Functional and Phenotypic Differences of Pure Populations of Stem Cell-Derived Astrocytes and Neuronal Precursor Cells

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Availability of homogeneous astrocyte populations would facilitate research concerning cell plasticity (metabolic and transcriptional adaptations; innate immune responses) and cell cycle reactivation. Current protocols to prepare astrocyte cultures differ in their final content of immature precursor cells, preactivated cells or entirely different cell types. A new method taking care of all these issues would improve research on astrocyte functions. We found here that the exposure of a defined population of pluripotent stem cell derived neural stem cells (NSC) to BMP4 results in pure, nonproliferating astrocyte cultures within 24–48 h. These murine astrocytes generated from embryonic stem cells (mAGES) expressed the positive markers GFAP, aquaporin 4 and GLT 1, supported neuronal function, and acquired innate immune functions such as the response to tumor necrosis factor and interleukin 1. The protocol was applicable to several normal or disease prone pluripotent cell lines, and the corresponding mAGES all exited the cell cycle and lost most of their nestin expression, in contrast to astrocytes generated by serum addition or obtained as primary cultures. Comparative gene expression analysis of mAGES and NSC allowed quantification of differences between the two cell types and a definition of an improved marker set to define astrocytes. Inclusion of several published data sets in this transcriptome comparison revealed the similarity of mAGES with cortical astrocytes *in vivo*. Metabolic analysis of homogeneous NSC and astrocyte populations revealed distinct neurochemical features: both cell types synthesized glutamine and citrate, but only mature astrocytes released these metabolites. Thus, the homogeneous cultures allowed an improved definition of NSC and astrocyte features.

Key words: astrocytes, neural stem cells, differentiation, metabolic flux, transcriptome

Introduction

The definition of astrocyte functions and developmental potential, in particular in relation to neural stem cells, is still a dynamic area of research (Bayraktar et al., 2015; Gotz et al., 2015; Robel et al., 2011). Astrocytes and neural stem

cells (NSC) share several phenotypic and functional features (Doetsch et al., 1999; Kriegstein and Alvarez-Buylla, 2009; Levitt and Rakic, 1980; Seri et al., 2001). Moreover, some types of NSC (radial glia or astrocytes from the subventricular zone) have been categorized as members of the

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heterogeneous group of astrocytes, due to their expression of the glial fibrillary acidic protein (GFAP), the most frequently used astrocyte marker (Eng et al., 2000; Ihrie and Alvarez Buylla, 2008; Steindler and Laywell, 2003). Under pathophysiological conditions, astrocyte behavior shows high plasticity (Parpura et al., 2012) and reversion to an NSC like precursor state has been observed (Gotz et al., 2015; Sirko et al., 2013).

Astrocytes play a pivotal role in brain metabolism (Amaral et al., 2011; McKenna et al., 2012; Waagepetersen et al., 2009) and in linking neurons and other brain cells to blood supply (Kacem et al., 1998). Beside their role in metabolic homeostasis, astrocytes efficiently take up neurotransmitters like glutamate (Zhou and Danbolt, 2013), and thus modulate synaptic function (Perea et al., 2009). In this context, glial glutamine synthetase (Glul) catalyzes the condensation of ammonia to imported glutamate to produce glutamine, which is then released, and taken up by neurons to produce glutamate again (Daikhin and Yudkoff, 2000). This dynamic recycling mechanism of glutamate, considered essential to prevent neuronal excitotoxicity (Delaney et al., 1996), is thought to be astrocyte specific. However, NSC also express the glutamate aspartate transporter GLAST (Liu et al., 2006; Ullensvang et al., 1997) and glutamine synthetase (Hernández et al., 1999; Monzon Mayor et al., 1990; Suarez et al., 1997) and their potential role in glutamate metabolism therefore deserves attention.

For the study of astrocyte metabolism and function, primary monolayer cultures have become the dominating model system. However, contamination with other cell types such as microglia (Saura, 2007), requires stringent controls and the contribution of immature cells with high levels of nestin expression to standard primary cultures has often been neglected (Hansson, 1986; Sergent Tanguy et al., 2006; Stahlberg et al., 2011). An alternative approach to obtain astrocytes is to use neurospheres from embryonic stem cells (ESC) (Kuegler et al., 2012) or brain (Crocker et al., 2008) as starting material for astrocytogenesis. The resulting astrocyte population is free of microglia, but it may still contain nestin positive precursors and various proliferating cell types. The same applies to the generation of human astrocytes from pluripotent stem cells. Several corresponding protocols generate GFAP positive cells (Krencik et al., 2011; Roybon et al., 2013; Shaltouki et al., 2013), but they all still contain sizable subpopulations of immature or contaminating cells that might spoil results of gene expression analysis, identification of metabolic pathways, or cell fate examinations.

Compared with astrocytes, hardly any data is available on metabolism or inflammatory reactions of their precursor cells and of NSC. A major reason for this paucity of information may be the limited access to suitable experimental systems. Iso-

lation of defined and sufficiently large populations of primary cells is difficult, and NSC amplified *in vitro* in the form of neurospheres often comprise heterogeneous subpopulations that are challenging to handle for biochemical studies.

A new option to study NSC was provided by the discovery that such cells may be cultured and propagated as highly homogeneous monolayer cultures (Conti et al., 2005). This seminal work mainly described the use of the cells to study neurogenesis, but it was also shown that this NSC cell population differentiated to GFAP positive cells within 48 h of exposure to serum (Conti et al., 2005). These data suggested an attractive way of producing astrocytes (Schneider, 2014; Schneider and d'Adda di Fagagna, 2012; Sparmann et al., 2013), but more characterization of the resultant cells is necessary, and the original protocol requires adaptations and further specifications before pure cultures of astrocytes can be obtained reproducibly.

Since there is a great, yet largely unrecognized, potential in the astrocyte generation from NSC, we set out to (i) define the critical steps of the procedure, (ii) to characterize the resulting cells, and (iii) to demonstrate the usefulness of a protocol adapted from Conti et al. (2005) for generating NSC and astrocyte populations. This allowed us for the first time a direct comparison of NSC and homogeneous, nonproliferating astrocytes concerning differentiation response, neuronal support, immune activation, differentially expressed genes, and metabolic features with clear differences identified between the two cell types.

Materials and Methods

Maintenance of Murine Embryonic Stem Cells

The murine embryonic stem cell (mESC) lines CGR8.0, E14, BTBR T+ Itpr3tf/J PB60.6 (The Jackson Laboratory, 002282C01), DO335.45 (Diversity Outbred strain, Jax number 009376), and O9 iPS (Wernig et al., 2007) were maintained in Glasgow Minimum Essential Medium (GMEM) containing high glucose (4.5 g/L), 10% fetal bovine serum (FBS), 2 mM Glutamax, 2 mM sodium pyruvate, 2 mM nonessential amino acids, and 50 μ M β mercaptoethanol (materials are listed in the Supp. Info.). Medium was changed every day with freshly added 1,000 U/mL leukemia inhibitory factor (LIF). Cells were passaged every other day with 0.05% trypsin and replated on NunclonTM Delta flasks (Thermo Scientific) coated for 1 h with 0.1% gelatin dissolved in water.

Differentiation of Neural Stem Cells From mESC

Cultures of mESC were used when they reached 80% confluency. Cells were harvested with 0.05% trypsin, pelleted and replated as single cells in "N2B27 medium" [1 part DMEM/F12 + 1 part Neurobasal medium, supplemented with N2 and B27, 2 mM Glutamax, 100 μ M β mercaptoethanol, 7.5 μ g/mL insulin, 50 μ g/mL bovine serum albumin (BSA)] at a density of 10,000 cells/cm² on 10 cm Nunclon dishes coated for 2 h with 0.1% gelatin. Medium was changed every other day. On Day 7, cells were treated with 0.05% trypsin for 1 min, resuspended with N2B27 medium and filtered through a 70 μ m cell strainer into 50 mL polypropylene tubes

prefilled with phosphate buffered saline (PBS) to dilute trypsin. After centrifugation at 500g for 3 min, all cells were plated onto gelatin coated T75 Nunclon flasks in N2B27 medium supplemented with 20 ng/mL FGF2 and 20 ng/mL EGF. For cryopreservation, cells were trypsinized and cryopreserved in FBS containing 10% DMSO at 2×10^6 cells/mL.

Maintenance and Selection of NSC From Freshly Prepared d7NSC

After 7 days neural induction of mESC, cells were grown in N2B27 medium supplemented with EGF and FGF2. The cells in these initial cultures had different morphologies and cell cycling behavior, some of them growing faster than others. Since the desired bipolar, RC2 positive NSC can be rare at Passage 1, cultures were not passaged until they reached full confluency (~1 week). To get rid of aggregating cells, which sit on top of other cells, plates were tapped several times to detach them mechanically before trypsinization. Some aggregating cells are usually in closer contact with other cells as well as the coated surface; therefore they cannot be removed by tapping the flasks. They were manually scratched with an aspiration pipette (aggregates are visible with the naked eye) and the borders of the flask were scratched as well. To separate bipolar NSC from triangular flat cells, cultures were treated with trypsin for only 10–15 s. Under these conditions, NSC detach in contrast to the more adherent triangular cells. Pictures before and after mechanical cleaning and selective trypsinization are shown in the Supporting Information Fig. S2. After trypsinization, the flask surface was washed only once with N2B27 medium to harvest bipolar NSC and avoid detaching triangular cells. The harvested cells were thoroughly resuspended in one corner of the flask to obtain single cells. Since NSC are very sensitive to trypsin, the cell suspension was directly diluted in about 20 mL PBS (through 70 μ m cell strainer), centrifuged rapidly afterward, and resuspended in medium. After several passages, the aggregating and the triangular cells disappeared. At about Passages 8–12, cultures purely consisted of bipolar NSC. Sometimes aggregating cells reappeared after Passage 8, but they were easily removed as described above. Notably, NSC do not tend to aggregate when passaged twice a week at the right density. They should not be seeded at less than 10,000 cells per cm^2 and should be passaged at maximum 90% confluency (70–80% confluency is better). For maintenance culture, flasks with at least 75 cm^2 should be used, since NSC tended to clump in smaller flasks.

Astrocyte Differentiation

NSC cultures from Passage 8 or later at 80% confluency were treated with 0.05% trypsin for 10–15 s, resuspended in N2B27 medium and filtered through a 70 μ m cell strainer prefilled with PBS, centrifuged (500g, 3 min), and resuspended in medium. Cells were plated at a density of 30,000 (e.g., for NFkB translocation) to 50,000 (e.g., for metabolic studies) cells/ cm^2 in N2B27 medium supplemented with 20 ng/mL bone morphogenetic protein 4 (BMP4). Nunclon dishes or plates were coated with 10 μ g/mL poly-L-ornithine hydrobromide in PBS for 2 h at 37°C, washed twice with PBS, and coated with 2 μ g/mL laminin in PBS overnight.

Laminin was aspirated and the cell suspension was added to dishes/plates. Medium was changed every other day from Day 1 and experiments were performed at Day 3 or 5 of differentiation. If cells were cultured for longer periods, medium was changed every other day using N2B27 medium with 10 ng/mL BMP4.

Preparation and Maintenance of Primary Astrocytes

BALB/c mice were bred at the animal facility of the University of Konstanz (Konstanz, Germany). All mice were housed at 22°C and 55% relative humidity in a 12 h day/night rhythm with free access to food and water according to national regulations and EU guide line 2010/63/EU. Primary astrocytes were prepared from 1 to 2 days old mouse pups as described earlier (Henn et al., 2011). Briefly, cells were prepurified by a percoll density gradient centrifugation right after dissociation of the cortices. The enriched astrocyte fraction was kept in tissue culture flasks for two weeks in medium containing 20% FBS. Then, they were trypsinized and plated for experiments.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated using PeqGold TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions and 1 μ g of RNA was reverse transcribed to complementary DNA (cDNA) using the iScript reverse transcription polymerase chain reaction (RT-PCR) Supermix kit (Biorad). Quantitative RT-PCR (qPCR) to measure mRNA expression levels was performed using a CFX96TM Real Time System (BioRad, Hercules, CA) and SSO Fast EvaGreen Supermix (Biorad) according to the manufacturer's protocol. The cDNA levels relative to the housekeeping gene *Gapdh* were calculated using the delta C_T method. Primers used are listed in the Supporting Information.

Immunofluorescence Staining and EdU Labeling

Cells were fixed for 10 min with ice cold 90% methanol at -20°C, permeabilized with 0.1% Triton X 100 in PBS at RT for 10 min, and blocked for 30 min in 1% BSA in PBS. Cells were stained with primary antibodies for 2 h and the respective secondary antibodies (Invitrogen, Darmstadt, Germany) for 50 min. 1 μ g/mL H 33342 was added during the last 20 min to counterstain DNA. Primary antibodies used are listed in the Supporting Information. To detect DNA synthesis, cells were incubated for 48 h with the thymidine analogue EdU (Bioss, BCK EdU555), fixed and permeabilized as described above, and stained according to the manufacturer's instruction.

Microarray Profiling and Data Analysis

RNA of four independent cultures and differentiations of mESC, NSC, and 5 day old mAGES was isolated from three pooled technical replicates as described for qPCR. Microarray analysis was conducted as described earlier (Krug et al., 2013). Briefly, 12.5 μ g fragmented aRNA was hybridized with Affymetrix Mouse Genome 430 2.0 arrays according to the manufacturer's instructions. All reagents and instruments were acquired from Affymetrix (Affymetrix, Santa Clara, CA). The generated CEL files were used for further statistical analysis. All microarray datasets were preprocessed using the GC RMA procedure for background correction, quantile

normalization and probe replicate summarization (Wu et al., 2004). All statistical analyses were performed in the R Statistical Programming Environment (Ihaka and Gentleman, 1996). Differentially expressed genes between pairs of conditions were determined using the Empirical Bayes moderated *t* statistic (Smyth, 2004) and adjusted for multiple hypothesis testing using the Benjamini Hochberg method (Benjamini and Hochberg, 1995). A false discovery rate threshold of 0.05 was chosen to determine the significantly differential genes.

Protein Measurement and LDH Release

Cells were lysed in 2% sodium dodecyl sulfate (SDS) and protein content of lysates was measured using PierceTM BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. For the release of lactate dehydrogenase (LDH), 10 μ L (one tenth of total volume) of supernatant and cell lysates (20 min 0.1% Triton X100) were used together with 200 μ L reaction mix containing 1:50 pyruvate to NADH reagent (68 μ g/mL pyruvate in KPP buffer (40.24 mM K₂HPO₄, 9.7 mM KH₂PO₄, pH 7.5) and 8.47 mg/mL NADH in 1% NaHCO₃). NADH oxidation was measured at 340 nm with BioTek EL808 Absorbance Reader for 20 min. LDH release in percent was calculated from ΔOD_{340} (supernatant)/(ΔOD_{340} (supernatant) + ΔOD_{340} (lysate)).

Glutamate Uptake Measurement

Cells were preincubated with Hanks' Balanced Salt Solution (HBSS, Gibco) containing 1 g/L glucose. After 30 min, 10 μ M L-glutamic acid (G1624, Sigma) was added supplemented with 0.12 μ Ci L-[3,4-³H] glutamic acid (PerkinElmer, NET490250UC) per sample. After 8 min, cells were washed 4 times with HBSS and lysed with 0.5% Triton X100 for 30 min. Lysates were mixed 1:1 with Ultima Gold AB scintillation cocktail (PerkinElmer, G013309) and measured with the LS 6500 scintillation counter (Beckman Coulter).

Culture Condition for Metabolic Flux Analysis and Metabolite Extraction

NSC and mAGES were seeded in six well plates in their normal condition. NSC were maintained 2 days until they were about 60% confluent and mAGES were differentiated until Day 5. Cells were washed twice with prewarmed PBS and incubated in N2B27 medium without glucose [1:1 Neurobasal A and Advanced DMEM/F12] supplemented with 10 mM D-[1-¹³C] glucose for 0, 0.3, 3, 12, and 24 h. Medium was collected, centrifuged at 200g for 10 min, and supernatant was frozen at -20°C until further processing. Cells were washed twice with ice cold PBS, frozen in liquid nitrogen, and extracted with 70% ethanol. After centrifugation of the cell extracts for 15 min at 20,000g, supernatants were collected and stored at -80°C until further analysis. Pellets were stored at -20°C for protein determination.

Measurement of Glucose, Lactate, and Amino Acids in Supernatant

Glucose and lactate concentrations in samples of cell supernatants were determined using an automated YSI 7100 Multiparameter Bioanalytical System (Dayton, OH). Extracellular concentrations of

amino acids were quantified by HPLC using a precolumn derivatization method based on the Waters AccQ Tag Amino Acid Analysis method as described elsewhere (Amaral et al., 2010).

Quantification of Mass Isotopomers by GC-MS

Analysis of ¹³C percent enrichment in metabolites was carried out by redissolving lyophilized extracts in 0.01 M HCl. Samples were extracted in multiple steps into an organic phase of ethanol and benzene and lyophilized before derivatization with *N*-methyl *N*-(*t*-butyldimethylsilyl)trifluoroacetamide + 1% *t*-butyldimethylchlorosilane (MTBSTFA). The samples were analyzed on an Agilent 6890 gas chromatograph (GC) connected to an Agilent 5975B mass spectrometer (MS) (Agilent Technologies, Palo Alto, CA). The parent ion (*M*) and atom percent excess for one, two, *n* ¹³C atom (*M*+1, 2, *n*) values for metabolites were calculated from GC-MS data using MassHunter software supplied by Agilent (Agilent Technologies) and corrected for the naturally abundant ¹³C by using nonenriched standards (Walls et al., 2014).

¹H-NMR Spectroscopy for Citrate Determination in Cell Culture Supernatants

Citrate concentration in samples of cell supernatants was determined by ¹H-NMR spectroscopy, in a 500MHz Avance spectrometer (Bruker, Billerica, MA) with a 5 mm QXI probe. Spectra were recorded at 25°C, using a NOESY-based pulse sequence with water presaturation, performing 256 scans, with 4 s acquisition time and 2 s relaxation delay. DSS-d₆ (Sigma Aldrich, St. Louis, MO) was used as internal standard. Samples were mixed with phosphate buffer (pH 7.4) prepared in D₂O at a 2:1 ratio. Each spectrum was phased, baseline corrected, and integrated using the Chenomx NMR Suite 7.1 (Chenomx, Canada) software.

Cell Stimulation, NFκB Translocation, and interleukin 6 ELISA

Cells were stimulated with the complete cytokine mix (CCM) containing 10 ng/mL tumor necrosis factor α (TNF α), 10 ng/mL interleukin 1 β (IL 1 β), and 20 ng/mL IFN γ (R&D Systems, Wiesbaden, Germany) or with its single components for 30 min in case of NFκB staining, 4 h for qPCR measurements, or 8 h for IL 6 measurement in the respective medium. For NFκB measurement, cells were fixed, permeabilized and stained for NFκB. NFκB translocation was measured with the high throughput device Cellomics ArrayScan or CellInsightTM CX5 High Content Screening (Thermo Scientific) using the nuclear translocation algorithm as described previously (Henn et al., 2011). Supernatants for IL 6 ELISA were collected and stored at -80°C until further processing. IL 6 was measured in supernatants diluted 1:5 in assay diluent and mouse IL 6 ELISA Ready SET Go was performed according to the manufacturer's protocol (eBioscience).

Results

Pivotal Conditions for the Generation of Astrocytes from ESC-Derived Neural Stem Cells

Embryonic stem cells were differentiated within 7 days into neural stem cells of distinct morphologies, when plated on

gelatin coated dishes in N2B27 medium without growth factors. The cells had a high nestin expression and neurogenic potential as described previously (Conti et al., 2005; Zimmer et al., 2011a,b). However, they hardly generated astrocytes when exposed to bone morphogenetic protein 4 (BMP4) (Supp. Info. Fig. S1A), serum, CNTF, or combinations thereof (not shown). The morphologically heterogeneous neural stem cell population contained bipolar cells in addition to other cell types (Supp. Info. Figs. S1B and S3). Maintenance of the cells in EGF and FGF2 containing medium for several passages, and removal of aggregate forming as well as flat, triangular cells (selective trypsinization/mechanical cleaning) at each passage step produced a homogeneous population of bipolar cells (Supp. Info. Fig. S2). These “secondary NSC” could be completely converted to GFAP positive, nestin negative (about 80% of the cells only showed background staining after 3 days) astrocytes upon BMP4 exposure (Supp. Info. Fig. S1A). The pivotal step in the production of “murine astrocytes generated from embryonic stem cells” (mAGES) was the selection of an astrogenic cell population, which could then be easily maintained for at least 40 passages or frozen, banked, and re thawed for subsequent differentiation (Fig. 1A).

The secondary NSC (=high passage NSC) still retained their potential to generate mature neurons within 14 days, when growth factors were withdrawn from the medium (Supp. Info. Fig. S4). Thus, NSC are multipotent neural stem cells, which give rise to astrocytes as well as neurons. This also held true for single cell clones: Ten out of 10 clones generated mAGES after 5 days of BMP4 exposure, with only slight morphological differences (Supp. Info. Fig. S5). Moreover, when differentiated without growth factors, all of the clones generated about 60% neurons.

Astrogenic NSC have been produced five times from the mESC cell lines CGR8.0 and E14, and astrocytes have been generated from these with the same efficiency (not shown). Furthermore, mESC lines from a different mouse background (DO335 and BTBR) or murine induced pluripotent stem cells (iPSC) have been used successfully for mAGES generation (Supp. Info. Fig. S6). Expression of GFAP, Aqp4, and nestin in mAGES produced therefrom was similar to the one found in the control line CGR8.0. This broad applicability confirmed the robustness of the astrocyte differentiation protocol.

Rapid Generation of Mature Astrocytes From NSC

To characterize the cells, well known markers were quantified for NSC as well as mAGES on mRNA level by quantitative PCR. Expression levels were compared with those of primary murine astrocytes. As previously reported, NSC expressed the neural stem cell markers *Nestin*, the brain lipid binding pro-

tein *Blbp*, and the oligodendrocyte transcription factor *Olig2* (Conti et al., 2005). All three genes were downregulated in mAGES after differentiation for 5 days with BMP4 (Fig. 1B). The expression of *Blbp* and *Olig2* was similar in primary astrocytes and mAGES, while *Nestin* expression was more than 100 fold lower in mAGES compared with NSC or primary astrocytes. Other genes, which are normally expressed in both neural stem cells and astrocytes, like the glutamine synthetase (*Glu1*) and the glutamate aspartate transporter (*Glast*), were similarly expressed in all three cell types (Fig. 1B).

The astrocytic glial fibrillary acidic protein (*Gfap*) was highly expressed in mAGES as well as in primary astrocytes, whereas it was absent in NSC (Fig. 1C). The calcium binding protein *S100b*, another well known astrocyte marker, was highly expressed in all three cell types. Aquaporin 4 (*Aqp4*) and the glutamate transporter *Glt-1*, both specific markers for mature astrocytes, were upregulated in mAGES and primary astrocytes compared with NSC. Also, the aldehyde dehydrogenase *Aldh1L1*, which is reported to be astrocyte specific in the brain (Cahoy et al., 2008), was expressed at eightfold higher levels in mAGES compared with NSC (Fig. 1C).

When the time course of marker expression was followed during the conversion of NSC to mAGES, we found that dramatic changes (up to 1,000 fold) occurred within 24 h after BMP4 addition. *Gfap* and *Glt-1* reached their maximum level already after 1 day, whereas *Aqp4* and *Nestin* further increased/decreased from Day 1 to Day 3 of BMP4 exposure. No changes were observed from Day 3 to Day 5. This suggests that astrocyte differentiation was completed after 3 days (Fig. 1D). The cells could be easily maintained in this stage for at least 4 weeks (not shown).

As further differentiation characteristic, we investigated the proliferation state of mAGES. This endpoint was chosen, because fully differentiated, mature astrocytes hardly undergo spontaneous cell division (i.e., without stimulation by growth factors or serum), while immature cells and astrocytic precursors (as sometimes present in primary cultures or stem cell derived populations) have a relatively high proliferation rate. DNA replication was followed on single cell level by incorporation of the thymidine analogue 5 ethynyl 2' deoxyuridine (EdU) into newly synthesized DNA. EdU was easily detected in the nuclei of NSC, whereas mAGES did not show any EdU incorporation at all during a 48 h incubation (Fig. 1E). Exact quantification showed that more than 90% of NSC incorporated EdU (proliferating cells), while < 0.1% of mAGES proliferated (Fig. 1F).

Factors Affecting Differentiation Efficacy

Since a single positive marker, such as *Gfap* mRNA or GFAP protein expression is not sufficient to characterize the extent

FIGURE 1: Rapid generation of nonproliferating, mature astrocytes from neural stem cells. (A) A heterogeneous population of “neural stem cells” (d7NSC) was generated within 7 days by spontaneous differentiation of murine embryonic stem cells (mESC). Selective passaging (at least 8–12 times) in the presence of 20 ng/mL EGF and FGF2 on gelatine produced a homogeneous population of NSC that could be frozen for later use or maintained by further passaging. Transfer of the cells to poly ornithin/laminin (P/L) coated dishes and exposure to BMP4 (20 ng/mL) resulted in the generation of murine astrocytes generated from mESC (mAGES). (B and C) For measurements of gene expression by qPCR, mRNA was prepared from mAGES (after 5 days of differentiation), NSC or primary murine astrocytes. All data are given in fold difference (x fold) relative to the housekeeping gene *Gapdh* (note logarithmic axis scaling). The horizontal brackets in the plots indicate the fold difference of mAGES vs. NSC (B) Nestin, Blbp (FABP7, brain lipid binding protein), Olig2 (oligodendrocyte lineage transcription factor; neuroectodermal marker), Glul (Glutamine synthetase) and Glast (Slc1A3; glutamate transporter) were used as NSC markers; (C) *Gfap* (glial fibrillary acidic protein), *S100b* (*S100*beta), *Aqp4* (aquaporin), *Glt-1* (Slc1A2, glutamate transporter), and *Aldh1L1* (aldehyde dehydrogenase) were used as astrocyte markers (D) NSC were exposed to 20 ng/mL BMP4 and left to differentiate to mAGES. At the times indicated, mRNA was prepared to quantify the expression of marker genes by qPCR. All data are given relative to the expression in NSC ($\log_{10}$ scaled), based on the $\Delta\Delta C_T$ method. Data are means $\pm$ SEM from three experiments (some error bars are smaller than the symbols). *** $P < 0.0001$; ** $P < 0.001$; * $P < 0.01$ (one way ANOVA with Dunnett’s post hoc test). (E) NSC and mAGES (3 days old) were incubated with the nucleoside analogue EdU (10 μ M) for 48 h, before cells were fixed. Then, EdU incorporation was visualized by immunocytochemistry, and nuclei were counterstained with H 33342 (note that EdU staining (dark green) appears yellow to green, depending on the strength of the always underlying H 33342 (red) stain) (F) The number of nuclei that were EdU positive were counted in NSC and mAGES cultures by an automated screening microscope (1,000 nuclei/condition). The data displayed are means $\pm$ SEMs from three independent experiments. In mAGES cultures less than 1 cell per condition was found to be EdU positive (<0.1%). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of astrocyte differentiation, we chose here to measure GFAP, nestin (protein expression) and EdU incorporation (DNA synthesis) to compare different inducers of differentiation. BMP4, as used in our optimized protocol, led to a complete

cell cycle exit, a high percentage of GFAP positive cells and a >80% reduction of nestin positive cells. When fetal bovine serum (FBS) was used instead of BMP4, NSC differentiated to mAGES with a similar GFAP expression, but nestin

expression was higher (Supp. Info. Fig. S7). Moreover, some mAGES were still proliferating, as indicated by EdU incorporation. Other cytokines, such as LIF or CNTF were not able to induce complete mAGES differentiation. Although GFAP was upregulated under these conditions to a high extent, the cells had a high nestin expression and proliferation rate. Moreover, the morphology was not typical of astrocytes (Supp. Info. Fig. S7). These findings showed that GFAP alone is not sufficient to judge the differentiation to mature astrocytes, and that BMP4 was the most efficient agent, when several parameters were considered.

Finally, we were interested in whether the protocol allowed the identification of factors that negatively affect astrocyte differentiation (e.g., developmental toxicants). Several poisonous compounds known to affect neurodevelopment (e.g., methylmercury, manganese) were added during the differentiation procedure, and the expression (immunocytochemistry) of GFAP, Aqp4, and nestin was evaluated as endpoint. None of the toxicants affected mAGES generation, when used at noncytotoxic concentrations. The same applied to small molecules, which are known to inhibit BMP4 induced SMAD signaling (e.g., dorsomorphin or LDN 193189), indicating that canonical SMAD signaling was not required for the differentiation of mAGES. From this we conclude that the protocol developed here is very robust toward many external disturbances. In the search for positive examples of developmental disturbances, we used two harsh approaches: (i) addition of noggin, which neutralizes BMP4, inhibited upregulation of GFAP as well as downregulation of nestin (data not shown) and (ii) very high concentrations of valproic acid (VPA, 10 mM), an HDAC inhibitor and neurodevelopmental toxicant, reduced mAGES generation, although they were not directly cytotoxic to astrocytes (Supp. Info. Fig. S8). Thus, identification of chemicals or hormonal factors that inhibit astrocytogenesis seems to be feasible in this system.

Basic Metabolic Features of mAGES vs. NSC

Relatively little is known yet about metabolic differences and similarities of astrocytes and their immediate precursors. In 2004, Brunet and others used murine neural stem cells differentiating to astrocytes in order to ask the question, at which developmental stage typical metabolic features of astrocytes are developed (Brunet et al., 2004). This elegant study provided initial evidence on metabolic differences between NSC and astrocytes. For example the maximum glutamate transport capacity changed during astrocytogenesis, but the line of work was unfortunately not continued and metabolic consequences for the cells are not known. As both mAGES and NSC expressed glutamine synthetase, we were interested in its cellular metabolic integration. As an integrative endpoint for this, we studied cell survival in medium supplemented

with glutamine or not. In mAGES, the protein content per well (as viability parameter) and LDH release (as direct cell death measure) did not change within a 24 h period in either condition. Thus, the absence of glutamine did not trigger cell death (Fig. 2A,B). In fact, mAGES could be cultured for at least 4 days in glutamine free medium without changes in viability (data not shown), confirming their independence from exogenous glutamine. By contrast, NSC were strongly dependent on glutamine, i.e., protein content per well decreased and LDH release increased already at 12 h after withdrawal from glutamine.

To get an overview on central energy metabolism, glucose uptake and lactate release rates were determined. NSC had nearly a twofold metabolic rate (increased glucose consumption as well as lactate release) compared with mAGES (Fig. 2C,D). Altogether, both cell types had a high glucose to lactate conversion rate, and thus a minor contribution of mitochondrial metabolism (Fig. 2E,F), which is typical for stem cells (Candelario et al., 2013; Teslaa and Teitell, 2015), and astrocytes (Amaral et al., 2011; Magistretti et al., 1999; Waagepetersen et al., 1998). Thus, mAGES and NSC share some basic metabolic features, while differences became evident in glutamine free environment, where only mAGES survived.

Immunocytochemical Phenotyping of NSC and mAGES as Homogeneous Populations of Neural Stem Cells and Astrocytes

After having established basic metabolic features and mRNA marker expression patterns in NSC and mAGES cell populations, we investigated the homogeneity of the two cultures on single cell resolution by immunocytochemistry. While all NSC expressed NESTIN, most mAGES (>80%) downregulated its expression to background levels, and some cells retained a weak expression. All NSC stained positive for the radial glia marker RC2, while this protein was absent in mAGES. GLAST was expressed to varying levels in NSC (heterogeneous staining pattern), and to a much lower extent in mAGES. The calcium binding protein S100B was detected in both cell types, consistent with similar mRNA levels (Fig. 3A).

The “astrocytic” intermediate filament protein GFAP was strongly and ubiquitously (>99%) expressed in mAGES, but not in NSC (<1%). The same was observed for the glutamate transporter GLT1. About $70 \pm 7\%$ of mAGES expressed AQP4 (to various extents), while this protein was absent in NSC. The morphology of NSC changed dramatically when exposed to BMP4 for 3 days. All of the small, bipolar NSC cells adopted the typical star shaped/feathery morphology of astrocytes with radial processes and a flattened appearance, when differentiated to mAGES (Fig. 3B). To

FIGURE 2: General metabolic features of mAGES vs. NSC. (A) Cells (mAGES/NSC) were plated in six well plates at a density of 500,000 cells/well for mAGES and 80,000 cells/well for NSC. After 48 h (5 days for mAGES) the plating medium was changed, and cells were cultured further in medium containing either 2 mM glutamine (Gln) or no Gln. At the times indicated, medium was removed, cells were lysed and the protein content was measured. (B) The experiment was performed as in (A), and at the times indicated the percentage of LDH released from the cells into the medium was measured as cell death parameter. (C and D) The glucose concentration or lactate concentration in the medium of NSC and mAGES cultures was measured over time, and the overall protein content of the cultures was determined. From these data, normalized uptake/release rates were calculated. Data presented are means $\pm$ SEMs of three separate experiments. (E) The central carbon metabolism was schematically summarized to indicate that the uptake of 1 mol of glucose would result in the average release of 2 mol lactate, if only glycolytic catabolism was involved; and less than 2 mol lactate if some of the glucose metabolites were used in the tricarboxylic acid cycle (TCA) to produce CO₂. (F) The data from (C + D) were used to calculate the lactate to glucose ratio of NSC and mAGES metabolism.

more closely examine the presence of astrocytic precursors, cells were stained for CD44 and A2B5, and both markers were undetectable in mAGES, while low levels of both proteins could be detected in NSC (Fig. 3C). The absence of the proliferation marker Ki67 in mAGES as well as the upregulation of p27, a cell cycle inhibitor in postmitotic cell, further confirmed their cell cycle exit and the absence of proliferating cells in this population. This characterization of the two cell populations, based on 12 different parameters, suggested that

NSC were converted to a highly homogeneous population of astrocytes, containing no detectable proliferating precursor cells, or GFAP negative cells, and displaying heterogeneity for Aqp4, as observed in brain tissue.

Whole Transcriptome-Based Characterization of mAGES and Their Relationship to NSC

Only relatively recently, transcriptome data for astrocytes have become available to characterize their gene expression levels in different brain regions, culture conditions, and as opposed to neurons or oligodendrocytes (Cahoy et al., 2008; Lovatt et al., 2007; Shaltouki et al., 2013). However, little is known about the relationship of astrocytic and NSC gene expression, in particular for murine cells as major model system. Therefore, expression of over 34,000 genes (covered by 45,000 probesets) was measured here by microarray profiling. For each cell type (mAGES, NSC, mESC), mRNA was prepared from four independent differentiations, and principal component analysis of global gene expression revealed significant differences between all three cell types (Fig. 4A).

Quantitative comparison of gene expression allowed the identification of differentially expressed genes (DEG) for the different developmental stages, and more than 3,000 genes differed at least twofold between the cell types with an adjusted *P* value cutoff <0.05 . The most substantial change occurred during mESC to NSC differentiation with more than 10,000 DEG between the two cell populations. Most importantly for the present study, we found that NSC and mAGES differed in more than 7,000 genes. Thus, although mAGES and NSC are developmentally and functionally related, these data show their considerable disparity (Fig. 4B).

To identify marker genes, we performed pairwise comparisons of mESC, NSC, and mAGES, and the top 20 up or downregulated genes of each comparative analysis, sorted by *P* value significance or fold change, were compiled (Supp. Info. Fig. S10). During development of mESC to NSC, many genes involved in nervous system development were upregulated, some of them (e.g., *Olig1/2*, *Ptn* (pleiotrophin) or *Gpm6a* (calcium channel related to neurogenesis)) more than 1,000 fold. The top 20 downregulated genes coded for factors essential for the maintenance of embryonic stem cells, such as *Zfp42*, *Nanog*, *Oct4* (*Pou5f1*), *Fgf4*, and *Lefty2* (Supp. Info. Fig. S9).

The top 20 genes (according to the significance of regulation) in mAGES compared with NSC included *Id1* and *Id4*. These may not necessarily be cell type specific markers, as they are triggered by the BMP4 present in our cultures, but they are also known to play a role in astrocytogenesis (Fig. 4C). The highest upregulated gene in mAGES compared with NSC was fibromodulin (*Fmod*), which was more than 6,000 fold changed. The corresponding protein has

FIGURE 3: Immunocytochemical characterization of marker expression and cell culture homogeneity of NSC and mAGES. NSC and mAGES (3 days old) were fixed and permeabilized. Marker proteins (green) were visualized by immunocytochemistry and nuclei were counterstained with H 33342 (red). (A) NESTIN, RC2 (radial glia marker), GLAST (glutamate transporter), and S100B were used as NSC marker proteins. (B) GFAP (glial fibrillary acidic protein), AQP4 (aquaporin 4), and GLT 1 (glutamate transporter) were used as markers for mature astrocytes. Phase contrast pictures were taken from living cells before fixation. (C) CD44 and A2B5 were used as astrocyte precursor markers. The cell cycle inhibitor (p27) and the proliferation marker Ki67 were used to examine the cell cycle status of NSC and mAGES. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

metabolic functions and is involved in extracellular matrix organization. We also found genes involved in development (*Aplnr*, *Ndr4*), chemotaxis (*Cxcr7*, *Cmtm6*), and immune response (*Lyz1*, *Lyz2*, *Peli2*). The top 10 upregulated genes were further validated by qPCR and the data confirmed that all of them were much higher expressed in mAGES compared with NSC (Supp. Info. Fig. S12A,B). When DEG of mAGES against NSC were sorted by fold change, then the top 20 list comprised the well known astrocyte marker genes *Gfap* and *Aqp4*. Furthermore, we found again genes involved in the heterogeneous functions of mature astrocytes, such as immune response, extracellular matrix organization, metabolic homeostasis, or angiogenesis. More than 70% of the genes, which were downregulated in mAGES vs. NSC, were related to the cell cycle (Fig. 4C,D). This is well in concordance with the cell cycle exit of mAGES. Since the top 20 DEG expressed genes in mAGES comprised several transcripts, which were not previously recognized to be cell type specific, we investigated their *in vivo* expression by using published microarray data on parenchymal astrocytes and adult neural stem cells (aNSC) from the subventricular zone (Beckervor dersandforth et al., 2010). Many of the genes upregulated in mAGES were also more prominent in mature astrocytes (Supp. Info. Fig. S13A). Moreover, all genes found to be downregulated in our study were much higher expressed in aNSC than in parenchymal astrocytes. Thus, the set of DEG found in our study seems to be suitable to distinguish astrocytes and NSC also *in vivo*. In summary, the gene expression profiles provide cell type specific signatures that enable a clear distinction between NSC and mAGES, and thus also a refined definition of respective cell specific functions.

Comparison of mAGES Gene Expression With Different Other Cell Populations

For further characterization of mAGES and NSC, we compared their gene expression levels to legacy data and marker genes available from the literature. First, we compiled a list of genes specific for mESC, neural stem cells, and astrocytes (Cahoy et al., 2008; Kuegler et al., 2010). Amongst the 94 markers, 29 were for embryonic stem cells, 29 were considered specific for neural stem cells, and 36 for astrocytes.

Comparison of mAGES, NSC and mESC expression levels for these markers showed that all embryonic stem cell marker genes were indeed expressed most strongly in our mESC cultures (Fig. 4E). About half of the genes considered to be specific for murine neural stem cells had their highest expression in NSC. However, the other half was expressed to similar levels in mAGES. Almost all astrocyte markers had indeed their highest expression in mAGES (compared with mESC or NSC), only *Glul* expression was more prominent in NSC (Fig. 4E).

These findings corroborate the formation of genuine astrocytes from NSC by the mAGES differentiation protocol presented here, and they also confirm a close biological relationship between astrocytes and NSC. The need for redefinition of an optimized marker set is also obvious from the data presented. For instance *Fgf2* should rather be considered an astrocyte marker than an NSC marker.

To compare the microarray data of *in vitro* generated NSC and mAGES to primary brain cells, a heatmap of Spearman correlations was generated to illustrate relative similarities and differences of the expression profiles of the 94 marker genes described above. First, data on different primary cultures of astrocytes, neurons, or oligodendrocytes, prepared at different days of development (Cahoy et al., 2008), were correlated with NSC and mAGES. The mAGES correlated best with primary astrocytes, and less with primary neurons or oligodendrocytes. The highest correlation was found with astrocytes from postnatal brains at Days 7–8 *post partum*. The NSC expression patterns only showed a modest correlation with any of the primary brain cells. They were most similar to developmentally early brain cells like postnatal astrocytes from *post partum* Day 1 mice or oligodendrocyte precursor cells, while the greatest difference was found between NSC and mature astrocytes or neurons (Fig. 5A).

As an alternative approach, expression profiles of mAGES were also compared with data obtained previously on primary cells from different brain regions (Doyle et al., 2008). Here, the highest correlation was observed for cortical astrocytes, while intermediate correlations were seen for cerebellar astrocytes and Bergmann glia, and the lowest correlation was obtained for neuronal cultures (Fig. 5B).

Divergent Biosynthetic Metabolism of NSC and mAGES

A direct metabolic comparison of neural stem cells and astrocytes has been difficult so far, especially as different populations of surrounding cells (in their tissue context or in conventional cultures) make biochemical approaches very challenging. We used here the availability of the homogeneous and well characterized mAGES and NSC populations to obtain first evidence on divergent metabolic features of such cells. As an established approach to study central carbon metabolism, we followed the incorporation of the nonradioactive ^{13}C isotope from D [1- ^{13}C] glucose into different cellular metabolites (Fig. 6A). As default condition, NSC and mAGES were cultured in medium, which was adapted to the respective basic cell functions *in vivo*: mAGES were cultured without glutamine, similar to conditions chosen for metabolic flux analysis with glutamine producing primary astrocytes earlier (Amaral et al., 2011); NSC were cultured in the presence of 2 mM glutamine, according to their metabolic requirement for an external

FIGURE 4: (Continued)

glutamine source (Fig. 2). The measurement of label incorporation into the cellular glucose pool and glycolytic metabolites (3-phosphoglycerate, phosphoenolpyruvate) confirmed for both cell types the high glycolytic rate: the equilibrium state for label incorporation rates (90% for glucose, 45% for the tricarboxon metabolites) was reached within the first hour.

Analysis of the TCA cycle intermediate citrate showed a delayed equilibration of label incorporation at 12–24 h (not shown), which is mainly due to a lower flux of glucose-derived pyruvate entering the TCA cycle compared with the high glycolytic flux, but also due to the large pools of (unlabeled) glutamate and aspartate in rapid exchange with TCA cycle intermediates. Still, the labeling of the citrate pool was faster and higher for NSC, suggesting differences in the metabolism of both cell populations (Fig. 6B). As citrate release is a well-known peculiar property of astrocytes (Westergaard et al., 1994), we quantified the citrate release rate from mAGES and NSC by quantitative ^1H NMR spectroscopy (Fig. 6C). NSC did not release significant amounts of citrate, while mAGES released citrate into the medium. A direct comparison with primary cortical astrocytes indicated that the overall range of their release rate is similar to the one of mAGES (albeit still somewhat higher) (Fig. 6C).

To investigate whether the observed differences in citrate release may have been due to medium conditions, we repeated experiments with mAGES in medium supplemented with 2 mM glutamine. Under these conditions, citrate release was even enhanced, compared with glutamine-free medium (used for the initial comparison). Conversely, NSC kept in glutamine-free medium (instead of medium with 2 mM glutamine) did not show any citrate release (Supp. Info. Fig. S14). Thus, citrate release appears to be a unique cell intrinsic difference in metabolism between mAGES and NSC.

For further characterization of the net uptake or release of medium components, the amino acid content of the supernatant was quantified 24 h after addition of fresh medium to the cells. NSC took up significant amounts of serine

(Fig. 6D), while mAGES did not affect the concentrations of this amino acid to a measurable extent. The same pattern was observed for all branched chain amino acids: leucine, isoleucine (Fig. 6D), and valine were taken up by NSC, while mAGES did not contribute to a significant change in extracellular concentration. Net glutamate uptake was, however, more pronounced for mAGES than NSC.

The latter finding, based on long-term net uptake, was investigated in more biochemical detail, since the glutamate–glutamine shuttle between astrocytes and neurons plays an important role in brain physiology. Expression of glutamine synthetase and of the glutamate transporter GLAST by neural stem cells would in theory enable them to recycle glutamate, as in astrocytes, but this has not been tested directly. We evaluated therefore, as a first step, the glutamate uptake capacity by following the uptake of radiolabeled glutamate over a short period of time (8 min), and determining the initial speed for mAGES, NSC, and primary astrocytes. All cells transported the amino acid to a relatively similar extent (Fig. 7A).

To obtain a measure of cellular glutamine synthesis under steady-state conditions, we studied incorporation of ^{13}C from D-[1- ^{13}C] glucose into glutamine. The incorporation of parts of the glucose carbon skeleton into glutamine occurs via the citric acid cycle, α -ketoglutarate formation, transamination of this metabolite to glutamate and finally the glutamine synthetase reaction (Fig. 7B). This biosynthetic pathway is competitive with i) the direct uptake of glutamine and ii) with the uptake of glutamate and use of this nonlabeled metabolite in the glutamine synthetase reaction. Thus, the relative extent of label incorporation from glucose allows conclusions on the existence and competition of these three reactions in the test cell population. For glutamine-free medium conditions (no glutamine uptake possible), we found that both NSC and mAGES synthesized glutamine (as indicated by ^{13}C incorporation from glucose in about 20% of all glutamine). Thus, both cell types were capable of glutamine synthesis. When glutamine (2 mM) was added to the

FIGURE 4: Transcriptome data of mESC, NSC, and mAGES and comparative analysis of gene expression to identify mature astrocyte marker genes. RNA was prepared from mESC, NSC, and mAGES (5 days old) in four independent experiments and used for Affymetrix Mouse Genome 430 2.0 arrays. (A) To visualize the grouping patterns across different samples, a principal component analysis of global gene expression was performed for samples of 4 independent differentiations, and data (color coded) with their 95% confidence interval (gray shading) were plotted along the first two principal components (PC). (B) Gene expression datasets from (A) were used to compute differentially expressed genes (DEG) by applying the Empirical Bayes moderated *t* statistics and adjusting for multiple hypothesis testing using the Benjamini–Hochberg method. Genes with ≥ 2 fold changes and an adjusted *P* value < 0.05 were considered as significant (with duplicates or nonannotated transcripts removed). The number of DEG is shown for different pairwise comparisons (e.g., mAGES vs. NSC) separately for upregulations (up, red) and downregulations (down, blue). (C) The gene list generated in (B) was sorted by adjusted *P* value and the top 20 genes that were differentially expressed in mAGES relative to NSC are displayed. Bars indicate fold changes of genes, which were upregulated (red) or downregulated (blue). (D) The gene list generated in (B) was sorted by fold change and the top 20 DEG are presented. The adjusted *P* value of all selected genes was $< 10^{-5}$. (E) The gene expression values were retrieved for 94 genes selected from the literature as being specific for embryonic stem cells, neural stem cells, or astrocytes (listed vertically). The normalized expression data on log scale obtained here (four times mESC, 4 $\times$ NSC, 4 $\times$ mAGES) are plotted as horizontal lines of the heatmap in the form of *z* scores (blue color representing low and red color high expression). The corresponding gene expression values are listed in Supporting Information Fig. S11. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5: Correlation of mAGES microarray profiles with published data on astrocytes and other brain cell types. (A) The heatmap shows the Spearman correlation coefficient between NSC and mAGES (vertical axis) and data published by Cahoy et al. (2008) on primary astrocytes, neurons, or oligodendrocytes, isolated at different postnatal days (horizontal axis). (B) The heatmap shows the Spearman correlation coefficient between mAGES (vertical axis) and data from Doyle et al. (2008) on primary astrocytes from different brain regions, neurons, or oligodendrocytes (horizontal axis). The Spearman correlation was performed over the 94 selected genes (Fig. 4E). Blue color represents low correlation and red color high correlation (highest red value = 0.8 in both cases). Samples are grouped by cell type across both studies; within the sample groups, the ordering was determined by a standard average linkage hierarchical clustering using the Euclidean distance metric. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

medium, neither mAGES nor NSC produced significant amounts of glutamine from labeled glucose, i.e., direct glutamine uptake was the dominating mechanism for filling the cellular glutamine pool (Fig. 7C). Thus, the cellular capacity of NSC (and mAGES) to synthesize glutamine (and the pathway employed for it) depends on the overall metabolic situation. When we re examined the microarray data for hints of metabolic differences relevant to glutamate metabolism, we found that pyruvate carboxylase expression was 19 fold upregulated in mAGES compared with NSC. This enzyme catalyzes the anaplerotic process of oxaloacetate formation from pyruvate to replenish α ketoglutarate in the TCA cycle, when this is used for glutamate and glutamine synthesis. In this context, pyruvate carboxylase has been reported to be astrocyte specific (Schousboe et al., 2013; Shank et al., 1985), as it allows a higher efficacy of glutamine synthesis from glucose and TCA cycle metabolites in astrocytes.

As both NSC and mAGES were able to take up glutamate and to synthesize glutamine, we were interested in their ability to release glutamine to the medium. The net release/uptake was measured first in medium containing glutamine during a 24 h incubation period for the two cell types. Under these conditions, both mAGES and NSC showed a net consumption of the amino acid of similar magnitude (Fig. 7D). By contrast, cell type differences became obvious in glutamine free medium: only mAGES released glutamine, whereas NSC did not release detectable amounts. Therefore, it appears that NSC cannot support the full glutamate glutamine recycling between astrocytes and neurons, and they therefore differ in this respect from astrocytes/mAGES.

Functional Maturity of mAGES concerning Innate Immune Response and Neuronal Support

We tested also directly, in how far mAGES were able to provide neuronal support. Human neurons, differentiated from the conditionally immortalized LUHMES cell line (Schildknecht et al., 2013; Scholz et al., 2011), were plated on top of mAGES. Within few days, stable co cultures formed and mAGES assumed an extreme star shaped morphology that is usually also observed for astrocytes present in primary rodent neuronal cultures (Supp. Info. Fig. S15A). When medium changes were stopped 2 days after plating the neurons, all LUHMES in monocultures died within 10 days, whereas virtually all cells survived (and continued to do so for at least 4 weeks), when co cultured with mAGES (Supp. Info. Fig. S15B). Direct cell contact (of the somata) did not seem to be necessary for this effect, and a mAGES:LUHMES ratio as low as 1:5 seemed to be sufficient to support at least 80% neuronal survival (Supp. Info. Fig. S15C). NSC did not appear to support LUHMES neuron survival, although the data interpretation from such experiments is difficult due to differentiation processes in the NSC population during the culture period. Moreover, LUHMES cultured on mAGES had an increased synaptic density compared with LUHMES monocultures (Supp. Info. Fig. S16).

Besides metabolic functions pivotal for brain homeostasis, important roles of astrocytes also involve their activation in inflammatory situations (Falsig et al., 2006, 2008), and neuronal support (Kuegler et al., 2012). Therefore, we examined here whether mAGES acquire similar properties, and in how far they differ from NSC. First, mAGES and NSC were

FIGURE 6: Differences in metabolic flux between NSC and mAGES. (A) Schematic representation of ¹³C incorporation into citrate from D [1-¹³C] glucose. The carbon skeleton of selected metabolites is shown. Citrate can incorporate ¹³C at one carbon position (M+1), which is C2, from condensation of [2-¹³C] acetyl CoA with unlabeled oxaloacetate. The M+2 citrate isotopomer is generated, when [2-¹³C] citrate is used in the tricarboxylic acid (TCA) cycle to generate [2-¹³C] oxaloacetate, which condenses with [2-¹³C] acetyl CoA, yielding double labeled [2,3-¹³C] citrate. NB: Not shown here is that citrate might alternatively be labeled at C4 depending on label position in oxaloacetate at C3 (due to symmetric structure of fumarate). (B) Cells (mAGES/NSC) were plated in 6 well plates at a density of 500,000 cells/well for mAGES and 80,000 cells/well for NSC. After 48 h (5 days for mAGES), medium was changed to glucose free medium supplemented 10 mM D [1-¹³C] glucose. Supernatant as well as cell extracts were harvested at indicated time points. ¹³C incorporation into citrate was measured in NSC and mAGES cell extracts after derivatization and analysis by gas chromatography mass spectrometry (GC MS). Isotopomer fractional enrichment above natural abundance of 1.2% (i.e., the percentage of the respective isotopomer of the total citrate pool) was measured for M+1 (=citrate with one ¹³C), and M+2 (=citrate with two ¹³C atoms). (C) Experiments were performed as in (B) and citrate concentrations were measured in the supernatants of NSC and mAGES using ¹H NMR spectroscopy. For comparison, data from cortical astrocyte cultures were included. The overall protein content was measured in pellets of cell extracts, and the citrate release was normalized accordingly. (D) Experiments were performed as in (B) and serine, leucine, and isoleucine were measured in NSC and mAGES supernatants by HPLC. The overall protein content was measured in pellets of cell extracts, and amino acid uptake or release rates were calculated. All data are means ± SEM from duplicate determinations in three independent experiments. ****P* < 0.001 (One way ANOVA with Dunnett's post hoc test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

exposed to a mix of cytokines (complete cytokine mix (CCM): consisting of 10 ng/mL TNFα, 10 ng/mL IL 1β, and 20 ng/mL IFNγ), which is normally found during brain inflammation, and is well established to activate the inflammatory master transcription factor NFκB in glial cells (Henn et al., 2011; Kuegler et al., 2012). CCM triggered pronounced translocation of NFκB from the cytosol to the nucleus in mAGES, but showed no effect at all in NSC (Fig. 8A, Supp. Info. Fig. S17). A more detailed investigation of the individual cytokines showed that translocation of NFκB was induced by TNFα as well as IL 1β, but not by IFNγ (Fig. 8B). This activation pattern was similar to the one

found before in primary astrocytes (Falsig et al., 2004; Henn et al., 2011), and in mAGES from different mESC and iPSC lines (Supp. Info. Fig. S6).

Translocation of NFκB is an inflammation event easily quantifiable on single cell level, and indicating cytokine receptor activation. To obtain information on the actual inflammatory activation, it is necessary to measure relevant mRNA levels directly. Indeed, we found that CCM induced the expression of the cytokine *IL-6*, the inducible nitric oxide synthase *Nos-2*, and the toll like receptor *Tlr-2* (Fig. 8C). All three exemplary marker genes used here, were upregulated in mAGES at 4 h after incubation with CCM at least 100 fold.

FIGURE 7: Similarities and differences of NSC and mAGES concerning metabolic processes related to the glutamate-glutamine cycle. (A) NSC, mAGES (5 days old), and primary astrocytes were incubated with glutamate (10 μM , radiolabeled (^3H) with 0.12 $\mu\text{Ci}/\text{well}$) in PBS for 8 minutes. After 3 rapid washing steps with PBS, cells were lysed and the cellular glutamate content was measured using a scintillation counter. The uptake data were normalized to mAGES as 100% reference point. (B) Schematic representation of ^{13}C incorporation into glutamate and glutamine from $[1-^{13}\text{C}]$ glucose: the carbon skeleton of selected metabolites is shown. Direct metabolism of glucose to pyruvate & conversion to acetyl CoA & conversion to citrate & conversion to α -keto-glutarate can lead to the formation of glutamate with one ^{13}C atom incorporated at position C4 (M+1). Use of the already singly labeled tricarboxylic acid (TCA) cycle metabolite $[2-^{13}\text{C}]$ oxaloacetate in the citrate synthase reaction can lead to the formation of double labeled $[2,3-^{13}\text{C}]$ citrate. This can then be further metabolized to double labeled α -keto-glutarate, $[3,4-^{13}\text{C}]$ glutamate and $[3,4-^{13}\text{C}]$ glutamine (M+2). (C) Cells (mAGES/NSC) were plated in six well plates at a density of 500,000 cells/well for mAGES and 80,000 cells/well for NSC. After 48 h (5 days for mAGES), medium was changed to glucose free medium supplemented with 10 mM D $[1-^{13}\text{C}]$ glucose with either 2 mM glutamine (Gln) or no Gln. Supernatants and cell extracts were harvested at 0.3, 3, 12, and 24 h. ^{13}C incorporation into glutamine was measured by gas chromatography mass spectrometry (GC/MS) in NSC and mAGES cell extracts. Isotopomer fractional enrichment (i.e., the percentage of the respective isotopomer of the total glutamine pool) was measured over time for M+1 (=glutamine with one ^{13}C), and M+2 (=glutamine with two ^{13}C atoms). It is displayed here for the 12 h incubation (=steady state level). (D) Experiments were performed as in (C) and supernatants were harvested at 12 h. Glutamine concentrations were measured by HPLC. The overall protein content was measured in pellets of cell extracts, and the uptake or release rate were calculated (similar results were obtained at 24 h). All data are means $\pm$ SEMs from three independent experiments, $*P < 0.01$ (One way ANOVA with Dunnett's *post hoc* test). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

NSC did not upregulate any of the genes when exposed to CCM (data not shown). For data on an actual functional response to inflammatory stimulation, we chose to measure IL 6 secretion by ELISA. Again, TNF α as well as IL 1 β , but not IFN γ , induced the production and the release of IL 6. On the level of protein secretion, there was an additive effect of the cytokines (Fig. 8D), exactly as described for primary astrocytes earlier (Falsig et al., 2004; Henn et al., 2011).

NSC did not release any IL 6 when exposed to CCM (not shown). In summary, mAGES, but not NSC, responded to inflammatory cytokines, and the response pattern was consistent with that of primary astrocytes.

Discussion

The study of metabolic and functional features of astrocytes, in their resting state or in different defined activation

scenarios, faces a number of challenges: (i) measurements *in vivo* require a distinction of astrocytes from surrounding cells for the analytical endpoints chosen; (ii) *ex vivo* studies, using, e.g., FACS purified adult astrocytes, suffer from a compromised viability of the obtained cells, and from undefined activation states, when put in culture; (iii) *in vitro* studies mainly rely on studies of mixed populations, prepared from relatively immature cells. They may contain precursor cells, reactive astrocytes and other cell types, such as microglia. Additional approaches would thus be desirable to further explore astrocyte biology. In this study, we present such an alternative strategy that allows generation of a pure population of non proliferative, nonactivated astrocytes suitable for functional studies (neuronal support, inflammatory activation), investigation of metabolism (metabolite exchange, metabolic flux), and direct comparison to their immediate precursors, i.e., neural stem cells (NSC). The simultaneous availability of NSC, and of a population of nonproliferating astrocytes (mAGES) derived directly therefrom allowed us a direct comparison of the two cell types, and the identification of differences and similarities on the level of gene expression and intermediary metabolism (Fig. 9).

The heterogeneity of astrocytes derived from rodent brains or from stem cells has been underestimated in the past, as it has been common in the field to strongly rely on few single markers, such as GFAP. Nowadays, it is well described that there exist a subpopulation of mature GFAP negative astrocytes, which express astrocyte marker genes apart from GFAP and reveal functional inflammatory capacity (Cahoy et al., 2008; Kuegler et al., 2012; Pekny et al., 1995; Walz and Lang, 1998). For many studies, a quantification of negative markers indicative of contaminating or immature cells is often missing. It is clear now that a single marker cannot define the cells (Gotz et al., 2015), and that functional properties (e.g., cell cycle progression, glutamate conversion or response to cytokines) need to be combined with an extensive set of markers. For instance, we found that GFAP positive cells may co express nestin or not, and also be proliferative or not, depending on the culture conditions. Such observation by us and others are in good agreement with findings that astrocytes generated from NSC by LIF, CNTF or FBS all expressed GFAP, but otherwise strongly differed from one another (Brunet et al., 2010). Here we found that the most complete conversion to nondividing astrocytes occurred in the presence of BMP4, while addition of serum (FBS), which is frequently used in other protocols, exposes the cells to an undefined mixture of growth factors, some of which (e.g., EGF) also stimulate proliferation. The inevitable use of FBS in conventional primary astrocyte cultures might therefore explain the heterogeneity of developmental stages in such cultures. Moreover many studies are not truly based on primary cells, but rather secondary or tertiary cultures. The associated

selection and expansion of subpopulations, and cell activation may be the reason for contradictory studies on astrocytic inflammatory functions and makes it impossible to attribute specific metabolic functions unambiguously to primary mature astrocytes.

The absence of immature or reactivated cells in our cultures was indicated by the very strong downregulation of nestin as well as markers of immature astrocytes (A2B5, CD44). The maturity of mAGES was further confirmed by a cell cycle arrest (which persisted over weeks) in all cells in mAGES cultures. Moreover, we exclude the generation of quiescent stem cells, which may occur in some biological context as a consequence of the addition of BMP4 (Andreu et al., 2015; Martynoga et al., 2013; Mira et al., 2010): The quiescent stem cell marker CD133/prominin1 was not expressed in mAGES (Supp. Info. Fig. S18) and the cells did not re enter the cell cycle when exposed to EGF or sonic hedgehog (SHH) within 10 days of exposure, as it would be expected from quiescent stem cells (Codega et al., 2014). As the procedure we describe here was reproducible for several lines of ESC or for iPSC, and as large numbers of cells can be produced by this protocol in a fast and cost efficient way, mAGES are suggested here as valuable additional model system to study the cell biology (e.g., epigenetics, metabolic adaptation, inflammation, gene regulation, or cell cycle re activation) of astrocytes. Generation of mAGES from BTBR ESC, i.e., from a mouse strain used for autism studies (McFarlane et al., 2008), shows that the protocol is suitable also for the study of genetic modifications underlying disease.

For the study of human genes contributing to disease, it would be desirable to transfer the protocol to human cells. At the present stage, we cannot predict whether this will be possible with the same speed and efficiency as found in the murine system. The key factor for the efficient and rapid differentiation of mAGES was the generation of the right type of precursors. To our knowledge, the human equivalent has not yet been found, despite large efforts. Several protocols to generate astrocytes from human ESC or iPSC have been published (Gupta et al., 2012; Juopperi et al., 2012; Krencik et al., 2011; Sun et al., 2008). They often require excessively long differentiation times (> 3 months), and the obtained cell populations usually contain substantial amounts of precursors or other cell types. For instance, a recent, very efficient protocol developed by experienced specialists in the field generated purities of 70–80% at best (Shaltouki et al., 2013). Such cells have been used successfully for transcriptome profiling, but long term studies and investigations in metabolic pathways might be complicated by other cells present. Even cells offered commercially from the best known source for stem cell derived products (Cellular Dynamics iCell astrocytes; www.cellulardynamics.com/products/astrocytes.html) are very strongly proliferative.

FIGURE 8: Response of mAGES, but not NSC, to inflammatory stimulation. (A) NSC and 5 days old mAGES were exposed to complete cytokine mix (CCM, 10 ng/mL TNF α , 10 ng/mL IL 1 β , and 20 ng/mL IFN γ) for 30 min. Then, cells were fixed, permeabilized, and immuno stained for the p65 unit of the inflammatory transcription factor NF κ B (NFkB). Nuclei were counterstained with H 33342. The ratio of NFkB in the cytosol and the nucleus was measured in NSC and mAGES cultures by an automated screening microscope (1,000 nuclei/condition) and an image data processing procedure based on a validated algorithm. The percentage of cells with NFkB translocation into the nuclei was calculated and presented. See Supporting Information Fig. S17 for representative images. (B) NFkB translocation was determined as in (A) for mAGES exposed to CCM or its single components for 30 min. (C) Primary astrocytes and mAGES were stimulated with CCM, and mRNA was isolated 4 h later from control and stimulated cells. The expression of three inflammatory markers (IL 6; inducible nitric oxide synthase (iNOS); toll like receptor 2 (TLR2) was analyzed by qPCR. Data are given for CCM exposed cells relative to the expression in unstimulated control cells. (D) mAGES were exposed to CCM for 8 h. Supernatants were harvested, and IL 6 protein was measured by ELISA. All data are means $\pm$ SEMs from three independent experiments.

A recent advance may be the generation of “late radial glia cells” as precursor population. This protocol allowed then the generation of up to 88% GFAP positive cells within 7 days of the addition of BMP4 (Duan et al., 2015). Although this latter procedure, close to the one presented here for mAGES, sounds promising, there are some issues that need to be resolved: the generation of the right precursors takes over 100 days; it is not yet clear, whether these precursor cells have sufficient proliferative and self renewal capacity to allow astrocyte generation at a reasonable cost; and it is not clear whether the astrocytes generated show the required functional features and are nondividing. An exact definition of the required precursor cell population in the mouse system may help to guide protocol optimization for human cells. To obtain the required data, it may be helpful to compare the transcript profile of NSC of the present study to NSC we have used earlier for the generation of mESC derived astrocytes (MEDA). The latter protocol (Kuegler et al., 2012) generated astrocyte cultures with a large proportion of mature astrocytes, but also with subpopulations of immature cells and rapidly dividing cells (i.e., similar to the situation usually observed in the currently available human stem cell derived astrocytes). A better selection of the precursors by repeated selective passaging of the desired subpopula

tion, made all the difference in the speed, efficacy and homogeneity of the mAGES generation. Thus, knowledge of differentially expressed genes or surface markers for the desired start population is expected to accelerate such a process for human cells. We hope that the detailed description of the pivotal protocol steps of mAGES generation not only helps the implementation of mAGES in the research community, but would also support the optimization of protocols for human astrocytes.

The availability of mAGES and NSC in highly homogeneous cultures allowed comparisons of their metabolic characteristics. Earlier studies found expression of glutamate transporters as well as the glutamine synthetase in both cell types. The functionality of NSC glutamate transporters was then shown in short term biochemical D aspartate uptake experiments (Brunet et al., 2004). Our cultures provided now an experimental basis for steady state studies (as part of cellular homeostasis) on the role of glutamate uptake/glutamine release during prolonged culture times. This allowed conclusions on the role that NSC may take part in glutamate glutamine recycling. The finding that both NSC and mAGES were able to take up glutamate and convert it to glutamine suggests that neural stem cells resemble astrocytes in this metabolic function.

FIGURE 9: Summary of differences and similarities between NSC and mAGES. NSC (blue) and mAGES (red) show functional differences as well as similarities (intersection, purple). Gene expression profiles based on microarray profiling are listed below. The specific marker genes suggested below are (i) known NSC or astrocyte markers (see Fig. 4E); (ii) had a logarithmic mean expression value higher than 4 in one of the cell types (to avoid genes with borderline expression); (iii) and showed a difference in their logarithmic mean expression values (mAGES vs. NSC) higher than 1.8 (3.48 fold difference). "New genes" comprise the top DEG between NSC and mAGES, which were validated by qPCR. *: genes labelled with asterisk were found here to show expression differences of 3.5 fold between mAGES and NSC, but they have previously been discussed as potential markers for the other cell type than the one listed here (i.e., their specification was revised by our study). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

However, NSC did not release glutamine to the extracellular space as mAGES did. Thus, NSC are probably not involved in the glutamate glutamine shuttle between astrocytes and neurons. However, glutamine synthetase activity is also essential for detoxification of excessive ammonia in the brain. NSC may take part in this important process.

A further metabolic difference of mAGES and NSC concerned the release of citrate to the extracellular space. This peculiar metabolic feature has been known for astrocytes (Westergaard et al., 1994), and was now confirmed for the highly homogeneous mAGES cultures, while this process was not detected in NSC. Furthermore, NSC clearly differed from mAGES by their net exchange pattern for various amino acids. The interpretation of such metabolic studies requires homogeneous populations to exclude subpopulations responsible for altered overall metabolite patterns. The data generated here suggest that the cultures are suitable for more extensive mapping of overall cellular metabolic fluxes, and for

the comparison of such data to primary astrocyte cultures (Amaral et al., 2011) and other cell types.

In the fields of immunology and stem cell biology, it has long become a rule that cells cannot be characterized by single markers, but rather by signatures across multiple positive and negative markers. The recognition of astrocyte heterogeneity has now led to a critical reexamination of the specificity of established marker genes in the field of neurosciences. Microarray studies provide new insight in corresponding multifactorial marker patterns of glial cells (Beckervordersandforth et al., 2010; Gotz et al., 2015). Classical single markers, such as GFAP, may still play a role in the distinction of neurons or microglia from astrocytes, but multiple markers and full expression profiles are required to characterize closely related cell populations. This is the case for a comparison between astrocytes and their self renewing precursors, as presented here. The gene expression data generated in this study can be used to define a predictive set of differentially expressed genes for in vitro

differentiation experiments in different settings. Such marker selection benefits largely from earlier gene expression studies of different murine cell populations that helped to define commonalities and differences of astrocytes from different brain regions, developmental stages and activation states (Beckervordersandforth et al., 2010; Buffo et al., 2008; Cahoy et al., 2008; Dimou and Gotz, 2014; Lovatt et al., 2007). *In vitro*, such studies have in the past been mainly used to define cell activation states (Falsig et al., 2006; John et al., 2005), while less information is available on the ground state of murine astrocytes cultures as opposed to other cell types. Here, we used microarray analysis to explore differentially expressed genes between NSC, mAGES and pluripotent ESC similarly to what has been done for human cells (Shaltouki et al., 2013). Moreover, the data were used for comparisons to some available data sets on astrocytes. As our system provides the opportunity to directly compare mAGES to their immature precursors, new RNA markers of mature cells such as fibromodulin (*Fmod*) could be readily identified (Fig. 9). The cognate protein (fibromodulin) is involved in extracellular matrix organization, which is one of the first recognized and accepted functions of astrocytes that has been described in connection with astrocytes functioning as “brain glue” (Volterra and Meldolesi, 2005).

Since differentiation of mAGES is very fast, and all steps are easily accessible at every stage, it offers the possibility to investigate factors and, e.g., toxicants or drugs that disturb astrocyte generation. For instance, valproic acid (VPA) has been shown to disturb astrocyte generation, while it favors neurogenesis (Su et al., 2014). We confirmed this property in a study that examined the generation of mAGES with readouts of maturity markers on the single cell level. Our system can thus be used for high throughput screenings of developmental toxicants, or for general studies of astrocyte development, for instance with regard to gene regulation, acquisition of immune functions and of biological features controlling the expression of cytokine receptors.

The differentiation of mAGES from different iPSC lines offers new possibilities to investigate the involvement of astrocytes in processes leading to disease establishment or progression. Such approaches have already been taken with astrocyte enriched cultures derived from human iPSC with a Huntington's disease genetic background (Juopperi et al., 2012), or a Rett syndrome genotype (Lioy et al., 2011). The availability of a wealth of transgenic and gene targeted mice, or the possibility of direct manipulation of NSC or mAGES offers multiple opportunities for the study of disease related genes. Besides such studies, the inflammation competence of mAGES might be utilized to study the causes and consequences of astrocyte activation in multiple neurological diseases. Moreover, the easy availability of the cells, and their compatibility with, e.g., human neuronal cultures allows also investigations

of neuroprotective or neurodamaging roles of astrocytes (Efremova et al., 2015; Schildknecht et al., 2015).

In summary, our exact characterization of the differentiation requirements, together with data on the phenotype and function of the resultant cells, suggests that generation of astrocytes from pluripotent stem cells (mAGES) is a viable alternative to the preparation of primary astrocytes for biological, pharmacological and toxicological studies. The direct comparison of mAGES and NSC performed here provided new insights into astrocyte and neural stem cell metabolism. In the future, the generation of mAGES with different genetic backgrounds opens up new possibilities in biomedical research.

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