFs, was highly expressed in the DG as opposed to non-neurogenic tissues (17), IGFBP6 is a negative regulator of IGF signaling with highest specificity to IGF-II (44). Overexpression of IGFBP6 in astrocytes of transgenic mice (GFAP promoter-IGFBP6 transgenic mice) leads to decreased IGF levels and brain size (44). The function of IGFBP6 in adult neurogenesis has not been shown previously. In our functional assays, neither IGFBP5 nor IGFs had significant effects on NSPC neuronal differentiation, probably due to the fact

that our NSPC culture medium contained a high concentration of insulin. By contrast, IGFBP6 did inhibit neuronal differentiation of adult NSPCs co-cultured with NBH astrocytes in our study, possibly by inhibiting the effect of insulin expressed by NBH astrocytes. Our finding suggests that the high levels of IGFBP6 expressed by ADS astrocytes may contribute, at least partially, to the inhibitory effect of ADS astrocytes on NSPC neuronal differentiation in the adult spinal cord. It will be interesting to know whether GFAP promoter-IGFBP6 transgenic animals have reduced adult hippocampal neurogenesis. In addition, TGF β s have been shown to inhibit cell proliferation but promote differentiation (45). We previously found that TGF β 2 was expressed at higher levels in neurogenic tissues than in non-neurogenic tissues (17). However, when we applied TGF β 2 alone to NSPCs, we did not observe any effect on either NSPC proliferation or differentiation, but TGF β 2 was a necessary component of the combined factors (Combo) that exhibited the strongest effects on promoting NSPC neuronal differentiation in our assay. Interestingly, in this study we found that decorin, an extracellular proteoglycan that can bind and inhibit several cytokines including TGF β 2 (33), was expressed at higher levels in ADS astrocytes. We then confirmed that decorin could significantly inhibit NSPC neuronal differentiation promoted by NBH astrocytes (Fig 3a). Our data suggest that IGF/IGFBPs and TGF β 2/decorin pathways may be involved in regulating NSPC neuronal differentiation in the adult stem cell niche. Additional studies at molecular and cell signaling levels and further in vivo assays will unveil how these two pathways regulate adult neurogenesis.

Traditionally, cytokines and chemokines have been shown to be responsible for damaging neuroinflammation during diseases and CNS injuries (46). Transgenic mice that chronically overexpress IL-6 under the GFAP promoter (GFAP promoter-IL-6 mice) exhibit CNS damage, with the severity of the damage correlating with levels of IL-6 expression (46). Cytokines and chemokines can have distinct biological effects when present at different concentrations and in various combinations (47). On the other hand, recent findings indicate cytokines can also have neuroprotective and regenerative effects (7,48). The effects of cytokines on adult neurogenesis have recently triggered great attention because of their potential roles in regenerating damaged adult CNS. To date, experimental evidence supports the hypothesis that cytokines play inhibitory roles in adult neurogenesis. For example, both IL-6 and LIF instruct embryonic NSCs into the astrocyte lineage (49). GFAP promoter-IL-6 transgenic mice exhibit a 63% reduction in adult hippocampal neurogenesis and significantly reduced neuronal differentiation of NSCs in vivo (14). IL-6 inhibits RA-initiated neuronal differentiation of adult NSPCs and inflammation blockade restores hippocampal neurogenesis in irradiated adult brains (15). The role of cytokines in promoting neuronal differentiation of adult CNS stem cells or progenitor cells have not been shown previously. The fact that cultured adult NSPCs express receptors for both IL-1 and IL-6 indicates that these cells are ready to respond to these stimulations. To our surprise, our gene expression analysis indicated that neurogenesis-promoting astrocytes express many cytokines, chemokines and inflammation-related proteins at higher levels than did neurogenesis-inhibitory cells. Specifically, we found that IL-1 β and IL-6 promoted NSPC neuronal differentiation, contradicting the current theory that inflammatory cytokines, such as IL-6, inhibit adult neurogenesis and NSPC neuronal differentiation (15). To address this apparent inconsistency, we performed additional parallel experiments using both our condition and published conditions, and found that in the presence of RA, high levels of IL-6 [50 ng/ml used by Monje et al (15)] inhibited NSPC neuronal differentiation. However, in the absence of RA, relatively low levels of IL-6 (20 ng/ml, used in this study) promoted neuronal differentiation of adult NSPCs (Fig. 4 and Fig 5). Since IL-6, like many cytokines, can have distinct physiological effects at different concentrations and in different biological contexts (47), the differences between our results and published work could be due to the differences in the amounts and conditions that we used in the experiments. It is also possible that IL-6 modulates adult NSPC fate differently depending on the context. When other neurogenic cues (such as RA) are present, IL-6 inhibits neuronal differentiation of adult

NSPCs (15), but in the absence of other neurogenic cues, IL-6 promotes neuronal differentiation of adult NSPCs (this study). The results of our present study suggest that the effects of cytokines on adult NSPCs are complex and are likely context- and concentration-dependent.

Interestingly, we found that even though TGF β 2, IP-10, Cathepsin S, and VCAM1 did not display any effect on NSPC neuronal differentiation when applied alone, the combination of these factors and IL-1 β and IL-6 could significantly promote neuronal differentiation of NSPCs. This is consistent with our finding that IL-1 β and IL-6 antibodies partially blocked the effects of co-cultured NBH astrocytes on NSPCs. IP-10 is a chemokine induced by several cytokines and has chemoattractant effects on immune cells such as monocytes and lymphocytes (50). VCAM-1 is an extracellular matrix adhesion protein that regulates cell-cell interaction and can be induced by both IL-1 β and IL-6 during neural inflammation (51). Cathepsin S belongs to a group of Cysteine proteases of the papain family and is involved in MHC presentation during immune responses. Cathepsin S degrades proteins in the extracellular matrix (52) and may modulate cell surface receptors and hence cellular functions. The role of these factors that are normally involved in inflammatory or immune responses, to promote neuronal differentiation of NSPCs is unclear. Extensive studies have demonstrated that injuries can induce neurogenesis in adult brain regions that are normally non-neurogenic; however, the underlying mechanism is not clear. Our current findings provide a new perspective on understanding injury-induced neurogenesis by identifying the molecular cues, which will facilitate the understanding of adult neurogenesis and the development of NSC-based therapies. The fact that we did not observe a synergistic effect between these factors and RA suggests that these factors may act through the same pathways as RA to promote NSPC neuronal differentiation. The signaling mechanisms underlying the neuronal differentiation effect of these factors are currently under investigation.

In summary, we have performed the initial identification and functional analysis of a group of positive and negative regulators expressed by astrocytes that can modulate adult NSPC differentiation, and we have provided molecular evidence that astrocytes play critical roles in modulating adult NSPC fate determination. Further mechanistic studies using antibodies and signaling pathway inhibitors will unveil the molecular mechanisms underlying these cytokines regulate NSPC proliferation and fate choice both in normal brains and after injuries.

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Abbreviations

NSPC	neural stem/progenitor cells
NSC	neural stem cells
NBH	newborn hippocampal
ADH	adult hippocampal
NBS	newborn spinal cord
ADS	adult spinal cord
ASF	adult skin fibroblasts

Fig. 1. Astrocytes derived from adult and newborn hippocampus and spinal cord expressed different sets of genes

(a) Sample correlation coefficient (cc) plots of signal intensity (representing gene expression levels) obtained by using MAS4.1 software (Affymetrix); Note that replicates (NBH1 vs. NBH2, ADS1 vs. ADS2) had similar gene expression profiles (high cc values), whereas samples from different tissues (NBH vs. ADS, NBH vs. ASF) had distinct gene expression profiles (low cc values); (b) A modified Venn Diagram showing numbers of genes that are unique or common among these astrocytes derived from different tissues; Among 5,190 genes that are “Present” in at least one astrocyte sample, 3,394 (65.4%) genes were shared by all four types of astrocytes, 914 (17.6%) genes were shared by 3 types of astrocytes, 537 (10.3%) genes

were shared by two types of astrocytes, and 527 (10.2%) genes were only “Present” in one type of astrocyte; **(c)** Among the 3,394 common genes shared by the astrocytes, 3,231 genes are also shared with ASF.

Fig 2. Gene expression profiles of sample candidate genes were confirmed using real time quantitative PCR
 (a) IL-6; (b) IL-1 β ; (c) VCAM-1; and (d) IGFBP6. The relative mRNA levels of each candidate genes were obtained by normalizing to an internal control, GAPDH. PCR results were consistent with the results obtained by microarray analysis. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (unpaired t-test).

Fig. 3. Factors expressed at higher levels in neurogenesis-inhibiting astrocytes and non-neurogenic regions inhibit NSPC neuronal differentiation promoted by NBH astrocytes

(a, b, c) GFP-expressing NSPCs (green) differentiate into TuJ1+ neurons (red) when co-cultured with NBH astrocytes for 4 days, in the absence (a,b) or presence of 50ng/ml IGBP6 (c). (d) Quantification of immunofluorescent staining indicated that IGBP6, decorin (Dcn) and enkephalin (Enk) significantly reduced neuronal differentiation (represented by the percentage of TuJ1+ neurons) of NSPCs that were co-cultured with NBH astrocyte; (e) Dose-dependent inhibition of NSPC neuronal differentiation by IGBP6. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ (unpaired, 2-tailed student's t-test).

Fig 4. Inflammatory cytokines, IL-1 β and IL-6, could modulate affect NSPC differentiation
 (a) Both NRG/ED ($p < 0.05$, $n = 8$) and IL-6 ($P < 0.05$, $n = 14$) increased NeuroD1 promoter activity in NSPCs; (b) IL-6 treatment increased GFAP promoter activity in NSPCs ($p < 0.05$, $n = 6$); (c) Both IL-1 β and IL-6 promote the neuronal differentiation of NSPCs ($p < 0.01$, $n = 7$ for both) and such effect could be blocked by their specific blocking antibodies ($p < 0.05$, $n = 3$ for both); (d) Antibodies to IL-6 and IL-1 β blocking the neurogenic effects of NBH astrocytes on co-cultured NSPCs, control un-treated NSPCs co-cultured with NBH astrocytes. (e) IL-6, but not IL-1 β also increased GFAP+ astrocyte differentiation of NSPCs ($p < 0.05$). *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$ (unpaired, 2-tailed, student's t-test).

Fig 5. A combination of factors expressed at higher levels by neurogenesis-promoting astrocytes and tissues promote neuronal differentiation of NSPCs
(a) The combination of IL-1 β , IL-6, VCAM1, IP-10, Cathepsin S, and TGF β 2 (Combo) enhanced NeuroD1 promoter activity in NSPCs, analyzed by luciferase activity assays ($p < 0.01$, $n = 14$); **(b)** Immunofluorescent images showing TuJ1+ neurons (red) differentiated from NSPCs that were treated by IL-1 β , IL-6, Combo and RA in the absence of FGF-2. Such fluorescent staining results were quantified using unbiased stereology quantification to generate data shown in Fig 4c and Fig 5d. **(c)** A Z-stack confocal image of a TuJ1+ neurons differentiated from IL-6 treated NSPCs; **(d)** IL-1 β , IL-6 and Combo could promote neuronal differentiation of NSPCs indicated by the percentage of TuJ1+ neurons. Combo was significantly more potent

than IL-1 β and IL-6 alone. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ (unpaired, 2-tailed, student's t-test).

Table 1
Genes that are expressed at higher levels in neurogenesis-promoting astrocytes

Gene Symbol	Gene Description	dChip Fold Change* (Range)	Drop Confidence# (Range)	Detected by Affymetrix Probe Sets &
Fabp7	fatty acid binding protein 7, brain	16.62	98%	U02096_at
MMP-9	matrix metalloproteinase 9 (gelatinase B)	16.27	100%	U24441_at
Tf	Transferrin	12.34 - 18.98	99.8% - 100.0%	U31866_g_at, D38380_g_at
Cxcl10	chemokine (CXC motif) ligand 10	15.3	100%	U17035_s_at
Best5	Best5 protein	9.97 - 17.19	98.3% - 100.0%	Y07704_g_at, Y07704_at
Cd74	CD74 antigen	7.79 - 17.08	98.3% - 99.0%	X13044_at, X13044_g_at
GFAP	glial fibrillary acidic protein	7.75 - 15.49	99.6% - 99.6%	AF028784mRNA [#] 1_s_at, AF028784cds [#] 1_s_at
MMP-12	matrix metalloproteinase 12	9.49	99%	X98517_at
Ccl3	chemokine (CC motif) ligand 3	8.55	100%	U22414_at
Cxcl2	chemokine (CXC motif) ligand 2	8.29	100%	U45965_at
IL-1β	interleukin 1 beta	5.31 - 10.36	99.6% - 100.0%	M98820_at, M98820_g_at, E01884cds_s_at
Ass	arginosuccinate synthetase	7.15	100%	X12459_at
VCAM-1	vascular cell adhesion molecule 1	6.11 - 7.14	99.9% - 100.0%	M24067_at
Serpine1	serine (or cysteine) proteinase inhibitor, member 1	5.62	100%	M58364_at, E03424cds_s_at
Gch	GTP cyclohydrolase 1	3.78 - 7.16	71.5% - 99.5%	D28560_at, D28560_g_at
Enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2	5.06 - 5.79	99.6% - 99.6%	rc_AA892775_at
Lyz	lysozyme	5.28	87%	rc_AA799861_at, rc_AA799861_g_at
IRF-7	interferon regulatory factor 7	4.76 - 5.54	97.2% - 100.0%	L03201_at
Cathepsin S	cathepsin S	4.84	94%	rc_A1235890_s_at, AF029240_at
RT1.S3	MHC class Ib RT1.S3	4.81 - 4.83	99.7% - 99.9%	AB005900_at, rc_A1071531_s_at
Olr1	oxidised low density lipoprotein (lectin-like) receptor 1	1.41 - 7.76	98.1% - 99.2%	L21711_s_at
Lgals5	lectin, galactose binding, soluble 5	3.88	100%	M57664_at, M57664_g_at
Ckb	creatine kinase, brain	3.5 - 4.16	96.9% - 97.2%	AF097593_g_at, AF097593_at
Cdh2	cadherin 2	1.92 - 5.2	71.5% - 98.9%	M26745cds_s_at, M26744_at
IL-6	interleukin 6	1.66 - 5.01	90.2% - 100.0%	X52713_at
Mx2	myxovirus (influenza virus) resistance 2	3.1	100%	U16025_g_at
RT1-M3	RT1 class Ib, locus M3	3.07	97%	U72741_g_at
Lgals9	lectin, galactose binding, soluble 9	3.03	98%	rc_AA799560_at
Ndrp2	N-myc downstream-regulated gene 2	2.89	97%	X74293_s_at, X65036_at, X65036_g_at
Igfa7	integrin alpha 7	1.69 - 3.77	96.9% - 99.6%	rc_AA891194_s_at, AF026505_at
Argbp2	Arg/Abi-interacting protein 2	2.3 - 2.98	21.9% - 99.2%	rc_A1230614_s_at, rc_A112173_at
Alplb1	ATPase, Na+/K+ transporting, beta 1 polypeptide	1.64 - 3.04	99.3% - 99.9%	X96437mRNA_at
Ler3	immediate early response 3	2.11	92%	U17604_at, X52817cds_s_at
Rtn1	reticulon 1	1.21 - 2.95	19.0% - 62.2%	rc_AA891797_at
---	Unknown EST	1.67	100%	X71127_g_at, X71127_at
Clqb	complement component 1, q subcomponent, beta polypeptide	1.2 - 1.28	2.2% - 2.8%	U02320_s_at, U02315_g_at, U02322_s_at
Nrg1	neuregulin 1	1.86 - 1.57	0.6% - 60.2%	

Reference fold changes were obtained by dChip analysis;

[#] Confidence values were obtained by Drop analysis were indicated; the ranges are the maximum and minimum if multiple probe sets were returned.

& probe sets in italic were identified by only one data analysis software.

Table 2
Genes that are expressed at lower levels in neurogenesis-promoting astrocytes

Gene Symbol	Gene Description	dChip Fold Change* (Range)	Drop Confidence# (Range)	Detected by Probe Sets&
Sepp1	selenoprotein P, plasma, 1 EGF-containing fibulin-like	-5.77	100%	rc_A1230247_s_at
Fib13	extracellular matrix protein 1 precursor (Fibulin-3) (T16 protein)	-4.78 - -5.01	99.9% - 99.9%	D89730_g_at, D89730_at
Den	decorin	-3.54 - -5.42	99.8% - 99.9%	Z12298cds_s_at, X59859_i_at, X59859_r_at
Sfp4	secreted frizzled-related protein 4	-4.21	100%	AF012891_at
Anpep	alanyl (membrane) aminopeptidase	-1.84 - -5.26	25.1% - 97.2%	AF039890mRNA_s_at, M25073_at
IGFBP-6	insulin-like growth factor binding protein 6	-2.83	97%	M69055_at
LOC191574	3-alpha-hydroxysteroid dehydrogenase	-1.92 - -3.27	97.8% - 98.3%	D17310_s_at, S35751_f_at
Bst1	bone marrow stromal cell antigen 1	-2.41	100%	D49955_at
Col5a1	Collagen, type V, alpha 1	-1.94 - -2.56	97.5% - 99.6%	rc_AA859757_g_at, AJ005394_at, rc_AA859757_at
Maf	v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog (c-maf)	-2.17	100%	U56242_at
Sc65	SC65 synaptonemal complex protein	-2.02 - -2.16	97.2% - 100.0%	X65454_at, X65454_g_at
---	Unknown EST	-2.02	97%	rc_AA893180_at
FGFR1	Fibroblast growth factor receptor1	-1.96 - -2.03	91.1% - 99.9%	D12498_s_at, S54008_i_at
---	Similar to cDNA sequence BC019776	-1.84	99%	rc_A1639012_at
---	Unknown EST	-1.83	100%	rc_AA893082_at
Npr2	natriuretic peptide receptor 2	-1.46	-95%	M26896_at
---	Unknown EST	-1.38	93%	rc_AA799299_at

Reference fold changes were obtained by dChip analysis;

Confidence values were obtained by Drop analysis were indicated; the ranges are the maximum and minimum if multiple probe sets were returned.

& probe sets in italic were identified by only one data analysis software.