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Nuclear Factor I isoforms regulate gene expression during the differentiation of human neural progenitors to astrocytes

Katarzyna M. Wilczynska[#], Sandeep K. Singh[#], Bret Adams[#], Lauren Bryan[#], Raj R. Rao[‡], Kristoffer Valerie^{†,§}, Sarah Wright^{§§}, Irene Griswold-Prenner^{§§}, and Tomasz Kordula[#]

[#]Department of Biochemistry and Molecular Biology, Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia 23298

[†]Department of Radiation Oncology, Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia 23298

[‡]Chemical and Life Science Engineering, Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia 23298

^{§§}Elan Pharmaceuticals, South San Francisco, California 94080

Abstract

Even though astrocytes are critical for both normal brain functions and the development and progression of neuropathological states, including neuroinflammation associated with neurodegenerative diseases, the mechanisms controlling gene expression during astrocyte differentiation are poorly understood. Thus far, several signaling pathways were shown to regulate astrocyte differentiation, including JAK-STAT, BMP-2/Smads, and Notch. More recently, a family of Nuclear Factor-1 (NFI-A, -B, -C, and -X) was implicated in the regulation of vertebral neocortex development, with NFI-A and -B controlling the onset of gliogenesis. Here, we developed an *in vitro* model of differentiation of stem cells towards neural progenitors and subsequently astrocytes. The transition from stem cells to progenitors was accompanied by an expected change in the expression profile of markers, including Sox-2, Musashi-1, and Oct4. Subsequently, generated astrocytes were characterized by proper morphology, increased glutamate uptake, and marker gene expression. We used this *in vitro* differentiation model to study the expression and functions of NFIs. Interestingly, stem cells expressed only background levels of NFIs, while differentiation to neural progenitors activated the expression of NFI-A. More importantly, NFI-X expression was induced during the later stages of differentiation towards astrocytes. In addition, NFI-X and -C were required for the expression of GFAP and SPARCL1, which are the markers of astrocytes at the later stages of differentiation. We conclude that an

Address correspondence to: Dr. Tomasz Kordula, Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, Virginia 23298, tel. (804) 828-0771, fax. (804) 828-1473, tkordula@vcu.edu.

Author Contributions:

Katarzyna M. Wilczynska: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing.

Sandeep K. Singh: collection and assembly of data.

Bret Adams: collection and assembly of data.

Lauren Bryan: manuscript writing, final approval of manuscript.

Raj R. Rao: provision of study material, collection and assembly of data.

Kristoffer Valerie: final approval of manuscript.

Sarah Wright: provision of study material, final approval of manuscript.

Irene Griswold-Prenner: provision of study material, final approval of manuscript.

Tomasz Kordula: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

expression program of NFIs is executed during the differentiation of astrocytes, with NFI-X and -C controlling the expression of astrocytic markers at late stages of differentiation.

Keywords

neural progenitors; astrocytes; differentiation; NFI; GFAP; SPARCL1

INTRODUCTION

Astrocytes are crucial for the normal functions of neurons; however, they are also responsible for the development of many pathological states in the brain, including neuroinflammation associated with both neurodegenerative diseases and progression of brain tumors (1-3). In fact, loss of expression of astrocyte-specific markers is the hallmark of gliomas, suggesting that glioma cells have exited the astrocyte-differentiation program (4). Despite the fundamental role of astrocytes in the brain, the mechanisms controlling their differentiation and astrocyte-specific gene expression are only partially understood. The data accumulated over the years suggest that several signaling pathways are indispensable for astrocyte differentiation (5-7). These include the JAK-STAT (8) and the BMP-2-Smad pathways (9), the activation of Notch signaling (10), and the expression of Nuclear Factor-1 (NFI) family of transcription factors (11). During vertebrate embryonic development, gliogenesis follows neurogenesis, and is likely triggered by the activation of the JAK-STAT signaling in neural precursors by neuron-derived cardiotrophin-1 (12,13). Specifically, STAT3 is believed to be critical for the generation of astrocytes, since it induces the production of bone morphogenic protein-2 (BMP-2) (14), subsequent activation of Smad1, and the formation of STAT3/Smad1 complex bridged by the p300 coactivator, which then results in the induction of astrogliogenesis (15). In addition, activation of Notch also affects gliogenesis via the formation of a complex containing a transcriptional regulator RBP-J κ ; however, this promotes gliogenesis only when the JAK-STAT pathway is on, while simultaneously inhibiting neurogenesis (10,16,17). Recently, it became apparent that the family of NFI transcription factors also regulates gliogenesis, but their precise role is not known. These phylogenetically conserved proteins regulate the expression of various cellular and viral proteins, as well as viral DNA replication (18). In vertebrates, NFIs are encoded by four, highly conserved genes: *Nfia*, *Nfib*, *Nfic*, and *Nfix*, and their products (NFI-A, -B, -C, and -X) share a conserved N-terminal sequence containing both DNA binding and dimerization domains (18). However, significant variations occur within the C-terminal transactivation domains of the NFI proteins. In addition, transcripts of all four genes can be alternatively spliced, yielding as many as nine different proteins from a single gene.

In mammals, the NFI genes are expressed in complex, overlapping patterns during embryogenesis, with particularly high levels of expression of NFI-A, -B, and -X in the developing neocortex (19,20). More recently, *Nfia*, *Nfib*, *Nfic*, and *Nfix* genes have been successfully disrupted in mice. Disruption of either the *Nfia* or *Nfib* gene causes late gestation neuroanatomical defects, including agenesis of the corpus callosum, size reduction in other forebrain commissures, loss of specific midline glial populations, and a 5-10 fold decrease in the expression of glial fibrillary acidic protein (GFAP) (21-23). In addition, *Nfib*-deficient mice show aberrant hippocampus and pons formation, and defects in lung development. In contrast, disruption of the *Nfic* gene results in early postnatal defects in tooth formation, including the loss of molar roots and aberrant incisor development (24). The *Nfix* gene knockout is postnatally lethal in most of the animals, and leads to hydrocephalus and partial agenesis of the corpus callosum (25). In addition, mice also develop a deformation of the spine with kyphosis, due to a delay in ossification of vertebral

bodies and a progressive degeneration of intervertebral disks. However, another group reported that *Nfix* knockout mice survive on a soft chow diet but have an increased weight of the brain, its expansion along the dorsal ventral axis, and aberrant formation of the hippocampus (26). Nevertheless, abnormalities in the nervous system are apparent in the *Nfix* deficient mice. Collectively, the knock-out data suggest that NFI-A, -B, and -X are important for brain development. In fact, both NFI-A and -B affect gliogenesis in the chick embryo, with NFI-A being also necessary for the maintenance of neural precursors (11). Interestingly, NFI-A expression precedes NFI-B expression in the developing chick embryo. Therefore, NFI-A and -B may be critical in the initial stages of gliogenesis, while the functions of NFI-C and -X are not known.

Here, we developed an *in vitro* differentiation model to study astroglialogenesis using neural progenitors derived from human BG01V embryonic stem cells. We find that NFI- X is expressed later during the astrocyte differentiation process, and both NFI-X and -C are required for the expression of astrocyte markers, including GFAP and SPARCL1.

MATERIALS AND METHODS

Human embryonic stem cell culture

Human BG01V embryonic stem cells (ATCC, Rockville, MD) were cultured on mitomycin C-inactivated mouse embryonic fibroblasts (MEF) layer. Cells were cultured in DMEM/F12 medium, supplemented with 20% knockout serum replacement (KSR) (Gibco, Grand Island, NY), 1 mM L-glutamine, 0.1 mM nonessential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin, 4 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, NJ), and 0.1 mM β-mercaptoethanol. Cells were propagated in 4 day cycles, and enzymatically passaged with collagenase and trypsin.

Human primary astrocytes culture

Human cortical astrocyte cultures were established using dissociated human cerebral tissue as previously described (27). Cortical tissue was provided by Advanced Bioscience Resources (Alameda, CA), and the protocol for obtaining postmortem fetal neural tissue complied with the federal guidelines for fetal research and with the Uniformed Anatomical Gift Act. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, sodium pyruvate, and non-essential amino acids.

Generation of neural progenitors

In order to generate neural progenitors, BG01V cells were allowed to proliferate for 7 days as described previously (28). Briefly, cells were grown in DMEM/F12, supplemented with 15% defined FBS (Hyclone, Logan, UT), 5% KSR, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 U/ml penicillin, 50 µg/ml streptomycin, 4 ng/ml bFGF, and 10 ng/ml leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA). Subsequently, cells were grown for an additional 7 days in differentiation medium composed of DMEM/F12, N2 supplement (Gibco, Grand Island, NY), 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 4 ng/ml bFGF. Following this stage, the MEF layer was removed and cells were dissociated by DPBS without calcium and magnesium, followed by collagenase and trypsin incubation. Cells were then plated on laminin and poly-L-ornithin coated dishes, propagated in NB27 medium consisting of neurobasal medium, B27 supplement (both from Gibco, Grand Island, NY), 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 20 ng/ml bFGF, and 10 ng/ml LIF. Cells were maintained by passaging using trypsin.

Differentiation into astrocytes

To generate astrocytes, neural progenitors were cultured on laminin and poly-L-ornithin coated dishes in DMEM, supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and nonessential amino acids for 21-24 days.

Down-regulation of target genes

Expression of NFI-A, -B, -C, -X mRNAs was down-regulated using SmartPool siRNAs from Dharmacon (Dharmacon, Int., Lafayette, CO). siRNAs were transfected into neural progenitor cells on day 15 of differentiation using Dharmafect 1, according to the manufacturer's instructions. Subsequently, efficiency of target gene downregulation was assayed by quantitative PCR 6 days post-transfection.

RNA isolation and Quantitative PCR

Total cellular RNA was prepared by Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Subsequently, 1 µg of total RNA was reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). GFAP, CD44, GLAST, SPARCL1, S100B, BFABP, NFI-A, -B, -C, -X, and GAPDH mRNA levels were measured using pre-mixed primer-probe sets, and TaqMan Universal PCR Master Mix according to the supplier's instructions (Applied Biosystems, Foster City, CA). The cDNAs were diluted 10-fold (for the target genes) or 100-fold (for GAPDH), and amplified using the ABI 7900HT cycler. Gene expression levels were normalized to GAPDH mRNA levels and presented as a fold induction with mean values $\pm$ standard deviation. Statistical analysis was performed by one-way analysis of variance. Differences were considered statistically significant when p-values were <0.05 .

Immunocytochemistry

For immunocytochemical staining, cells were grown in 4-well chamber slides (Nunc, Rochester, NY). Subsequently, cells were washed with PBS, fixed with 3% formaldehyde for 15 min at room temperature, and washed once more with PBS. Cells were then permeabilized in 0.3% TritonX-100 in PBS for 10 min at 20°C, except when later stained for O1 surface marker. Slides were washed twice in PBS and blocked in 10% milk/0.1% goat serum in PBS for 3 hours. Subsequently, cells were incubated with primary antibodies against O1 (1:500), GFAP (1:600), β III-tubulin (1:1000), Musashi-1 (1:500), Oct-4 (1:500), Sox2 (1:1000), nestin (1:500), or isotype control nonspecific sera (Chemicon, Temecula, CA) overnight at 4°C in the blocking solution. This was followed by 5 washes with PBS and a 3 hour incubation with the corresponding fluorescent secondary antibodies in the blocking solution. After washing 5 times with PBS, cells were refixed with 3% paraformaldehyde for 15 minutes at 20°C, washed with PBS and dried. Coverslips were mounted over the chamber slides using 1.5 µg/ml Vectashield Mounting Media with DAPI. Images were captured on a confocal LSM 510 Meta Zeiss microscope with a 100x objective using the appropriate laser excitation.

Western Blotting

Cells were lysed in 10 mM Tris pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.5% NP-40, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM PMSF, and protease inhibitor cocktail (Roche, Mannheim, Germany). Samples were resolved using SDS-PAGE, and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Polyclonal anti-Oct-4 (1:1000), anti-Sox2 (1:1000), and anti-Musashi-1 (1:500) antibodies were purchased from Chemicon (Chemicon, Temecula, CA). Anti- β -tubulin polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antigen-antibody

complexes were visualized by enhanced chemiluminescence using Immobilon Western blotting kit (Millipore, Temecula, CA).

Glutamate Uptake Assay

The uptake of [^3H]-glutamate (Amersham, UK) was determined as previously described (29). Briefly, cells were grown in 12-well plates, and the medium was replaced by fresh DMEM, supplemented with 10% FBS, non-essential aminoacids, penicillin/streptomycin, 50 μM glutamate (Sigma, St. Louis, MO), and 18.5 kBq (9.25 pmol) [^3H]-glutamate. Cells were incubated for 20 minutes at 37°C, and then washed three times with ice-cold PBS containing 5 mM glutamate. After cell lysis in 0.5 ml of 10 mM NaOH with 0.1% Triton X-100, 300 μl of the lysate aliquots were assayed for by liquid scintillation counting, while 100 μl were used for measuring protein concentration by the BCA method (Sigma, St. Louis, MO). Statistical analysis was performed by one-way analysis of variance. Differences were considered statistically significant when p-values were <0.05.

BrdU incorporation

Cells were plated and allowed to adhere for 12 hours. Subsequently, 10 μM BrdU was added to media and cells were incubated for the times indicated in the figure legends (2 or 6 hours). The cells were fixed, exposed to 2M HCl for 30 min at 37°C, washed with PBS, treated with Borax (pH 8.5), probed with anti-BrdU antibody (1:100) (Abcam, Cambridge, MA) overnight 4°C, and then incubated with anti-rat Alexa 488 antibodies (1:500). Coverslips were mounted over the chamber slides using 1.5 $\mu\text{g/ml}$ Vectashield Mounting Media with DAPI. Images were captured on a confocal LSM 510 Meta Zeiss microscope with a 40x objective using the appropriate laser excitation.

RESULTS

Generation of neural progenitors

Recently, human fetal brain neural progenitor cells have been efficiently differentiated *in vitro* into neurons or astrocytes (30). However, the supply of human fetal tissue is limited due to ethical issues. Therefore, an alternative emerged with the use of commercially available human embryonic stem cells (ESC), which can be differentiated into neural progenitors (NP), and further into astrocytes, neurons, and oligodendrocytes. We used these cells as an *in vitro* model to study the mechanisms of astrocyte differentiation. First, we successfully differentiated BG01V ESC into NP as previously described (28). These progenitors are characterized by rosette-forming structures, and are capable of growing in culture without the mouse feeder layer (Fig. 1A). To further define the obtained cultures, we have analyzed the expression of pluripotent stem cell marker Oct-4, and the neural progenitor marker Musashi-1 (Msi-1), both by Western blotting (Fig. 1B) and immunocytochemistry (Fig. 1C). In fact, the expression of Musashi-1 was greatly enhanced, while Oct-4 was no longer expressed in the NP cells. Furthermore, both Sox2 and nestin were abundantly expressed in both cell types, as it has been previously reported for ESC and NP (31).

Neural progenitors as a model for astrocyte differentiation

We aimed at developing an *in vitro* astrocyte differentiation model, which would allow for the easy and efficient use of molecular biology approaches, such as knock-down experiments. For this reason, ESC and generated NP should not express markers that are found during later stages of astrocyte differentiation. Therefore, we assessed the expression of multiple markers in ESC and NP, and compared these cells with primary human astrocytes (Fig. 2A). This analysis included previously described markers, such as glial

fibrillary acidic protein (GFAP) (32), glutamate transporter (GLAST) (33), CD44 antigen (34), calcium-binding protein S100 β (35), brain fatty acid binding protein (BFABP) (36), and secreted protein acidic and rich in cysteine-like protein 1 (SPARCL1/hevin) (37). The expression of GFAP, BFABP, and CD44 was very low in ESC and NP, but they were expressed at high levels in primary human astrocytes. In addition, NP expressed significant amounts of S100 β , GLAST, and SPARCL1, which is in accordance with the previously published data (11,36,38) that showed the presence of these markers in the neural-type cells in the brain. These data suggest that the generated NP may represent the undifferentiated progenitor cells of the brain, which may have the potential to differentiate into astrocytes, neurons, or oligodendrocytes.

Expression of NFI isoforms in stem cells and neural progenitors

Expression of NFI isoforms is developmentally regulated in the CNS, with NFI-A, -B, and -X knockout mice presenting severe neuroanatomical defects (21,23,25). However, specific functions of NFI isoforms in the brain are not known. Furthermore, it is not clear which isoforms are crucial for proper differentiation of individual brain cell types, including astrocytes. In order to determine whether ESC and NP can be used to study the role of NFI isoforms in the process of differentiation, we analyzed the pattern of NFI expression in these cells, and compared them to primary human astrocytes (Fig. 2B). Indeed, the ESC expressed only minute amounts of mRNAs for all NFI isoforms when compared to primary astrocytes, in which the NFI expression is tens- or hundreds- fold higher. More importantly, NFI-A expression was dramatically increased in NP (13-fold), as previously published for the chick NP *in vivo* (11), indicating that these cells underwent the expected differentiation process. In addition, the expression of NFI-B and -C increased 3.5- and 2.2-fold, respectively, suggesting that these isoforms may be the next NFIs expressed during differentiation. We conclude that the *in vitro* differentiation model may be used to study the role of NFIs in the differentiation of NP into astrocytes.

Generation of astrocytes

Recently, NP isolated from both rat and human brains were differentiated towards astrocytes *in vitro* (30,39,40). We have followed the published differentiation protocols with some modifications, and generated cells that were subsequently analyzed for the expression of specific markers. Morphologically these cells resembled primary human astrocytes, as they showed a spread-out flat body with characteristic projections, in contrast to the more compact and round NP forming the rosettes (Fig. 3A). In addition, these cells were GFAP- and CD44-positive but O1-, Msi-1-, SOX2-, and β III-tubulin-negative (Fig. 3B and C), and also increased glutamate uptake by 40% (Fig. 3D). Significantly, these results suggest that all of the NP differentiated towards astrocytes, but not oligodendrocytes or neurons. It is noteworthy that many cells died during the differentiation process, hence the overall survival was low (10-20%), and the differentiated astrocytes lost their proliferative potential with only 4% of cells still able to replicate DNA (supplementary Fig. 1). Subsequently, we analyzed the expression of the markers during the 24-days of the differentiation (Fig. 4A). The expression of BFABP and CD44 gradually increased throughout the entire differentiation process. In contrast, the expression of GFAP and SPARCL1 was relatively unchanged for the first 15 days of differentiation, but subsequently increased dramatically (45 and 22 fold increase, respectively), peaking at the final days of the differentiation. Interestingly, both S100 β and GLAST were expressed at similar levels by the differentiating cells and neural progenitors (Fig. 4A). Although S100 β is abundantly expressed by activated astrocytes *in vivo*, it is also present in neural cells in the brain (35). Similarly, GLAST is produced by neural progenitors fated to become glia *in vivo* (11). As a negative control, we analyzed the expression of myelin basic protein (MBP), the marker of differentiated oligodendrocytes; however, we did not detect any MBP expression (data not shown).

Furthermore, the expression of Olig2, needed for oligodendrocyte but not astrocyte differentiation, was drastically suppressed during the differentiation of NP cells (supplementary Fig. 2). The expression levels of markers were approximately 15-20% of those found in primary human astrocytes; however, this lower expression levels were not due to a fraction of cells differentiating towards astrocytes since all of the cells are GFAP- and CD44-positive (Fig. 3B). Collectively, these results indicate that NP cells differentiated towards astrocytes, and not neurons or oligodendrocytes, as indicated by the expression of the appropriate glial markers.

Regulation of NFI expression during astrocyte differentiation

In comparison to the primary human astrocytes, NP cells express significant amounts of NFI-A, low amounts of NFI-B and -C, but only minute amounts of NFI-X (Fig. 2). Therefore, we investigated whether the expression of NFI isoforms is regulated during the process of NP differentiation into astrocytes (Fig. 4B). Indeed, the expression of NFI-B and -C moderately increased (5 and 3 fold, respectively) within 3 days, and was almost unchanged for the remaining differentiation time. More importantly, abundance of NFI-X mRNA dramatically increased (40-50 fold) within 15 days, and remained elevated for the remaining period of the differentiation process. Surprisingly, already high levels of NFI-A mRNA further increased with the kinetics and magnitude similar for NFI-X increase (Fig. 4B). These data suggest that NFI-A may be crucial at the initial stages of astrocyte differentiation, while NFI-X may regulate gene expression during the final stages.

NFI-X and NFI-C control expression of astrocyte markers at the late stages of differentiation

To study the role of NFIs in the expression of astrocyte-specific markers, we knocked-down their expression during the differentiation of NP towards astrocytes. Since simultaneous down-regulation of NFI-C and -X isoforms is required to effectively diminish GFAP expression in primary human astrocytes (supplementary Fig. 3), we targeted both NFI-X and -C on day 15 of astrocyte differentiation. For comparison, we also included siRNAs targeting all NFI isoforms (PanNFI siRNA). Subsequently, the expression of NFIs (Fig. 5B) and marker genes (Fig. 5A) were analyzed on day 21. Significantly, the knockdown of NFI-X and -C drastically diminished the expression of GFAP and SPARCL1 (by 80% and 50%, respectively). However, there was limited or no effect on the expression of CD44, GLAST, S100 β , and BFABP. Furthermore, PanNFI siRNA exhibited similar effects to NFI-X and -C siRNAs, suggesting that NFI-X and -C are necessary to drive the expression of GFAP and SPARCL1 during the later stages of astrocyte differentiation.

DISCUSSION

Human stem and progenitor cells are currently considered as replacements for cells lost during various diseases, including Parkinson's disease, Multiple Sclerosis, and Cerebral Palsy (41). They are also viewed as tools to deliver drugs to the target sites of disease by 'homing in', in particular to target brain tumors, such as glioblastoma multiforme (42). Therefore, understanding the processes controlling differentiation of these cells may have critical consequences for future therapies. Thus far, rodent NP have been commonly used for differentiation and functional studies providing the critical basic knowledge. However, these data cannot be extrapolated to analyze regulation of genes, which are different between humans and rodents. This is particularly important for the neurodegenerative disorders, which are typically human, as rodents do not develop Alzheimer's disease, Multiple Sclerosis, or Parkinson's disease. Recently, human NP have been isolated from epileptic patients or human fetal tissue, and differentiated into different brain cell types (40,43).

However, these types of studies, are always controversial, and thus there is a need for new *in vitro* models recapitulating cellular differentiation in the brain.

Here, we have employed human embryonic BG01V stem cells to generate NP, which were further differentiated towards astrocytes. This *in vitro* astrocyte differentiation model allowed us to monitor gene expression during the differentiation process, and analyze the effects of specific gene knock-down. The model we developed recapitulates gene expression patterns that have been previously described for several markers of differentiation. These include GFAP, SPARCL1, CD44, BFABP, GLAST, and S100 β . GFAP is an intermediate filament that replaces vimentin and nestin during the differentiation of astrocytes *in vivo* (32). It is detected in the later stages of brain development, and its expression increases with time. Although GFAP can also be present in ESC, it is expressed by mature astrocytes at levels that are hundreds fold higher. Importantly, BG01V ESC and NP express only minute amounts of GFAP (Fig. 2); however, their differentiation *in vitro* resulted in approximately 50-100 fold increase in GFAP expression (Fig. 4 and 5). Accordingly, SPARCL1, a matricellular protein widely expressed in the embryonic and neonatal brain and vasculature (37), is gradually expressed at increasing levels during development, thus indicating the progression of differentiation. Consequently, very low SPARCL1 levels were found in the ESC, which increased in the NP cells (Fig. 2A), and peaked in astrocytes (Fig. 4). On the other hand, CD44 is a receptor for hyaluronic acid expressed by astrocyte-restricted precursors in the developing rodent spinal cord (34). Moreover, CD44 does not co-localize with neuronal or oligodendroglial markers. The increased expression of CD44 in the differentiating astrocytes versus NP and ESC (Fig. 4), suggests that these cells followed the astrocyte differentiation pathway. *In vivo*, expression of CD44 precedes the expression of GFAP (34), suggesting that the cells we generated are still immature since they abundantly express both CD44 and GFAP (Fig. 4A). This is further supported by the high levels of BFABP, which *in vivo* is abundantly expressed by radial glia (36), early precursors of astrocytes in the developing brain. In contrast, S100 β is produced by many cell types, including astrocytes, oligodendrocytes, microglia, and some neuronal subpopulations (35). Its expression defines cells that lost stem cell potential. Therefore, expression of S100 β in the NP and astrocytes, but not in the ESC (Fig. 2A and 4A), is a strong indicator that the expected progression of differentiation had occurred. One of the main physiological functions of astrocytes is to absorb glutamate from the perisynaptic regions to protect the neurons against its cytotoxicity (44). However, this function is fulfilled by the NP during the neurogenesis in the developing brain, which still lacks mature astrocytes. Accordingly, we found significant glutamate uptake by NP, which was further increased in the differentiated astrocytes (Fig. 3D). Moreover, expression of GLAST was evident in NP, and it further increased during their differentiation (Fig. 4A). These observations coincide with expression of GLAST in both cortical NP and mature astrocytes *in vivo*.

Nuclear Factor I family members are expressed during the development of the CNS (18). NFI-A and -B direct the onset of gliogenesis in the developing mouse and chick spinal cord, with NFI-A also required for the maintenance of NP, specification of oligodendrocyte fate, and the inhibition of neurogenesis (11). The precise roles of the other family members, including NFI-X, are not known. We have previously shown that down-regulation of NFI-X decreases the expression of GFAP in U373 glioma cells (45) but simultaneous down-regulation of NFI-X and -C is required to significantly affect GFAP expression in primary human astrocytes (supplementary Fig. 3). Moreover, GFAP levels are normal in both NFI-X and NFI-C knock-out mice (24,25), suggesting that these two isoforms, which share a high sequence homology, may substitute each other. Here, we knocked-down the expression of NFI isoforms during the differentiation of NP towards astrocytes. Significantly, simultaneous knock-down of NFI-X and -C expression was sufficient to downregulate the expression of GFAP and SPARCL1 by 80% and 50%, respectively (Fig. 5B). Interestingly,

SPARCL1 is a member of osteonectin family of proteins that regulate proper development of osteoblasts. In fact, the *Nfix*-deficient mice developed spinal deformations due to a delayed ossification of vertebral bodies (25), suggesting that SPARCL1 may be important in this process. In contrast, the expression of the other markers was not affected by the NFI knock-down, indicating that GFAP and SPARCL1 may be specifically regulated by these two NFI isoforms. Of note, in differentiated astrocytes the levels of NFI-C mRNA were relatively low in comparison to NFI-X mRNA levels (Fig. 4B). However, these low levels of NFI-C are important likely due to generation of multiple splice isoforms containing different transactivating domains (46), which can specifically bind to the target gene promoters, including GFAP. Collectively, our data suggest that the expression of NFIs is temporally regulated during differentiation, and this affects the expression of marker genes, including GFAP and SPARCL1, during the progression of gliogenesis. NFIs most likely cooperate with the JAK-STAT, BMP, and Notch pathways to promote astrocyte differentiation, which may be coordinated around the formation of the CBP/p300 complex (15).

CONCLUSIONS

In summary, we developed an *in vitro* astrocyte differentiation model, which provides the means to study the regulation of gene expression during differentiation of human NP into astrocytes. Using this approach, we show that NFI-X and NFI-C regulate the expression of astrocyte markers GFAP and SPARCL1 in the later stages of astrocyte differentiation. In the future, this *in vitro* model may be used to further study glial cell differentiation, and thus may provide insights into various aspects of stem cell-based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. BG01V embryonic stem cells differentiate into neural progenitors

(A) Contrast phase microscopic image of ESC grown on a MEF feeder layer (left), and generated NP grown on laminin (right). Arrows point to ESC colonies. (B) Cell lysates were prepared, and the expression of the indicated markers was analyzed by Western blotting as described in the M&M section. β -tubulin was used as a loading control. (C) Immunocytochemical staining of stem cells and differentiated neural progenitors with antibodies (Ab) against the cell specific markers (red/green) as indicated, and DAPI staining (blue) was performed as described in the M&M section. Differentiation experiments were repeated six times with the consistent results.

Fig. 2. Gene expression in BGO1V-derived NP

RNA was prepared from ESC, NP, and primary human astrocytes (Astr) as a positive control. Subsequently, expression of glial markers (A), and NFI isoforms (B) was measured by qPCR (Taqman). Data were normalized to GAPDH mRNA levels, and are represented as a relative fold induction in reference to embryonic stem cells. Experiments were performed three times in triplicates, and the standard deviation was calculated as indicated by the error bars.

Fig. 3. Differentiation of NP into astrocytes

NP were submitted to the astrocyte differentiation protocol as described in the M&M section for 21 days. (A) Contrast phase microscopic image of NP, generated astrocytes (Diff. Astr), and primary human astrocytes (Astr). (B) Immunocytochemical double-staining of differentiated astrocytes with anti-GFAP (green) and anti-CD44 (red). DAPI (blue) was performed as described in the M&M section. (C) Immunocytochemical staining of differentiated astrocytes with anti-GFAP, anti-SOX2, anti-Msi-1, anti-O1, anti- β III-tubulin, non-specific antibodies (NS), and DAPI (blue). (D) Glutamate uptake was assayed in ESC, NP, Diff. Astr., and Astr. as described in the M&M section. Data are expressed in picomoles of glutamate per mg of cellular protein. Experiments were performed at least twice using multiple samples (NEP, n=7, Diff. Astr., n=5; Astr., n=6, ESC, n=4), and error bars indicate the standard deviation values.

Fig. 4. Regulation of gene expression during astrocyte differentiation

Neural progenitors were differentiated into astrocytes for 24 days according to the protocol described in the M&M. RNA was isolated during this process every 3 days, reverse-transcribed, and the expression of glial markers (A) and NFI isoforms (B) was analyzed by qPCR. Target gene expression was normalized to GAPDH levels, and is represented as a fold induction in reference to NP cells. Experiments were performed three times in triplicates.

Fig. 5. NFI-X and NFI-C regulate the expression of GFAP and SPARCL1 during astrocyte differentiation

Neural progenitors were submitted to the astrocyte differentiation protocol for 21 days. On day 15 of the differentiation process, cells were transfected with non-targeting control siRNA, siRNA targeting NFI-C and NFI-X ((C+X) siRNA), or siRNA targeting all NFI isoforms (panNFI siRNA). Subsequently RNA was isolated on day 21, and the expression of (A) NFI isoforms and (B) glial markers were analyzed in triplicates. Data were normalized to GAPDH mRNA, and are presented as a fold induction in reference to untreated differentiated astrocytes.