Conversion of non-proliferating astrocytes into neurogenic neural stem cells: control by FGF2 and IFN-gamma

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Abstract (250 words)

Conversion of astrocytes to neurons, via de-differentiation to neural stem cells (NSC), may be a new approach to treat neurodegenerative diseases and brain injuries. The signaling factors affecting such a cell conversion are poorly understood, and they are hard to identify in complex disease models or conventional cell cultures. To address this question, we developed a serum-free, strictly controlled culture system of pure and homogeneous 'astrocytes generated form murine embryonic stem cells (ESC)'. These stem cell derived astrocytes (mAGES), as well as standard primary astrocytes resumed proliferation upon addition of FGF. The signaling of FGF receptor tyrosine kinase converted GFAP-positive mAGES to nestin-positive NSC. ERK phosphorylation was necessary, but not sufficient, for cell cycle re-entry, as EGF triggered no de-differentiation. The NSC obtained by de-differentiation of mAGES were similar to those obtained directly by differentiation of ESC, as evidenced by standard phenotyping, and also by transcriptome mapping, metabolic profiling, and by differentiation to neurons or astrocytes. The de-differentiation was negatively affected by inflammatory mediators, and in particular, interferon gamma (IFNγ) strongly impaired the formation of NSC from mAGES by a pathway involving phosphorylation of STAT1, but not the generation of nitric oxide. Thus, two antagonistic signaling pathways were identified here that affect fate conversion of astrocytes independent of genetic manipulation. The complex interplay of the respective signaling molecules that promote/inhibit astrocyte de-differentiation may explain why astrocytes do not readily form neural stem cells in most diseases. Increased knowledge of such factors may provide therapeutic opportunities to favor such conversions.

Introduction

Astrocytes are well-recognized for their inflammatory activation upon injury, but less is known on factors that affect their plasticity and lineage commitment. For example, neurogenesis from astrocytic neural stem cells has been demonstrated in certain niches of the adult mammalian brain [1-4]. Outside these niches, conversion of mature astrocytes into functional neurons requires expression of ectopic neurogenic transcription factors [5-13]. For therapeutic purposes, conversion of astrocytes to neurons without genetic reprogramming would be favorable.

During inflammation or disease, astrocyte activation is promoted by cytokines released from e.g., microglia [14-18], infiltrating leukocytes [19], or stressed neurons (ATP; reactive oxygen species) [20, 21]. The resultant 'astrogliosis' may have both detrimental and beneficial effects on neuron regeneration [22, 23]. Important features of severe astrogliosis are the re-entry of mature, non-proliferating astrocytes into the cell cycle, and the formation of glial scars [23-27]. For instance, activation of the transcription factor NF-kB by TNF has been shown to be associated with a de-differentiation of astrocytes [28, 29]. Some of these astrocytes re-express markers normally found in neural stem cells, such as nestin [20, 30, 31]. The potential of astrocytes to be transformed into cells capable of generating neurons may be used for future neuroregenerative therapies, if more becomes known ~~on~~ why they normally stay within their astrocytic lineage *in vivo* [24, 32, 33].

Mature astrocytes do not normally proliferate in the intact brain [23, 34], but several molecules that induce cell cycle re-entry and reversion to stem cells have been described [35]. These comprise nucleotides [36, 37], and growth factors such as EGF [38, 39], FGF2 [40-42], or sonic hedgehog (SHH) [43]. Pioneering work by Magdalena Götz and colleagues found that SHH also de-differentiated astrocytes in certain mouse injury models. Astrocytes exposed to this factor and isolated even from uninjured brains could be converted into self-renewing

and neurogenic neural stem cells *in vitro*, although neurogenesis could not be demonstrated *in vivo* [44].

The reason for the failure of astrocytes to form neurons *in vivo* might be the release of inhibitory factors during inflammation or disease [45]. Such signals could either prevent de-differentiation and cell cycle re-entry [46], or they may favor astrocyte lineage commitment over neurogenesis (e.g., bone morphogenetic proteins (BMPs) [47, 48] ,or notch signaling [49]). Several factors that promote [28] and inhibit de-differentiation and neurogenic conversion may be released simultaneously in disease models [44, 45], and this makes it hard to identify targets that would allow pharmacological triggering of endogenous de-differentiation and neurogenesis from astrocytes [10, 50].

An alternative approach to *in vivo* disease models are well-controlled cell cultures. We have recently established an *in vitro* system of pure and post-mitotic 'murine astrocytes generated from embryonic stem cells' (mAGES) [51]. These cells show transriptomic, metabolic, and functional (glutamate uptake, glutamine release, response to cytokines) properties similar to primary astrocytes. As they can be maintained in medium without serum, their inflammatory activations state is very low, and the culture system allows the addition or removal of single factors under strictly controlled conditions. We tested several factors for their capacity to de-differentiate mAGES to NSC and found that the addition of FGF2 induced cell cycle re-entry and a full conversion of astrocytes into neurogenic NSC. This system was used to explore underlying signaling pathways. Moreover, we used the system to identify inflammatory factors that inhibit neurogenic conversion of astrocytes. IFN-gamma and the JAK-STAT pathway triggered by this cytokine were identified as important counter-regulators of de-differentiation.

Materials and Methods

Full details are given in Supporting Information.

Differentiation/preparation of neural stem cells, mAGES, and primary astrocytes

NSC and mAGES were differentiated from murine embryonic stem cells (mESC) and maintained as described earlier [51]. In brief, NSC were maintained in N2B27-medium [1:1 DMEM/F12 and Neurobasal-medium, N2-supplement, B27-supplement, 2 mM Glutamax, 100 µM β-mercaptoethanol, 7.5 µg/ml insulin, 50 µg/ml BSA] supplemented with 20 ng/ml EGF and FGF2. NSC were re-plated in N2B27-medium supplemented with 20 ng/ml bone morphogenetic protein 4 (BMP4) and differentiated into mAGES for 5 days. If mAGES were cultured for longer periods, medium containing 10 ng/ml BMP4 was changed every other day. Preparation of primary astrocytes [14, 17] is described in Supporting Information.

De-differentiation of mAGES to NSC2

Five days-old mAGES were washed twice with PBS, and N2B27-medium supplemented with 20 ng/ml FGF2 was added. Medium was changed every other day. Experiments were performed on day 1-10. As control, cells were cultured in N2B27-medium without BMP4 and FGF2. To further maintain mAGES-derived NSC2, cells were re-plated at a density of 10,000 cells per cm^2 in N2B27-medium supplemented with 20 ng/ml FGF2 (± EGF) on gelatin-coated dishes. Medium was changed every other day, and cells were passaged twice a week equal to original NSC.

Differentiation of NSC/NSC2 into neurons

NSC2 were seeded at a density of 30,000 cells per cm^2 in N2B27-medium supplemented with 10 ng/ml FGF2 on poly-L-ornithine-hydrobromide/laminin coated plates. On day 2, FGF2 concentration was reduced to 5 ng/ml. On day 4, N2B27-medium without FGF2 was added. Medium was changed every other day. Experiments were performed on day 14-21.

Quantitative RT-PCR (qPCR), immunocytochemistry, and EdU labeling

Quantitative PCR and immunocytochemistry have been performed as described before [51]. Primers and antibodies are listed in Supporting Information. To detect DNA synthesis, cells were incubated for 48 h with 10 µM of the thymidine analogue 5-Ethynyl-2´-deoxyuridine (EdU) (Baseclick, BCK-EdU555), then fixed, permeabilized, and stained according to the manufacturer's instruction. Antigen-positive cells (1000 cells/condition) were counted by the automated screening microscope CellInsight™ CX5 (Thermo Scientific) using the pre-defined algorithm 'target activation'.

Measurement of viability/proliferation

Resazurin reduction and release of lactate dehydrogenase (LDH) were measured according to standard protocols [52].

Live imaging and single cell tracking

Dedifferentiation of mAGES was followed by time-lapse video microscopy on a Zeiss cell observer at 37°C. Phase contrast images were taken every 15 minutes from day 3-10 using a 20x phase contrast objective and an AxioCamHRm camera. In addition, mAGES were transfected for single cell tracking with a retroviral vector encoding DsRed, as described earlier [53]. Pictures of the red channel were taken every 0.5 h with a 10x objective. Single cell tracking and the generation of consecutive lineage trees was performed using Timm's Tracking Tool as described earlier [54, 55].

CFSE labeling and measurement of label dilution in adherent culture

Adherent cultures of mAGES were incubated with 10 µM CFSE (in PBS) for 15 min, and washed thereafter. De-differentiation was initiated by addition of 20ng/ml FGF2. At the end of the incubation, cells were stained with H-33342, and total CFSE intensity per cell was measured in live cells by an automated screening microscope (CellInsight™ CX5, Thermo Scientific). As control, CFSE-labeled astrocytes were maintained in medium without growth

factors. To define the classes of non-dividing and dividing cells, a threshold has been set, which comprises 95% of the cells in control cultures. To normalize the data with regard to the starting population (day 0), cells were grouped into cells that have divided once, twice, or three times. Label-dilution factors per cell were used to back-calculate numbers of divisions, assuming a serial log2 intensity reduction per cell division.

Statistical analysis

Experimental data are based on three 'biological replicates' (different cell lots/differentiations); each of these data points being the means of several 'technical replicates' (repeated measures/wells within one experiment), unless otherwise stated. Data are presented as means ± SEM. Statistical analysis was performed using ANOVA with GraphPad Prism, followed by Dunnett's post-hoc test, unless otherwise indicated.

Results

Cell cycle re-entry of mature astrocytes under defined conditions

To investigate cell cycle re-entry of murine astrocytes, cells were exposed to the growth factors EGF and FGF2, and incorporation of the thymidine analogue EdU was measured to detect DNA synthesis/proliferation. Standard primary astrocytes [14, 17], normally maintained in medium containing 10% serum, were cultured in N2B27-medium without serum to exclude effects of other growth factors. In such cultures, some basic proliferation was observed, and this was increased by exposure to EGF for 8 days (about 10% of cells). FGF2 induced proliferation of a significantly larger fraction of cells (Fig. 1A,B), and EGF attenuated the response to FGF2. Affected astrocytes under these conditions divided once or twice within 10 days, and then exited the cell cycle again. Observations beyond that time frame were not pursued, due to the difficulty to maintain our primary cultures without serum and endothelium-derived factors [56] for more than two weeks (data not shown).

As second experimental system, homogeneous cultures of 'murine astrocytes generated from embryonic stem cells' (mAGES) [51] were used. In such cells, no basic proliferation was observed at all, and added EGF had no effect. However, most of the cells started to proliferate when exposed to FGF2 (Fig. 1C,D), and EGF did not blunt this effect. Cell cycle re-entry was sustainable, as mAGES exposed to FGF2 for 8 days could be re-plated and further expanded in N2B27-medium containing FGF2 (Supporting_Information_Fig. 1). Thus, mAGES as well as primary astrocytes re-enter the cell cycle in response to FGF2, although the time course and re-entry efficiency differed between both cell types.

Conversion of mAGES to neural stem cells by FGF2

Phenotypic changes associated with cell cycle re-entry were investigated by immunocytochemistry. Standard primary astrocyte cultures, as also observed by others [57-59], always contained some nestin-positive cells (neural stem cell marker), and some glial fibrillary acidic protein (GFAP, astrocyte marker)-nestin double-positive immature astrocytes.

Upon FGF2 exposure, nestin-staining increased, while GFAP expression decreased. This switch from a genuine astrocytic phenotype to a more immature precursor cell type was also observed in mAGES (Fig. 1B,D; Supporting_Information_Fig. 2A,B). In the latter cultures, star-shaped astrocytes with fine radial processes adopted hypertrophic processes (typically seen in reactive astrocytes) within the first few days of FGF2 exposure. At day 6, they adopted a bipolar morphology with two elongated processes, and finally the typical morphology of neural stem cells (NSC) was observed (Supporting_Information_Fig. 3). Thus, astrocytes exposed to FGF2 did not only re-enter the cell cycle, but also generated neural stem-like cells (NSC2), which could be maintained (by FGF2 or by FGF2 *plus* EGF) in a self-renewing state for ≥14 passages (Fig. 1E, Supporting_Information_Fig. 1).

A quantification of this conversion showed that EdU incorporation and downregulation of GFAP started from day 5-6. Upregulation of nestin expression started directly after FGF2 exposure, and increased continuously (Fig. 2A,B).

The mAGES cultures were selected for further studies on cell cycle re-entry as they are more homogeneous, show no basic proliferation and no need for serum. To ascertain that all cells in these cultures were fully differentiated astrocytes, mAGES were cultured for one month in BMP4-containing medium, i.e., under conditions in which transiently-silenced precursor cells are unlikely to survive [60]. The subsequent withdrawal of BMP4 and exposure to FGF2 resulted in a conversion into NSC2 with the same time course and efficiency as with 5-days old mAGES (Fig. 2C). Removal of BMP4 did not induce a de-differentiation within 8 d as shown by a lack of EdU incorporation. Moreover, we repeated the process with mAGES derived from three single cell clones of NSC, and with iPS-derived mAGES. In all cultures nestin and EdU incorporation were upregulated by FGF2, while GFAP-expression was downregulated (Fig. 2D). Thus, conversion of mAGES to NSC2 required FGF2 and was generalizable for various NSC.

Need for FGF2 signaling, but not EGF, for mAGES de-differentiation

For investigation of the signaling events involved in the conversion of mAGES into NSC2, phosphorylation of key proteins was quantified in the absence or presence of pathway-specific kinase inhibitors (Fig. 3A). An increased phosphorylation of ERK (pERK) and AKT (pAKT) was observed in response to FGF2 (Fig. 3B); levels of phosphorylated p38, JNK, or c-jun did not change (Supporting_Information_Fig. 4A). SU5402 (inhibitor of the FGF receptor (FGFR1) tyrosine kinase) inhibited the FGF2-induced phosphorylation of ERK and AKT (Fig. 3B,C) at non-cytotoxic concentrations (Supporting_Information_Fig. 4B). This was paralleled by an inhibition of the de-differentiation of mAGES in the presence of FGF2, as seen from reduced proliferation, inhibited EdU incorporation, and reduced expression of nestin (Fig. 3D,E). Thus, FGF receptor kinase activity was necessary for the conversion of mAGES to NSC2.

While Ly294002 (inhibitor of AKT phosphorylation) did not affect the response of mAGES exposed to FGF2 (data not shown), the MEK1/2 inhibitor U0126 (prevention of pERK formation) (Supporting_Information_Fig. 5A) inhibited the FGF2-induced proliferation of mAGES (Supporting_Information_Fig. 5B). U0126 also inhibited DNA synthesis in mAGES exposed to FGF2 (Supporting_Information_Fig. 5C) at non-cytotoxic concentrations (Supporting_Information_Fig. 5D), while FGF2-induced upregulation of nestin was not affected (Supporting_Information_Fig. 5C). This suggests that FGF receptor-induced phosphorylation of ERK, but not AKT, drives cell cycle re-entry, but not necessarily acquisition of other stem cell properties (nestin regulation).

Since other studies described rather an involvement of EGF than FGF2 in the de-differentiation of astrocytes, we investigated a putative role of endogenously produced EGF [38, 39]. The EGF receptor kinase inhibitor gefitinib inhibited EGF-induced phosphorylation of ERK and AKT at non-cytotoxic concentration of 0.1 μM (Fig. 3F, Supporting_Information_Fig. 6A), while phosphorylation of ERK and AKT induced by FGF2

was not affected (Fig. 3F, Supporting_Information_Fig. 6D). To test if any indirect EGF receptor activation is necessary for the de-differentiation of mAGES, cells were co-exposed to FGF2 *plus* gefitinib (1 μM). The EGF receptor inhibitor had no effect on EdU incorporation, nestin expression (Fig. 3G), or proliferation (Supporting_Information_Fig. 6B,C). Thus, EGF is neither sufficient (Fig. 1C) nor necessary for the conversion of mAGES into NSC2.

Characterization of NSC2 as multipotent neural stem cells

After having found that NSC2 are able to self-renew (> 14 passages), we investigated their developmental potency with respect to neurogenesis and gliogenesis: when NSC2 were exposed to BMP4, they adopted the typical star-shaped morphology of astrocytes within 3 days, and expressed GFAP and aquaporin 4 (Aqp4) to a similar extent as mAGES (Fig. 4A,B). The resultant astrocyte population was therefore termed 'mAGES2'. To investigate the neurogenic potential of NSC2, they were re-plated on poly-ornithine/laminin, and FGF2 was gradually withdrawn from the medium to allow spontaneous differentiation. After 14 days, the culture consisted of 60% βIII-tubulin-positive (TUJ1) neurons, and about 30% GFAP-positive astrocytes (Fig. 4C,D). Nestin expression was found to decrease to 20% after an additional week's culture (not shown). Conversely, most TUJ1-positive cells expressed the post-mitotic neuronal marker NeuN in the nucleus (Fig. 4E). In summary, NSC2 were found to be multipotent stem cells, giving rise to astrocytes and neurons with the same differentiation efficiency as the original NSC population.

Comparison of NSC2 with NSC concerning gene expression and function

To further characterize the phenotype of NSC2, several cell type marker genes were selected, and their expression levels were compared with those found in mAGES and NSC (Fig. 5A). All astrocyte markers were lower in NSC/NSC2 than in mAGES. The glutamate transporter *Glt-1* (*SLC1A2*) and the calcium-binding protein *S100B* were even lower expressed in NSC2 than in NSC. Neural stem cell markers (*Nestin, Blbp, Olig2)* were upregulated in NSC2 (and

NSC) compared with mAGES. Thus, qPCR analysis confirmed the similarity of NSC2 with NSC.

Next, expression of over 34,000 genes (covered by 45,000 probesets) was measured in mESC, NSC, mAGES, and NSC2 by microarray hybridization. Principal component analysis of global gene expression showed clustering of NSC2 and NSC samples as opposed to distinct groupings for the mESC and mAGES samples, and thus demonstrated the close similarity of NSC2 and NSC (Fig. 5B). This was further confirmed by analysis of significantly differentially expressed genes (DEG): no DEG was found for the NSC/NSC2 comparison.

For a targeted comparison, genes identified previously to distinguish astrocytes from neural stem cells [51] were selected. A heatmap of their normalized expression values, extracted from the microarray data, confirmed the similarity of NSC2 and NSC, and their pronounced differences to mAGES (Fig. 5C).

The high proportion of astrocyte genes in the above comparisons may obscure minor differences between the NSC populations. Therefore, we used a recently-identified set of *in vivo* NSC-specific genes [32] to compare NSC2 with NSC and mAGES. More than 70% of these marker genes showed higher expression in NSC2/NSC than in mAGES (Supporting_Information_Fig. 8), demonstrating conformity of NSC2 and NSC. The remaining 30% showed high variability in mAGES samples, and were here not suitable to clearly distinguish NSC from mAGES.

After this detailed phenotyping, functional comparisons were performed on the basis of features already known to differ between NSC and mAGES [51]. First, inflammation-related signaling was explored. Cells were exposed to an inflammatory cytokine mix (TNFα, IL1β, and IFNγ) for 30 min, and NFkB translocation was measured. NSC2/NSC showed no response at all, while more than 90% of the cells in mAGES/mAGES2 cultures showed NFkB translocation into the nucleus (Fig. 5D). Thus, NSC2 are clearly not reactive astrocytes. A comparison of the metabolism showed that mAGES2/mAGES released citrate, and exhibited

similar glucose consumption and lactate release rates (Fig. 5E,F). In contrast, NSC/NSC2 did not release citrate and showed higher metabolic rates compared with mAGES/mAGES2. These data confirm a complete conversion from an astrocytic to a neural stem cell identity, when mAGES are de-differentiated to NSC2.

Evidence for direct NSC2 generation from differentiated astrocytes

Although the mAGES population comprises >99% astrocytes (GFAP-positive) [51], it cannot be entirely excluded that NSC2 arise from a minor subpopulation of non-differentiated cells that is re-activated by FGF2. Therefore, two competing hypotheses may explain the generation of NSC2 (Fig. 6A): the first assumes that some cells within the mAGES population did not differentiate into mature astrocytes upon exposure to BMP4, but rather stayed in a dormant stem cell state. They may start to proliferate upon exposure to FGF2, and overgrow the mAGES. The second hypothesis assumes that a large proportion of mAGES re-enters the cell cycle, and convert into NSC.

For hypothesis-1 to be true, the original mAGES would need to die upon exposure to FGF2, in order to make space for the expanding NSC, and to explain the observed loss of GFAP-positive cells. Therefore, we carefully examined signs of cell death, and we also measured release of LDH into the medium as integral measure of putatively ongoing cell death. No indication of cell death was observed (Supporting_Information_Fig. 10A). Hypothesis-1 would be strengthened, if quiescent stem cells could be identified. However, we did not identify any $CD133^+$ cells, and the mAGES cultures were homogeneously $Ki67^-$, $p27^+$, within the range of our detection limits [51].

Hypothesis-2 would be supported, if intermediate cell stages would be identified that combine properties of astrocytes and proliferating cells; i.e., GFAP- and/or aquaporin 4 (Aqp4) -positive astrocytes should be observed to re-enter the cell cycle and incorporate EdU into DNA upon exposure to FGF2. For this purpose, cells double positive for EdU and an astrocyte marker were identified at different time points during the 8-day de-differentiation

process (Fig. 6B). At all timepoints, most of the proliferating (EdU-positive) cells co-expressed GFAP and/or Aqp4 (Fig. 6C,D). After day 7, the proportion of GFAP and EdU double-positive cells decreased, consistent with the downregulation of GFAP after prolonged FGF2 exposure. These data indicate that mature, GFAP- and Aqp4-positive astrocytes re-entered the cell cycle in response to FGF2.

Three imaging approaches were used to gain further insight: first, we used an established method of fluorescent label (CFSE) dilution [61] (adapted to adherent culture) to calculate the percentage of dividing cells. A histogram of total CFSE intensity in 20,000 cells showed that label intensity decreased in 72% of the cells in FGF2-exposed cultures (to indicate at least one division), compared to control cells not exposed to FGF2. Taking into account the quantification of label dilution steps (2x, 4x, 8x) in cells, and calculating back the respectively increased cell numbers at the time point of analysis, we found that >50% of the mAGES originally present at day 0 had divided at least once (Fig. 6E, Supporting_Information_Fig. 10B).

Second, to confirm the CFSE-labeling data, and to directly follow mAGES division, live cell time lapse imaging was performed. The mAGES were transfected with DsRed to track single cells (using Timm's Tracking Tool, which has been developed to track freshly isolated neural stem cells *in vitro* [54, 55]) (Supporting_Information_Fig. 11B,D, Supporting_Information_Video 1). Lineage trees were produced for 84 cells, of which 42 (50%) divided during 10 days of FGF2 exposure. Many of the cells divided once (19%), while another large subpopulation (19%) divided ≥3 times (Supporting_Information_Fig. 10C). Most of the cells (60%) started their first cell division on days 6-8 of FGF2 exposure (Supporting_Information_Fig. 10D), which is consistent with EdU incorporation starting to increase at day 5.

As third imaging approach, we followed the cells by phase contrast time-lapse microscopy in low density cultures, to make sure that the entire population could be captured. This

independent set of experiments also showed that around 50% of the mAGES (flat cells with a star-shaped morphology) divided (Supporting_Information_Fig. 11A,C, Supporting_Information_Video 2). After two to three divisions, cells changed their appearance and adopted a morphology similar to that of NSC (video 2, Supporting_Information_Fig. 3). No cell division was observed in control cultures maintained in medium containing no factors (Supporting_Information_Video 3). Thus, a large body of evidence supports hypothesis-2.

Blocking the generation of NSC2 from mAGES by IFNγ signaling

To investigate how de-differentiation of astrocytes may be affected by mediators present in a pathological environment, mAGES were exposed to inflammatory cytokines (TNFα, IL1β, IFNγ) during de-differentiation with FGF2. For a first overview, resazurin reduction was measured to assess cell proliferation in response to FGF2. TNFα or IL1β alone did not reduce proliferation, but a combination of both cytokines was effective. IFNγ, alone or in combination with other cytokines, also attenuated the proliferation of mAGES (Fig. 7A). These data were confirmed, when EdU incorporation was measured: the complete cytokine mix (CCM) or various combinations of the three cytokines reduced DNA synthesis to a large extent (Fig. 7B). IFNα or IFNβ did not show any effect on the FGF2-induced de-differentiation of mAGES (not shown). Thus, the IFNγ receptor specifically affected astrocyte de-differentiation, in addition to its effect described directly on quiescent NSC [62]. Notably, the downregulation of GFAP and the upregulation of nestin during de-differentiation were affected by the same cytokine combinations as for proliferation/DNA synthesis, but the overall effect size was smaller (Supporting_Information_Fig. 12).

As IFNγ alone was sufficient to inhibit the de-differentiation of mAGES, its mechanism of action was further explored. Ruxolitinib (Rux), an inhibitor of JAK1/2 (Fig. 3A), was used to test the role of the JAK/STAT pathway. In mAGES exposed to FGF2 *plus* IFNγ or CCM, co-treatment with ruxolitinib prevented the attenuation of proliferation, and of EdU

incorporation, which is otherwise triggered by IFNγ or CCM (Fig. 7C,D, Supporting_Information_Fig. 13A,B). Western blot analysis revealed that the same concentration of Rux, which showed strong effects on cell fate (1 μM), also completely blocked phosphorylation of STAT1 after short-term exposure to IFNγ or CCM (Fig. 7E,F), or after prolonged exposure (Supporting_Information_Fig. 13C). We also found that neither IFNγ nor ruxolitinib affected upstream FGF2 signaling (phosphorylation of ERK) (Supporting_Information_Fig. 13D). From this, we conclude that the JAK/STAT pathway, triggered by IFNγ, attenuates astrocyte de-differentiation downstream of FGF2 receptor surface expression and proximal signaling (Fig. 3A).

Discussion

It is widely accepted that post-mitotic astrocytes may re-enter the cell cycle under certain conditions [20, 44]. However, de-differentiation of genuine astrocytes into neural stem cells and their conversion into neurons usually requires genetic reprogramming or pathological situations, like trauma, excitotoxicity, or stroke [50, 62-69]. It has been suggested that migration of cells from the stem cell niches to the lesion site also contributes [70] to the *in vivo* emergence of neurogenic cells (identified by *in vitro* neurosphere assays [44, 67]). Most likely, various mechanisms contribute to the astrocyte activation/de-differentiation observed *in vivo*, and they are paralleled by large heterogeneity within the astrocyte population and the NSC [22, 62, 68, 71, 72]. We chose here a simplified experimental approach that allowed direct and quantitative studies of cell cycle re-entry of mature astrocytes, not manipulated by molecular biology approaches. The high homogeneity of the mAGES astrocyte cultures used here allowed quantifications of cell type, function, and plasticity over long time spans and with little background noise. This facilitated the identification of an FGF2 receptor pathway (involving tyrosine kinase activation and ERK phosphorylation) as a signal for cells to resume proliferation. The neurogenic neural stem-like cells (NSC2) derived from mAGES astrocytes upon FGF2 exposure resembled NSC concerning self-renewal, phenotype, metabolism, proliferation behavior, and bi-potent differentiation capacity. Thus, the type of neurogenic cells generated here does not resemble reactive astrocytes (that may divide once or twice in the brain, and retain many astrocytic markers), but rather exemplifies full reversion to an NSC-like state. Notably, cultures of mAGES could be converted into neurons without any re-plating steps, and only by change of medium conditions within about three weeks (not shown). However, this conversion of astrocytes to neurons in a cell culture dish is not to be confused with trans-differentiation [73]; in our system, mAGES first de-differentiated into NSC2 (10 days; +FGF2), and afterwards generated neurons (14 days; -FGF2).

A central issue of all de-differentiation studies with primary cells or other complex cell cultures is the exclusion of a major role of an already present stem cell population, or of a transgene affecting the cell cycle [29]. Although mAGES have been carefully characterized and phenotyped for the absence of cells with quiescent NSC markers [51], it was important to provide direct evidence that a majority of astrocytes in the cultures transformed into NSC2. Co-staining of EdU and Aqp4 suggested that indeed mature astrocytes re-entered the cell cycle. CFSE-label dilution and live imaging of mAGES cultures, as a direct measure of cell division, further confirmed the cell cycle re-entry of about 50% of the cells (within the experimental time span observed). This high percentage i) excludes the proliferation of only a minor subpopulation within mAGES cultures as major cause of NSC2 formation, and ii) corroborates a high efficiency of astrocyte de-differentiation under optimal conditions. For comparison, 22.5% of the astrocytes isolated from murine brains after stab-wound injury were able to form neurospheres [44].

In some studies, EGF has been shown to induce proliferation in astrocytes [38, 39]. This was not confirmed here, although mAGES expressed functional EGF receptors (Fig. 3A). Even an indirect contribution was excluded, as de-differentiation of mAGES was possible in the presence of non-cytotoxic, but effective concentrations of the EGF receptor inhibitor gefitinib. In view of these findings, it may be worth investigating in models that have been assumed to be EGF-dependent [38], whether production of endogenous FGF2 after exposure to EGF [39] would provide the pivotal signal for proliferation of cells. In fact, primary astrocytes have been observed to re-enter the cell cycle in response to FGF2 [40-42]. Discrepancies between studies may be due to different signaling systems being employed, depending on the maturity status of cells: EGF may have a more important role for less differentiated cells, and such cells are found as a subpopulation in primary astrocyte cultures.

The findings of our study are consistent with the broad role of FGF2 in neurodevelopment [74] and acute CNS injuries [45]. The cytokine stimulates proliferation of NSC in stem cell

niches [45, 75-77], and it improves regeneration after traumatic injury or ischemia [78, 79]. The high levels of FGF2 found in acute neurotrauma correlate with a proliferation of astrocytes in such conditions, while low or absent regulation in chronic neurodegenerative diseases, such as Parkinson's disease [80], correlate with a failure of astrocytes to proliferate [44]. The importance of FGF2 (in contrast to EGF) on NSC maintenance and astrocyte differentiation inhibition is in good agreement with earlier findings on rat [81]. However, terminally-differentiated astrocytes generated by exposure of rat NSC to BMP4 did not de-differentiate upon exposure to FGF2, and thus behaved differently from mAGES.

To obtain a pure population of fully-differentiated astrocytes, an alternative to mAGES is the use of primary cultures in combination with FACS sorting or panning for genuine astrocyte markers [56, 82]. In our hands, the required manipulations and culture conditions led to a slight inflammation-like activation of (untreated) astrocytes, and such conditions largely change their properties [22, 23, 83]. The findings presented here, confirm the pronounced effects that external inflammatory factors may have: for instance, the model system established here allowed for the identification of IFNγ as one of the pathologically-relevant inflammatory factors likely to attenuate the de-differentiation of astrocytes in many disease models. IFNγ (and complete cytokine mix) inhibited the de-differentiation of mAGES by a phosphorylation of STAT1, while inhibition of the JAK/STAT pathway by ruxolitinib restored FGF2-induced proliferation. The exact interference between STAT1 phosphorylation and FGF2 signaling needs further examination (Fig. 3A). The astrocyte de-differentiation was not only attenuated by IFNγ, a cytokine produced *in vivo* from infiltrating cells [19, 84] and from microglia [85, 86], but also by the very common combination of microglia-derived [14-18] inflammatory factors TNF *plus* IL-1. In our study, IL-1 or TNF alone did not attenuate de-differentiation. This may be due to an intrinsic property of such NF-kB-inducing cytokines to rather support de-differentiation of astrocytes [28, 87].

In summary, we have presented here evidence for defined signaling pathways that antagonistically control the conversion of astrocytes to NSC, i.e. cells capable of forming new neurons. The experimental system developed on the basis of these findings may be used to identify targets, and possibly to screen for new drug candidates that foster the conversion of astrocytes to neurons in CNS pathologies, and that could then be tested *in vivo* for their efficacy.

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Figure legends

Figure 1: Cell cycle re-entry of mature astrocytes under controlled conditions.

(**A**): Primary murine astrocytes were exposed to 20 ng/ml FGF2 or 20 ng/ml EGF or combinations thereof for 8 d. The nucleoside analogue EdU (10 μM) was added during the last 48 h. EdU incorporation into the DNA was visualized by immunocytochemistry, and the number of EdU-positive nuclei was counted by an automated screening microscope (1000 nuclei/condition). Data are means ± SEM for three cell preparations. ***, $p < 0.0001$; *, $p < 0.01$ (Tukey's posttest). (**B**): Representative images are shown for cells treated as in (A). Left: EdU incorporation was visualized by immunocytochemistry (green), and nuclei were stained with H-33342 (red). Right: glial fibrillary acidic protein (GFAP, green) and nestin (red) were visualized by immunocytochemistry, and nuclei were stained with H-33342 (blue). (**C**): Three different lots of murine astrocytes generated from embryonic stem cells (mAGES) were treated and analyzed as in (A). In control cultures (N2B27, serum-free) medium without additional factors), less than one cell per condition was found to be EdU-positive (< 0.1%). Data for cells exposed to growth factors are as indicated (given as means ± SEM). ***, $p < 0.0001$; ns, not significant (Tukey's posttest). (**D**): The mAGES were immunostained as in (B); a scale bar and color keys are displayed on the images. (**E**): Summary of the experimental procedure and findings of A-D: FGF2 drives the transformation of GFAP-positive, non-proliferating astrocytes (here exemplified by the pure and defined population of mAGES to proliferating nestin-positive neural stem cells (here called NSC2).

Figure 2. Conversion of mAGES to neural stem cells by FGF2

(**A**): Cultures of mAGES were exposed to 20 ng/ml FGF2 for 1-9 days, before they were fixed. EdU was added 48 h prior to termination of the experiment. The percentage of EdU-positive nuclei was determined. (**B**): GFAP and nestin were visualized by immunocytochemistry in cells treated as in (A). The percentage of positive cells is displayed.

(**C**): As shown in the schematic, neural stem cells (NSC) were differentiated for 30 days to mAGES by exposure to BMP4, before they were exposed to FGF2 (0 or 20 ng/ml) for 8 days. EdU was added to the medium for the last 48 h. GFAP and nestin were visualized by immunocytochemistry. Nuclei were stained with H-33342. FGF2 induced a conversion of fully mature mAGES into neural stem-like cells (=NSC2). Note that exposure of NSC to BMP4 for 5 days leads to homogeneous cultures of non-proliferating mAGES; long-term differentiation was chosen to render unlikely the presence of remaining NSC. (**D**): Embryonic stem cell-derived NSC (pool), three single cells clones of these NSC (#1-3), and induced pluripotent stem cell (iPS)-derived NSC were differentiated to mAGES (the left side of the diagram shows a typical phenotypic outcome for pool cells). Such mAGES were exposed to FGF2 for 8 days, and incubated with EdU for the last 48 h. GFAP, nestin, and EdU-incorporation were visualized by immunocytochemistry, and the percentage of positive cells was determined by an automated screening microscope (means ± SEM). n.d., not detectable.

Figure 3. Need for FGF2 signaling, but not EGF, for mAGES de-differentiation.

(**A**): Overview of inhibitor targets and signaling pathways affecting de-differentiation from mAGES to neural stem-like cells (NSC2). (**B**): Cultures of mAGES were pre-incubated for 30 min with SU5402 (10 μM), and exposed to FGF2 (20 ng/ml) for 20 min. Cell lysates were analyzed by Western blot for AKT and ERK, and their phosphorylated forms (pAKT, pERK). (**C**): Densitometric quantification of the ratio between phosphorylated and un-phosphorylated proteins from three experiments as in (B). ***, $p < 0.0001$; **, $p < 0.001$ (versus FGF2). **(D)**: To measure proliferation, mAGES were exposed for 8 days to 0 (ctrl) or 20 ng/ml FGF2 *plus* increasing concentrations of SU5402, and resazurin reduction was used as surrogate measure of cell number. The data indicate the percentage of increased resazurin fluorescence signal (proportional to cell number) relative to ctrl conditions. (**E**): Nestin protein and EdU incorporation were visualized in mAGES treated as in (D), and incubated with EdU for the last 48 h. Positive cells were counted by an automated screening microscope. (**F**): The

mAGES were pre-incubated for 30 min with gefitinib (Gef), and exposed to 20 ng/ml EGF or FGF2 for 20 min, before AKT, ERK, and their phosphorylated forms were determined by Western blot. (**G**): The mAGES were exposed for 8 days to 0 (ctrl) or 20 ng/ml FGF2 *plus* increasing concentrations of gefitinib; EdU was added for the last 48 h. EdU incorporation and nestin protein were visualized by immunocytochemistry. Positive cells were counted by an automated screening microscope. Data are means ± SEM.

Figure 4. Characterization of NSC2 as multipotent neural stem cells

NSC2, i.e. neural stem-like cells obtained from mAGES, were differentiated into either astrocytes (mAGES2) with BMP4 for 3 days (A,B) or into neurons (C, D, E). (**A**): Phase contrast images show typical neural stem cell morphology of NSC and NSC2 and astrocytic morphology of mAGES and mAGES2. (**B**): Resentative immunofluorescence images of mAGES2, generated from NSC2 by exposure to BMP4 (for 3 days): glial fibrillary acidic protein (GFAP, green), and either aquaporin 4 (Aqp4) or nestin are shown (red). Nuclei were stained with H-33342 (blue). (**C**): NSC2 were differentiated into neurons by a stepwise reduction and subsequent withdrawal of FGF2 for 14 days. After fixation, βIII-tubulin (TUJ1, red) and either GFAP or nestin (green) were visualized by immunocytochemistry. Nuclei were stained with H-33342 (blue). (**D**): Either NSC or NSC2 were differentiated into neurons and stained as in (C). Then, the percentage of antigen-positive cells was counted by an automated screening microscope in three different experiments (data are means ± SEM). (**E**): Phenotyping of neurons generated from NSC2 by co-immunostaining for βIII-tubulin (TUJ1, red) and NeuN (green). Black/white images show individual fluorescent channels for clarity; the colored composite image combines both channels.

Figure 5. Comparison of primary (NSC) and secondary (NSC2) neural stem cells for gene expression, metabolism and inflammatory competence.

(**A**): Expression levels (mRNA, measured by qPCR) in NSC, NSC2, and mAGES of astrocyte markers (Gfap, Aqp4 (aquaporin), Glt-1 (Slc1A2, glutamate transporter), Kir4.1 (inwardly-rectifying potassium channel), Aldh1L1, S100b), or NSC markers (nestin, Olig2, Blbp). ***, $p < 0.0001$; **, $p < 0.001$; *, $p < 0.01$; ns, not significant (versus NSC2). (**B**): Transcriptome data (microarray) obtained for murine embryonic stem cells (mESC), NSC, mAGES, and NSC2. The 2D principal component analysis plot shows 4 biological replicates of each cell type and the 95% confidence interval as shaded ellipses. (**C**): Heatmap of gene expression values for known astrocyte (blue) and NSC (red) marker genes (listed vertically) [51]. Z-scores of normalized expression data plotted for four NSC, four mAGES, and three NSC2 samples (listed horizontally). Blue colors represent low, red colors high expression, with z-scores ranging from 1.4 to -1.4. Full gene names and corresponding expression values are listed in Supporting_ Information_Fig. 7. (**D**): Cells were exposed to a cytokine mix (=10 ng/ml TNFα, 10 ng/ml IL1β, 20 ng/ml IFNγ) for 30 min, and immunostained for the transcription factor NFκB. Cytosol/nucleus ratio of NFkB was measured by an automated screening microscope to identify cells with NFkB nuclear translocation. (**E**): Citrate was measured in the supernatant after 3 and 24 h to calculate citrate release (normalized to total protein content). (**F**): Glucose/lactate concentrations were measured in the supernatant after 3 and 24 h. Uptake/release rates (normalized to total protein content) were calculated. Data are means ± SEM from two biological replicates.

Figure 6. Evidence for generation of NSC2 from differentiated astrocytes.

(**A**): Two working hypotheses on the origin of NSC2 have been formulated for further testing. Hypothesis-1: putative nestin-positive quiescent stem cells (red) within the mAGES population (<1%) are stimulated by FGF2 to proliferate (green nuclei), while original mAGES (blue) die. Hypothesis-2: FGF2 induces cell cycle re-entry of a large subpopulation

of mAGES (green nuclei); these convert to $GFAP^+$/$nestin^+$-positive cells, and later to $nestin^+$/$GFAP^-$ NSC. (**B**): Experimental design to investigate the phenotype of transition cells: mAGES were exposed to FGF2 (0 (blue)/20 ng/ml (red)) for up to 7 days; EdU was added for the last 48 h before immunostaining. (**C**): Cells were treated as in (B). EdU^+/$GFAP^+$, and EdU^+/$GFAP^-$ cells were counted by high-content imaging. (**D**): The experiment described in (C) was performed with Aquaporin 4 (Aqp4)-staining instead of GFAP. (**E**): The mAGES were exposed to FGF2 (20 ng/ml) for 10 days, and the percentage cells (from original population) that divided at least once during this period was scored. To identify dividing cells, two different methods were used. Left: mAGES were stained with carboxyfluorescein (CFSE) before the experiment; quantification of CFSE-fluorescence per cell after the experiment was used to identify cells that had divided 0, 1, 2, 3 or several times, based on label dilution. Right: live-cell continuous time-lapse imaging (tracking of 84 DsRed-transfected single cells was used (details in Supporting_Information_Fig. 10,11). All data are means ± SEM of three (left) or two (right) biological replicates.

Figure 7. Block of the generation of NSC2 from mAGES by IFNγ signaling.

(**A**): The mAGES were exposed to 20 ng/ml FGF2 for 8 days, and resazurin reduction was used as surrogate measure of cell number. Data indicate the percentage of increased resazurin fluorescence signal (proportional to cell number) relative to ctrl conditions (no FGF2). FGF2-exposed cells were co-treated with various combinations of inflammatory cytokines (10 ng/ml TNFα, 10 ng/ml IL1β, 20 ng/ml IFNγ) or complete cytokine mix (CCM). Data are means ± SEM; ***, $p < 0.0001$; *, $p < 0.01$; ns, not significant (versus FGF2). (**B**): The experiment was performed as in (A), with EdU added during the last 48 h, and staining of nuclei with H-33342. Percentage of EdU-positive cells was quantified. (**C**): The mAGES were exposed to 20 ng/ml FGF2 for 8 days. FGF2-exposed cells were co-treated with 20 ng/ml IFNγ and various concentrations of ruxolitinib (Rux). EdU was quantified as in (B). (**D**): The experiment was performed as in (C), with CCM instead of IFNγ. (**E**): The mAGES were pre-

incubated for 30 min with ruxolitinib (1 μM), and exposed to FGF2 (20 ng/ml) and/or IFNγ (20 ng/ml) for 20 min. Phospho-STAT1 (pSTAT1) was analyzed by Western blot (actin as loading control). The experiment was repeated once with similar result. (**F**): The experiment was performed as in (E), with CCM instead of IFNγ. The experiment was repeated once with similar result.

Supplemental figure Fig. S1: Self-renewal and multipotency of NSC2

NSC2 derived from mAGES after 10 d exposure to FGF2 were re-plated and maintained in N2B27-medium supplemented with 20 ng/ml FGF2 until passage 14. At passage 14, cells were re-plated for differentiation to astrocytes (3 d, 20 ng/ml BMP4) or neurons (14 d). Cultures of NSC2, mAGES2 derived from NSC2, and neurons derived from NSC2 were fixed and stained for phenotypic markers. Nestin, GFAP or ßIII-tubulin (TUJ1) were detected by immunocytochemistry. DNA was stained with H-33342. NSC2 maintained in FGF2-containing medium for 14 passages had the same doubling time and differentiation capacity as fresh NSC2.

Supplemental figure Fig. S2: Single-channel images of GFAP and nestin in mAGES exposed to FGF2.

(A): Single-channel images of Fig. 1B. Primary murine astrocytes (ctrl, no factors) were exposed to 20 ng/ml FGF2 for 8 d. Right: glial fibrillary acidic protein (GFAP, green) and nestin (red) were visualized by immunocytochemistry. (**B**): Single-channel images of Fig. 1D. The mAGES (ctrl, no factors) were exposed to 20 ng/ml FGF2 for 8 d. GFAP (left) and nestin (right) were visualized by immunocytochemistry. (**C**): Single-channel images of Fig. 2C. NSC were differentiated to fully mature mAGES for 30 days with BMP4, before they were exposed to 20 ng/ml FGF2 or medium without additional factors (no factors) for 8 days. GFAP (left) and nestin (right) were visualized by immunocytochemistry. Note that exposure

of NSC to BMP4 for 5 days leads to homogeneous cultures of non-proliferating mAGES; long-term differentiation was chosen to render unlikely the presence of remaining NSC.

Supplemental figure Fig. S3: Morphological changes in mAGES exposed to FGF2.

The mAGES (ctrl, no factors) were exposed to 20 ng/ml FGF2 and cells were fixed and stained every 24 h for 8 days. GFAP and Nestin expression was detected by immunocytochemistry. DNA was stained with H-33342.

Supplemental figure Fig. S4: Block of FGF2-induced de-differentiation of mAGES by SU5402 and U0126.

(**A**): Cultures of mAGES were pre-incubated for 30 min with the FGF receptor inhibitor SU5402 (10 μM), and exposed to 20 ng/ml FGF2 for 20 min. Cell lysates were analyzed by Western blot for p38, its phosphorylated form (p-p38), phosphorylated JNK (pJNK), and phosphorylated c-Jun (p-c-Jun). (**B**): The mAGES were exposed to increasing concentrations of SU5402 in medium containing 10 ng/ml BMP4 for 8 days. Resazurin reduction was normalized to control without SU5402 as a measure of viability. (**C**,**D**): The mAGES were exposed to 10 ng/ml BMP4 (C) or 20 ng/ml FGF2 (D) for 8 days *plus* 10 μM SU5402 or the ERK inhibitor U0126 (10 μM). Cells were incubated with the nucleoside analogue EdU for the last 48 h, and EdU incorporation and Nestin protein (red) were visualized by immunocytochemistry. Nuclei were stained with H-33342 (blue).

Supplemental figure Fig. S5: ERK phosphorylation is required for de-differentiation of mAGES

(**A**): Cultures of mAGES were pre-incubated for 30 min with 10 μM U0126, and exposed to 20 ng/ml FGF2 for 20 min. Cell lysates were analyzed by Western blot for Akt and ERK, and their phosphorylated forms (pAkt, pERK). (**B**): The mAGES (ctrl, no factors) were exposed to 20 ng/ml FGF2 *plus* increasing concentrations of the ERK inhibitor U0126 for 8 days.

Resazurin reduction was used as surrogate measure of cell number. The data indicate the percentage of increased resazurin fluorescence signal (proportional to cell number) relative to ctrl conditions. (**C**): The mAGES were treated as in (A), and incubated with EdU for the last 48 h. Nestin protein and EdU incorporation were visualized by immunocytochemistry. Positive cells were counted by an automated screening microscope (1000 nuclei/condition). (**D**): The mAGES were exposed to increasing concentrations of U0126 in medium containing 10 ng/ml BMP4 for 8 days. Resazurin reduction was measured and normalized to control without U0126 as a measure of viability.

Supplemental figure Fig. S6: EGF signaling is not involved in mAGES de-differentiation.

(**A**): The mAGES were exposed to increasing concentrations of the EGF receptor inhibitor gefitinib (Gef) in medium containing 10 ng/ml BMP4 for 8 days. Resazurin reduction was measured and normalized to control without Gef as a measure of viability. (**B**): The mAGES (ctrl, no factors) were exposed to 20 ng/ml FGF2 *plus* increasing concentrations of gefitinib for 8 days. Resazurin reduction was used as surrogate measure of cell number. The data indicate the percentage of increased resazurin fluorescence signal (proportional to cell number) relative to ctrl conditions. (**C**): The mAGES were exposed to gefitinib (1 μM) in medium containing either no factors or 20 ng/ml FGF2 for 8 days, and incubated with EdU for the last 48 h. EdU incorporation, nestin or GFAP protein (green) were visualized by immunocytochemistry. Nuclei were stained with H-33342 (blue). Gefitinib (1 μM) revealed no influence on mAGES exposed to FGF2. (**D**) Densitometric quantification of the ratio between phosphorylated and un-phosphorylated proteins from three experiments as in Fig. 3F. ***, $p < 0.0001$; **, $p < 0.001$ (Tukey's posttest).

Supplemental figure Fig. S7: Display of the mean expression for the astrocytes or NSC marker genes depicted in the heatmap of Fig. 5C.

Gene expression analysis (Affymetrix Mouse Genome 430 2.0 arrays) of the previously identified astrocyte (blue) and NSC (red) marker genes [51] in NSC, mAGES, and NSC2 from 4 independent differentiations. The logarithmized (to the base of 2) mean expression values, standard deviation, and fold changes relative to NSC are listed.

Supplemental figure Fig. S8: NSC2 resemble NSC in gene expression of NSC-selectivity markers identified by Beckervordersandforth et al. 2010 [88].

Heatmap of gene expression values for the NSC selectivity markers identified by Beckervordersandforth et al. 2010 (listed vertically) [88]. The normalized (z-scores) expression data are plotted for four NSC, four mAGES, and three NSC2 samples (listed horizontally) for each of the marker genes. Blue colors represent low, red colors high expression, with z-scores from 2 to -6. Full gene names and corresponding absolute expression values are listed in Supporting_Information_ Fig. S9. Red highlighted genes were selective for NSC, while yellow highlighted genes showed high variation between mAGES samples.

Supplemental figure Fig. S9: Display of the mean expression values for the NSC-selectivity marker genes depicted in the heatmap of Supporting_Information_Fig. S8.

Gene expression analysis (Affymetrix Mouse Genome 430 2.0 arrays) of the previously identified NSC-selectivity marker genes [88] in NSC, mAGES, and NSC2 from 4 independent differentiations. The logarithmized (to the base of 2) mean expression values, standard deviation, and fold changes relative to NSC are listed. Red highlighted genes were selective for NSC, while yellow highlighted genes showed high variation between mAGES samples.

Supplemental figure Fig. S10: Detailed information on NSC2 generation from differentiated astrocytes.

(**A**): Cultures of mAGES (ctrl, no factors) were exposed to 20 ng/ml FGF2 for up to 8 days without medium exchange, and LDH release was measured to determine cell death during de-differentiation. (**B**): The mAGES were labeled with carboxyfluorescein (CFSE) and exposed to 20 ng/ml FGF2 for 10 days (red). As control, mAGES were cultured in medium without additional factors (ctrl, green). CFSE intensity of 20,000 viable cells per condition was measured with a high throughput imaging device. A histogram of CFSE intensity (note logarithmic axis scaling) against cell count was produced. A threshold comprising 95% of the cells in control cultures was used to distinguish between dividing and non-dividing cells. The percentage of non-dividing cells in FGF2-exposed cultures is indicated in red. Further thresholds produced by dividing the upper threshold by 2 (CFSE label is halved during cell division) were used to determine the amount of cells, which divided once (1x), twice (2x), or three-times (3x). To normalize the data and to determine the percentage of cells, which divided or not with regard to the starting population, the amount of cells, which divided once, was divided by 2, the amount of cells, which divided twice, was divided by 4, and so forth. The percentage of non-dividing cells after normalization is given in blue (**C,D**): The mAGES were transfected with DsRed and exposed to 20 ng/ml FGF2 for 10 days. Fluorescent pictures of the red channel were taken with a live imaging microscope every 5 h. 84 single cells have been tracked using Timm's Tracking Tool (TTT) and lineage trees of single cells have been generated. (**C**): The percentage of cells, which did not divide or which divided once, twice or three-times, has been calculated. Representative lineage trees for each group were chosen. (**D**): The day of the first cell division of the single cells has been determined and 4 groups have been chosen giving the percentage of cells in each group. Representative lineage trees for each group were chosen.

Supplemental figure Fig. S11: Time-lapse microscopy of FGF2-induced de-differentiation of mAGES.

(**A**,**C**): The mAGES were exposed to 20 ng/ml FGF2 and phase contrast pictures were taken with a live imaging microscope every 15 min from day 3-10. Videos of mAGES exposed to FGF2 (video 2) as well as of control cultures (no factors, video 3) are attached in the Supplemental figure. A picture sequence of dividing cells from the black edging in (A) is shown in (C). (**B**,**D**): The mAGES were transfected with DsRed and exposed to 20 ng/ml FGF2. Fluorescent pictures of the red channel were taken with a live imaging microscope every 5 h until day 10 (video 1). A picture sequence of dividing cells from the black edging (B) is shown in (D).

Supplemental figure Fig. S12: Block of the generation of NSC2 from mAGES by inflammatory cytokines.

(**A**): The mAGES (ctrl, no factors) were exposed to 20 ng/ml FGF2 plus various combinations of inflammatory cytokines (10 ng/ml TNFα, 10 ng/ml IL1β, 20 ng/ml IFNγ) or a complete cytokine mix (CCM) containing all three cytokines for 8 days. EdU incorporation and nestin/GFAP protein were visualized by immunocytochemistry. **(B**): The mAGES (ctrl, no factors) were treated as in (A), and nestin was visualized by immunocytochemistry. Positive cells were counted by an automated screening microscope (1000 nuclei/condition). ***, p <.0001; **, p <.001; *, p <.01; ns not significant (versus FGF2). (**C**): The mAGES (ctrl, no factors) were treated as in (A), and GFAP was visualized by immunocytochemistry. Positive cells were counted by an automated screening microscope (1000 nuclei/condition). ***, p <.0001; **, p <.001; ns not significant (versus FGF2).

Supplemental figure Fig. S13: Ruxolitinib prevents IFNγ-induced inhibition of NSC2 generation.

(**A)**: The mAGES (ctrl, no factors) were exposed to 20 ng/ml FGF2 *plus* IFNγ and increasing concentrations of ruxolitinib (Rux) for 8 days. Resazurin reduction was used as surrogate measure of cell number. The data indicate the percentage of increased resazurin fluorescence signal (proportional to cell number) relative to ctrl conditions. (**B**): The experiment was performed as in (A), with complete cytokine mix (CCM) instead of IFNγ. (**C**): Cultures of mAGES were pre-incubated for 24 h with 20 ng/ml INFγ and/or for 30 min with the JAK/STAT inhibitor ruxolitinib, and exposed to 20 ng/ml FGF2 for 20 min. Cell lysates were analyzed by Western blot for the phosphorylated forms STAT1 (pSTAT1, phosphorylation at either serine residue 727 or tyrosine residue 701). Actin was used as loading control. (**D**): Cultures of mAGES were pre-incubated for 30 min with the JAK/STAT inhibitor ruxolitinib (Rux) and/or 20 ng/ml INFγ, and exposed to 20 ng/ml FGF2 for 20 min. Cell lysates were analyzed by Western blot for the phosphorylated forms of ERK (pERK) and STAT1 (pSTAT1, phosphorylation at serine residue 727). Actin was used as loading control.

Supplemental Video 1: Time-lapse imaging of DsRed-transfected mAGES exposed to FGF2.

NSC were seeded at high density (50,000 cells/cm^2) and differentiated into mAGES with 20 ng/ml BMP4 for 3 days. Then, mAGES were transfected with DsRed, washed, and exposed to 20 ng/ml FGF2. Fluorescent pictures of the red channel were taken with a live imaging microscope every 5 h until day 10. The movie shows 2 frames per second.

Supplemental Video 2: Time-lapse imaging of mAGES exposed to FGF2 in low-density culture.

NSC were seeded at low density (25,000 cells/cm^2) and differentiated into mAGES with 20 ng/ml BMP4 for 3 days. Then, cells were washed and mAGES were exposed to 20 ng/ml

FGF2. Phase contrast pictures were taken with a live imaging microscope every 15 min from day 3-10. The movie shows 15 frames per second.

Supplemental Video 3: Time-lapse imaging of mAGES in low-density culture (no factors).
NSC were seeded at low density (25,000 cells/cm^2) and differentiated into mAGES with 20 ng/ml BMP4 for 3 days. Then, cells were washed and mAGES were maintained in N2B27-medium without any additional factors to exclude the induction of cell division by the withdrawal of BMP4. Phase contrast pictures were taken with a live imaging microscope every 15 min from day 3-10. The movie shows 15 frames per second.