

# ***SOCS3* promoter methylation is mutually exclusive to *EGFR* amplification in gliomas and promotes glioma cell invasion through STAT3 and FAK activation**

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**Abstract** The suppressor of cytokine signaling 3 (*SOCS3*) gene is one of eight structurally related genes of the SOCS family and has been suggested to function as a tumor suppressor by inhibition of the JAK/STAT signaling pathway. We investigated 60 human gliomas of different histological types for *SOCS3* alterations and found frequent *SOCS3* promoter hypermethylation and transcriptional downregulation. However, *SOCS3* promoter hypermethylation was virtually absent in primary glioblastomas, which are characterized by frequent epidermal growth factor receptor (*EGFR*) amplification and overexpression. Assessment of the relationship between *SOCS3* and *EGFR* aberrations revealed that *SOCS3* promoter hypermethylation was inversely related to both the *EGFR* gene dosage as well as the *EGFR* protein expression, thus suggesting *SOCS3* inactivation as a mechanism substituting for *EGFR* activation in a subset of gliomas. In support of this hypothesis, stable shRNA-mediated *SOCS3* knock-down in U251 glioblastoma cells resulted in an activation of *EGFR*-related signaling pathways, i.e. an increase in the activation levels

of STAT3, FAK and to a lesser extent MAPK, while the AKT phosphorylation levels remained unaffected. Functionally, *SOCS3*-depletion caused strongly increased tumor cell invasion with no obvious effect on tumor cell proliferation. In summary, our findings suggest that *SOCS3* inactivation by promoter hypermethylation is mutually exclusive to *EGFR* activation in gliomas and preferentially promotes glioma cell invasion through STAT3 and FAK activation.

**Keywords** Brain tumor · Glioblastoma · Astrocytoma · JAK · MAPK · AKT

## **Introduction**

*SOCS3* belongs to a family of eight structurally related proteins (*CIS* and *SOCS1-7*) that function as negative regulators of cytokine signaling [3, 8]. Cytokine-mediated activation of the JAK/STAT signal transduction pathway leads to a transcriptional increase of several genes, including *SOCS3* that, in turn, acts as part of a negative feedback loop by inhibiting JAK activity through a mechanism involving receptor association. *SOCS3* thus serves as a crucial regulator controlling for an excessive activation of cytokine-related JAK/STAT signaling [8].

In human cancers, JAK/STAT signaling is known to promote the oncogenic properties of human cancer cells [6, 32] and *SOCS3* as a negative regulator of this signaling pathway has been suggested to bear tumor suppressor function [1]. In fact, inhibition of *SOCS3* facilitates tumor formation and growth in multiple human cancers, including lung and liver carcinomas, breast and prostate cancer as well as squamous cell carcinomas [1, 16, 21, 28, 31]. Inactivation of *SOCS3* is commonly caused by promoter

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hypermethylation in various types of cancer [4, 5, 24, 26, 28]. In human gliomas, *SOCS3* promoter hypermethylation has been reported to associate with an unfavorable clinical outcome in a series of 46 glioblastoma patients [12].

Gliomas are the most common primary tumors of the central nervous system and they comprise a heterogeneous group of neoplasms, among which glioblastoma is the most frequent and most malignant tumor entity [11]. Two clinically and molecularly distinct subtypes of glioblastoma, namely primary and secondary glioblastoma, have been identified [7, 15]. While primary glioblastomas arise de novo and demonstrate frequent *EGFR* amplification and *PTEN* mutation, secondary glioblastomas develop by progression from a preexisting lower-grade precursor lesion and are molecularly characterized by frequent *IDH1* and *TP53* mutations, while *EGFR* amplification is virtually absent [18, 20].

As JAK/STAT signaling is initiated by EGFR signaling [17] and *SOCS3* functions as a regulator within this signaling pathway, we aimed to investigate the role of *SOCS3* in a panel of human gliomas comprising different histologically and molecularly defined glioma subtypes. We found that *SOCS3* inactivation by promoter hypermethylation was significantly less frequent in primary glioblastomas than in all other investigated glioma entities. Moreover, *SOCS3* promoter hypermethylation was significantly inversely related to both the *EGFR* gene dosage as well as the EGFR protein expression, thus suggesting *SOCS3* inactivation as a surrogate mechanism in those types of gliomas that lack aberrant EGF receptor signaling. Subsequent functional in vitro analyses supported this hypothesis by indicating increased STAT3 and FAK activation levels as well as a strong enhancement of tumor cell invasion in *SOCS3* knock-down glioma cells.

## Materials and methods

### Tumor samples and cell lines

Tumors were selected from the tumor tissue archive of the Department of Neuropathology, Heinrich-Heine-University, Düsseldorf, Germany and investigated according to protocols approved by the institutional review board. Tumors were classified according to the World Health Organization (WHO) 2007 classification of tumors of the central nervous system [11]. Parts of each tumor were snap-frozen directly after operation and stored at  $-80^{\circ}\text{C}$ . Only tissue samples with a tumor cell content of 80% or more were used for molecular analyses. The tumor series consisted of 60 gliomas, including 25 primary glioblastomas and 8 secondary glioblastomas, WHO grade IV (*GB*); 4 anaplastic astrocytomas, WHO grade III (*AA*); 3 diffuse

astrocytomas, WHO grade II (*A*); 6 anaplastic oligoastrocytomas, WHO grade III (*AOA*); 4 oligoastrocytomas, WHO grade II (*OA*); 7 anaplastic oligodendrogliomas, WHO grade III (*AO*); and 3 oligodendrogliomas, WHO grade II (*O*) (Fig. 1; Supplementary Table 1). We used ten non-neoplastic brain samples from different individuals (*NB1-NB10*) as reference samples (5 tissue specimens obtained by neurosurgical resection or open biopsy, two tissue specimens obtained at autopsy and three tissue specimens or nucleic acids obtained from commercial sources; Supplementary Table 1). As a positive control for the methylation studies we used commercially available hypermethylated DNA (CpG Genome<sup>TM</sup> Universal Methylated DNA, Cat.-No. S7821; Millipore, Billerica, MA). The human glioma cell line U251MG for stable transfection with shRNAs directed against *SOCS3* (see below) was obtained from the American Type Culture Collection (Manassas, VA, USA).

### DNA and RNA extraction

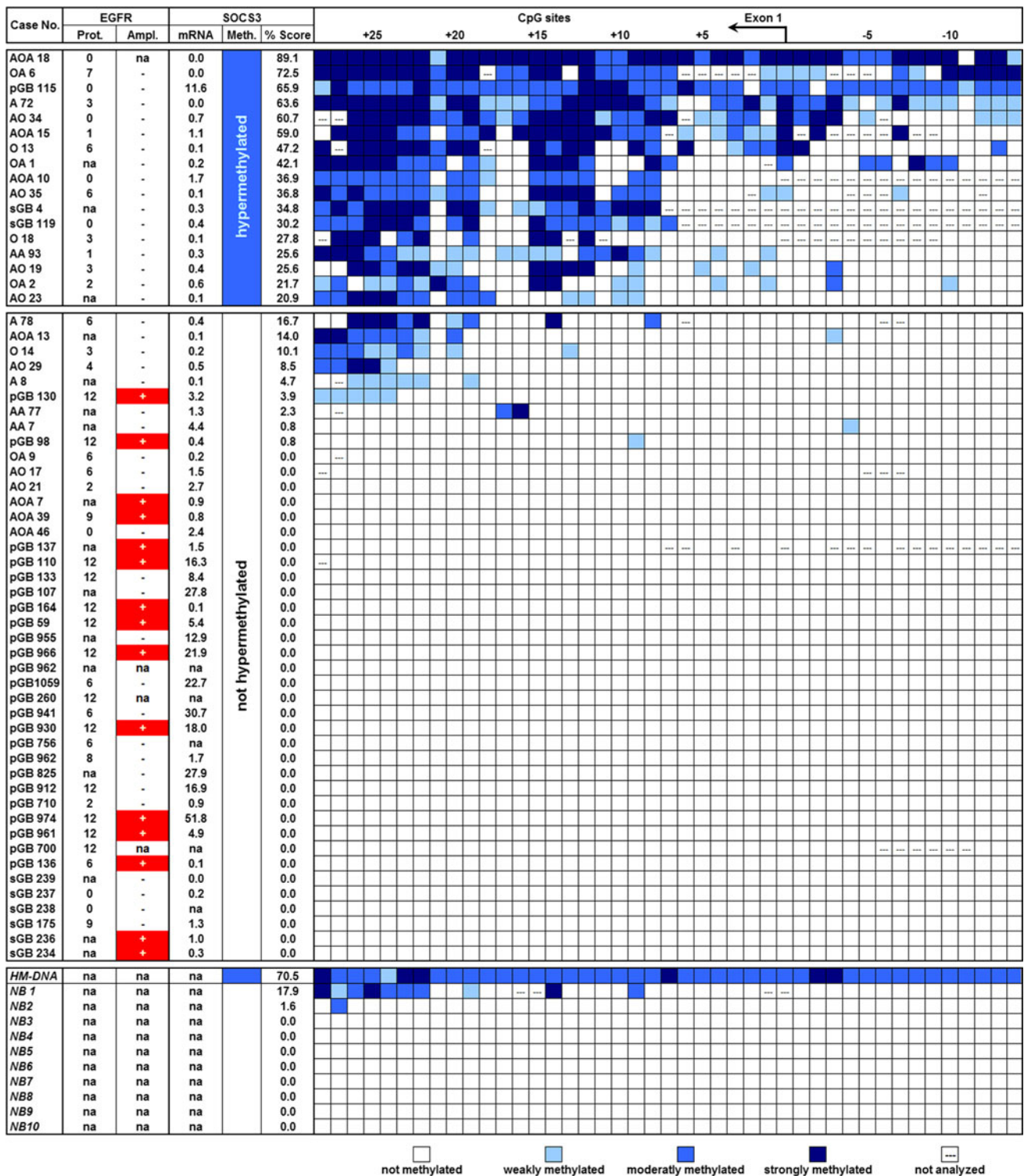
Extraction of DNA and RNA from frozen tumor tissue was performed by ultracentrifugation over a caesium chloride gradient as described elsewhere [27]. Total RNA from cell cultures was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

### Real-time (reverse transcription) PCR analysis

Three micrograms of total RNA extracted from tumor tissue or cell lines was reverse transcribed into cDNA using random hexanucleotide primers and SuperScript<sup>®</sup> Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. *SOCS3* mRNA expression levels were determined by real-time PCR on the StepOnePlus<sup>TM</sup> sequence detection system (Applied Biosystems, Foster City, CA) by means of incorporation of SybrGreen fluorescent dye as reported before [2]. Fold expression changes (for the tumor tissues relative to non-neoplastic brain tissue and for shRNA-transfected cell lines relative to corresponding control cells) were calculated with the  $\Delta\Delta\text{CT}$  method [10] using *ARF1* (ADP-ribosylation factor 1) as the reference transcript. For the assessment of *EGFR* gene copy number gains, we used a real-time PCR-based assay with the *EGFR* gene copy number being normalized to the copy number of the genomic marker D2S1743 (WI3306.1) at chromosomal band 2q21.2. Copy number gains of  $>5$  were designated as *EGFR* amplification. All primer sequences are listed in Supplementary Table 2.

### Methylation analysis using sodium bisulfite sequencing

Sodium bisulfite treatment of 1  $\mu\text{g}$  DNA was performed overnight (16 h) according to a standard protocol [13]. A



**Fig. 1** Synopsis of molecular aberrations of the *SOCS3* and the *EGFR* gene in human gliomas. Note that *SOCS3* is frequently inactivated by promoter hypermethylation (*blue*) in a panel of 60 human gliomas, however, in the subgroup of primary glioblastomas (pGB) that are characterized by a high frequency of activating EGF receptor aberrations *SOCS3* methylation is absent in all but one patient. Moreover, *EGFR* amplification (gene copy number > 5; *red*) is merely observed in tumors that lack *SOCS3* promoter

hypermethylation. *Prot.* EGFR protein expression, *Ampl.* relative *EGFR* gene copy number >5 (+) or ≤5 (-), *mRNA* relative *SOCS3* transcript levels, *Meth.* *SOCS3* methylation status, *% Score* % *SOCS3* promoter methylation score. The *SOCS3* promoter methylation pattern has been assessed by direct bisulfite sequencing and is depicted for each individual CpG site within the analyzed region spanning the transcription start site of *SOCS3*. *na* not analyzed

fragment of the *SOCS3* 5'-CpG island located at 17q25.3 between nucleotides 73,867,448 and 73,867,855 (numbering according to the UCSC genome browser; <http://www.genome.ucsc.edu>) was PCR-amplified from sodium bisulfite-modified DNA using the primers as reported in Supplementary Table 2. The amplified fragment covers 43 CpG sites and spans the transcription start site of *SOCS3* (Fig. 1). Direct bisulfite sequencing and semiquantitative calculation of a promoter methylation score were carried out as described [25]. In short, the methylation status at each of the analyzed CpG sites was semi-quantitatively rated using the following scale: 0, completely unmethylated; 1, a weak methylated signal detectable in the sequence; 2, methylated signal approximately equal to unmethylated signal; 3, methylated signal markedly stronger than unmethylated signal. Based on this rating, a promoter methylation score in percentage was calculated for each tumor as well as the non-neoplastic brain tissue controls by adding the figures determined at the individual CpG sites divided by the maximum possible methylation score at all analyzed CpG sites. Tumors with a methylation score exceeding that of non-neoplastic brain tissue were regarded as hypermethylated (methylation score > 18; Fig. 1). False-positive results due to incomplete bisulfite conversion were excluded by controlling for the conversion of cytosines entrapped in the respective sequences that were not part of CG dinucleotides. Only sequences with 100% conversion rates were used for evaluation.

#### Mutational analysis

Single strand conformation polymorphism (SSCP)/heteroduplex analysis was performed to detect mutations within the entire coding sequence of *SOCS3* (exon 2) in all 60 tumors. The respective primer sequences are listed in Supplementary Table 2. PCR products were separated by electrophoresis on 10–12% polyacrylamide gels at room temperature and at 4°C overnight. Gels were then silver stained to visualize band patterns and PCR products sequenced in case of aberrant band patterns.

#### Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections using a commercially available EGFR-specific mouse monoclonal antibody (Dako, Hamburg, Germany; 1:25 dilution). Immunoreactivity was detected with the EnVision™ Detection System (Dako) using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen. Negative controls without primary antibodies were performed for all reactions. Protein expression levels were semi-quantitatively assessed by a

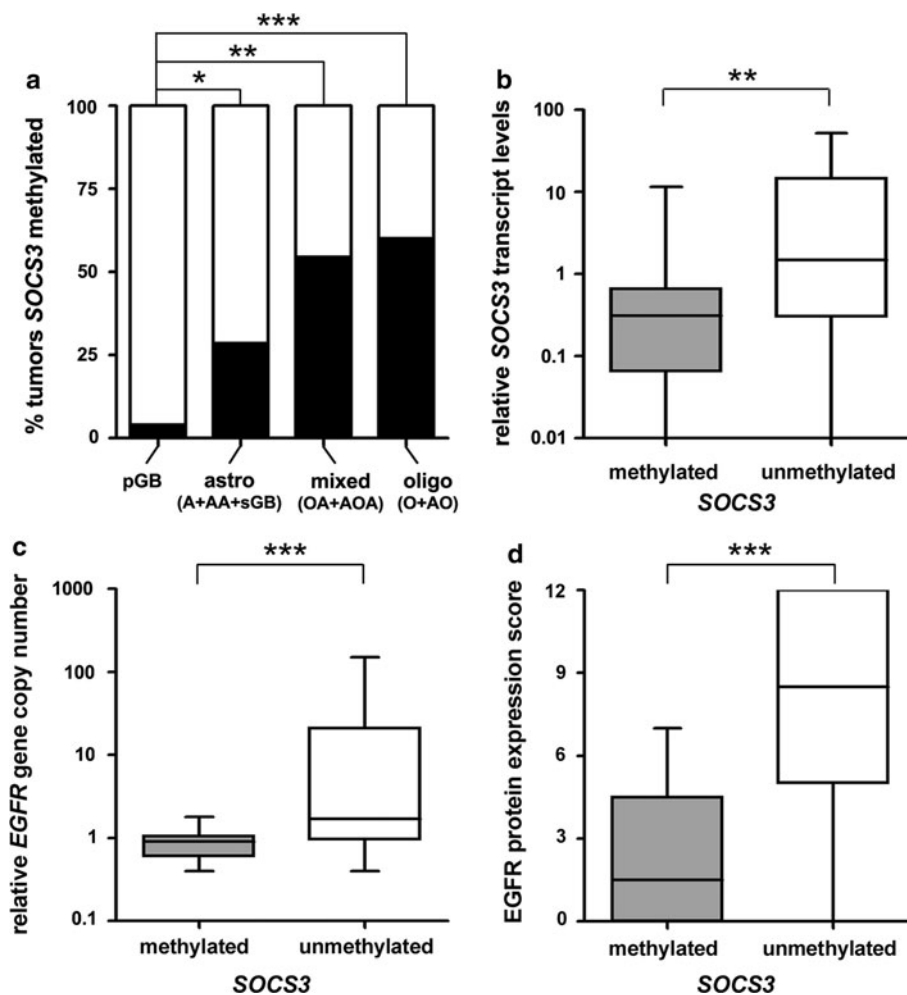
composite numerical score, based on the percentage of positively stained tumor cells multiplied by staining intensity, potentially ranging from 0 to 12 (Fig. 2d). The percentage of labeled cells was scored as follows: 0 (no or minimal reactivity, similar to non-neoplastic brain tissue), 1 (<10%), 2 (10–50%), 3 (50–90%) and 4 (>90%). Staining intensity was graded as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong).

#### Generation of glioblastoma cell lines with stable *SOCS3* knock-down

Glioblastoma cells with a stable *SOCS3* knock-down were generated using vector-based shRNA-mediated RNAi. To control for potential off-target effects, we used two different predesigned shRNA oligonucleotides (Qiagen, Hilden, Germany) with the following sequences: *sh1-sense*, TGACGGTCTTCCGACAGAGAT; *sh2-sense*, TTTCTCATAGGAGTCCAGGTG. shRNA oligonucleotides were cloned into the pcDNA™ 6.2-GW/EmGFP-miR vector (Invitrogen, Carlsbad, CA) containing the human CMV promoter, EmGFP for fluorescent detection as well as a Blasticidin resistance gene for selection of stable cell lines. DNA sequencing was performed to confirm the proper orientation of the inserts into the vector and to exclude mutations. U251MG cells were then transfected with the shRNA oligonucleotide-containing vectors using GeneJuice transfection reagent (Merck, Darmstadt, Germany) according to the manufacturer's recommendations. Cells were also transfected with the pcDNA™ 6.2-GW/EmGFP-miR-neg control plasmid containing an insert able to form a hairpin structure that is processed into mature miRNA, but is not predicted to target any known vertebrate gene. Stable transfectants were selected for by growing the cells under 20 µg/ml blasticidin. Blasticidin-resistant cells were then separated and individual cell clones were expanded for further analyses. Two different subclones of each of the two hairpins were investigated in the in vitro experiments to control for insertion-site-specific side effects (Fig. 3).

#### SDS-polyacrylamide gel electrophoresis (PAGE) and western blot analysis

Proteins (30 µg) extracted from U251MG transfected and control cells were denatured, electrophoretically separated on 10–12% gels and transferred to a nitrocellulose membrane (Whatman, Maidstone, UK). After blocking (5% BSA in TBS-T), the membrane was incubated overnight at 4°C with the following primary antibodies: phospho-STAT3 (phosphorylated on Tyr705; 1:500 dilution; #9138; Cell Signaling, Danvers, MA), phospho-MAPK (Thr202/Tyr204; 1:500; #9101; Cell Signaling), phospho-Akt



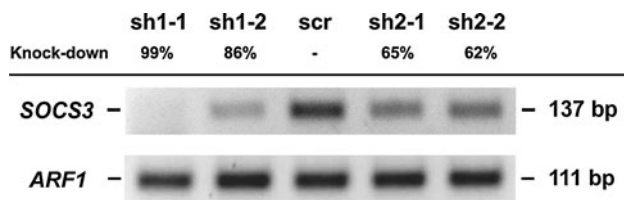
**Fig. 2** Statistical analyses of *SOCS3* promoter hypermethylation in human gliomas. **a** *SOCS3* promoter hypermethylation is significantly less frequent in primary glioblastomas than in all other investigated histological glioma subtypes (astrocytic tumors other than pGB, mixed oligoastrocytomas and oligodendrogliomas). **b** Tumors with *SOCS3* promoter hypermethylation show significantly lower *SOCS3* transcript levels than tumors without *SOCS3* promoter hypermethylation. **c–d** *SOCS3* promoter hypermethylation is inversely correlated with the presence of *EGFR* aberrations. Note that both the relative

gene copy number of *EGFR* (**c**) as well as the immunohistochemically assessed protein expression level of *EGFR* (**d**) are significantly lower in tumors with *SOCS3* promoter hypermethylation than in tumors lacking this molecular alteration. *Boxplots*: boxes extend from the 25th to the 75th percentile with the middle line at the 50th percentile, whiskers extend down to the lowest and up to the highest values. Asterisks indicate significant differences (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ )

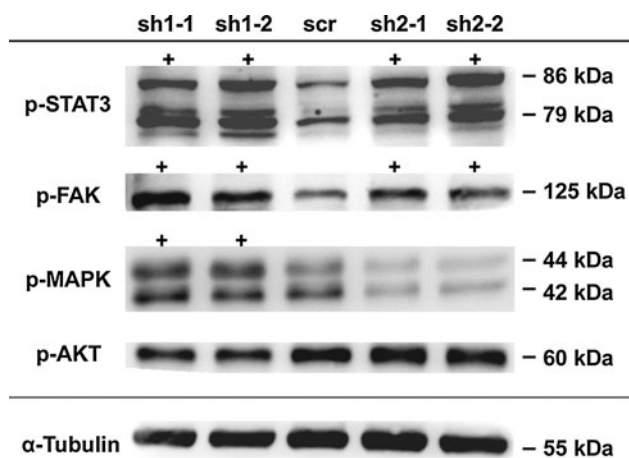
(Ser473; 1:1,000; #4060; Cell Signaling), phospho-FAK (Tyr397; 1:500; #3283; Cell Signaling) and  $\alpha$ -tubulin as a loading control (1:3000; #T5168; Sigma-Aldrich, Taufkirchen, Germany). Blots were then incubated for 2 h with HRP-linked anti-mouse or anti-rabbit antibodies, respectively (1:10,000 dilution; Thermo Fisher Scientific, Waltham, MA). To visualize primary antibody binding by chemiluminescence, the membranes were treated with Immobilon<sup>®</sup> Western HRP Substrate containing luminol and peroxide solution (Millipore, Billerica, MA) and recorded with the LAS-3000 mini system (Fujifilm Life Science, Stamford, CT). Results were reproduced in at least two independent experiments (Fig. 4).

#### Cell-based functional assays

The proliferation rate of *SOCS3* knock-down clones was compared to control cells using a commercially available BrdU incorporation assay (Roche, Mannheim, Germany). After incubation with BrdU labeling solution for 24 h at 37°C, cells were fixed and absorption values of the colorimetric reaction were measured. Differences in apoptotic activity between *SOCS3*-depleted cells and controls were assessed by a fluorometric caspase-3/7 assay (Promega, Mannheim, Germany) according to the manufacturer's protocol. Fluorimetric measurements were optimized for the U251MG cell line and performed after 150 min of

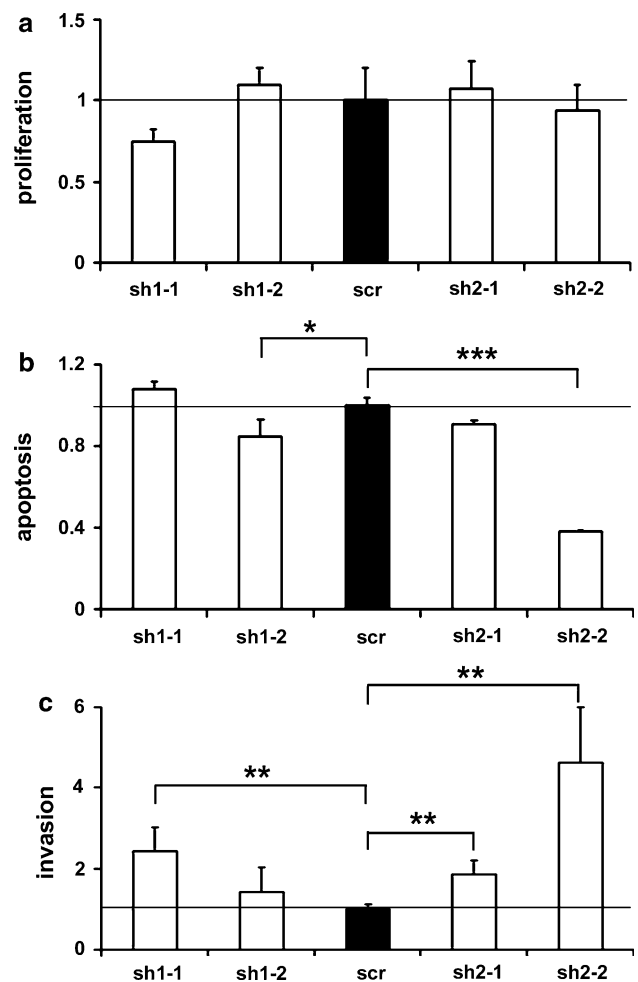


**Fig. 3** Generation of U251 glioblastoma cells with a stable shRNA-mediated *SOCS3* knock-down. Two different hairpins (*sh1* and *sh2*) were investigated to exclude off-target effects of the respective shRNAs. To correct for insertion-site-specific effects we investigated two different subclones of each hairpin (*second digit* within the respective designation). Note that in comparison to the scrambled negative control shRNA (*scr*) all four selected clones exhibit markedly reduced *SOCS3* expression levels relative to the *ARF1* reference gene (knock-down efficacy is provided in percentage on *top* of each band)



**Fig. 4** Western blot analysis for the activation of EGFR downstream signaling targets in U251MG *SOCS3* knock-down cells. Protein phosphorylation levels for both STAT3 and FAK are uniformly increased (+) in all four *SOCS3* knock-down clones relative to U251MG scrambled negative control cells (*scr*). The *SOCS3* knock-down effect on the activation levels of MAPK is inconsistently associated with an increase in protein phosphorylation (i.e. activation) in only one of the two investigated hairpins (clones *sh1-1* and *sh1-2*). p-AKT expression levels appear completely unaffected by the modulation of *SOCS3* expression in U251MG glioblastoma cells.  $\alpha$ -Tubulin was employed as a control for equal protein loading. Results were reproduced in at least two independent experiments

incubation with the caspase substrate at room temperature. For investigating the impact of *SOCS3* inactivation on the invasive properties of glioblastoma cells we used a 24-well modified Boyden chamber assay (BD Biosciences, San Jose, CA). After serum-starvation for 24 h, cells were seeded on top of a transwell insert containing a Matrigel<sup>TM</sup>-coated fluorescence blocking membrane with 8.0  $\mu$ m pores. Inserts were then transferred into a lower chamber filled with DMEM containing 10% fetal bovine serum as a chemoattractant and cells were allowed to invade along the serum gradient. Following incubation for 24 h, the cells on the membrane were fixed in paraformaldehyde (4% in PBS) and stained with 4'-6-diamidino-2-



**Fig. 5** Cell-based assays for the functional effects of a reduction of *SOCS3* expression levels in U251MG glioblastoma cells. Cell proliferation was measured using a BrdU incorporation assay (a), apoptosis was quantified using a fluorometric caspase-3/7 assay (b) and tumor cell invasion was assessed using a transwell assay with Matrigel-coated membranes (c). Note that inhibition of *SOCS3* does not appear to affect proliferation, slightly inhibits apoptosis in a subset of the investigated clones, but reveals the most pronounced overall effects on tumor cell invasion. All four *SOCS3*-depleted clones show an uniform increase in tumor cell invasion that is significant in three out of the four investigated clones. The average result in the negative control cells (*scr*) has been set to 1. Error bars indicate standard deviations and asterisks significant differences. All assays were performed in at least six replicates

phenylindole (DAPI). Membranes were then sliced out from the inserts and transferred upside down on glass slides. Cells that had invaded were counted on the underside of the membrane by means of a fluorescent microscope. All cell-based functional assays were performed in at least six replicates (Fig. 5).

#### Transient siRNA-mediated silencing of *SOCS3*

To independently validate the *SOCS3* effect on invasion observed in the stable U251MG knock-down clones, we

performed siRNA-mediated silencing of *SOCS3* in U251MG and A172 cells (Suppl. Fig. 1). Transfections were done with HiPerFect transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's protocol. For testing knock-down efficiency by quantitative real-time RT-PCR, transfections were performed in 24-well plates with 30,000 cells/well. The RNeasyPlus kit (Qiagen, Hilden, Germany) was used for RNA extraction. For invasion assays, cells were transfected in 6-well plates with 240,000 cells/well. All experiments were performed with 50 nM siRNA (Qiagen, Hilden, Germany). Sequences of siRNAs used were as follows: *siRNA1-sense*, CCAAGA ACCUGCGCAUCCATT; *siRNA2-sense*, GAAGAGCCU AUUACAUCUATT; *siRNA3-sense*, GGGAGUCCUG GACCAGUATT; *siRNA4-sense*, CGCUCAGCGUCA GACCATT.

### Statistical analyses

Fisher's exact test was used to assess frequency differences of *SOCS3* promoter hypermethylation between the different histological glioma entities (Fig. 2a). In this regard, we compared primary glioblastomas (pGB,  $n = 25$ ) to other astrocytic gliomas (A + AA + sGB,  $n = 14$ ), mixed oligoastrocytomas (OA + AOA,  $n = 11$ ) and pure oligodendroglial neoplasms (O + OA,  $n = 10$ ).  $P$  values were verified for significance by employing Benjamini-Hochberg corrections for multiple testing.

Mann-Whitney  $U$  test analyses were used to compare *SOCS3* mRNA expression (Fig. 2b), *EGFR* gene copy number (Fig. 2c) and EGFR protein expression scores (Fig. 2d) between tumors with and without *SOCS3* promoter hypermethylation. In the cell-based functional assays, two-sided Student's  $t$  test analyses were utilized to compare results between transfected and control cells (Fig. 5). All statistical analyses were computed using the Graph Pad Prism Software (Version 5). The  $P$  value cut-off level for significance was set to 0.05 (\*). (\*\*) indicates a  $P$  value of <0.01 and (\*\*\*) and a  $P$  value of <0.001.

## Results

### Promoter hypermethylation and transcriptional downregulation of *SOCS3* in human gliomas

We assessed the methylation status of the *SOCS3* promoter in a panel of 60 human gliomas of different histological subtypes and grades using direct bisulfite sequencing analysis (Fig. 1). Methylation of the *SOCS3* promoter in the tumor samples exceeded that of non-neoplastic brain tissue in 17 out of 60 glioma patients (28%). There was a significant inverse correlation between *SOCS3* promoter

hypermethylation and *SOCS3* transcript levels (Mann-Whitney  $U$  test,  $P = 0.001$ ). *SOCS3* mRNA expression levels were significantly lower in the group of gliomas with a hypermethylated *SOCS3* promoter (median 0.3; interquartile range, IQR 0.5) when compared to the group of tumors with promoter methylation scores equal to or lower than the reference brain tissue samples (median 1.5, IQR 11.4) (Fig. 2b).

When comparing the *SOCS3* methylation status between the different glioma entities represented in our panel, we found that *SOCS3* promoter hypermethylation—with a single exception (pGB115)—was absent in primary glioblastomas (1 out of 25 cases, 4%). Furthermore, *SOCS3* promoter hypermethylation was significantly less frequent in primary glioblastomas compared to all other glioma entities, i.e. diffusely infiltrating astrocytic (A + AA + sGB: 4 out of 14, 29%; Fisher's exact test,  $P = 0.047$ ), mixed oligoastrocytic (OA + AOA: 6 out of 11, 55%;  $P = 0.001$ ) and oligodendroglial (O + AO: 6 out of 10, 60%;  $P = 0.0008$ ) neoplasms (Fig. 2a).

To screen for potential alternative *SOCS3* inactivation mechanisms, we performed an additional mutation analysis of the entire coding sequence (exon 2) of *SOCS3* by means of SSCP/heteroduplex analysis but did not detect any tumor-associated (somatic) mutations in the 60 gliomas investigated.

*SOCS3* promoter hypermethylation is inversely correlated with *EGFR* gene dosage and EGFR protein expression

Following up on our observation of a significantly less frequent *SOCS3* promoter hypermethylation in primary glioblastomas, which usually contain a high fraction of activating *EGFR* aberrations, we assessed the *EGFR* gene dosage by real-time PCR analysis as well as the EGFR protein expression by immunohistochemistry in our tumor panel and correlated the results with the *SOCS3* promoter methylation status. A total of 15 tumors (13 glioblastomas and 2 anaplastic oligoastrocytomas) showed a more than fivefold increase in the *EGFR* gene dosage, consistent with *EGFR* amplification (Fig. 1) and none of these tumors exhibited concomitant *SOCS3* promoter hypermethylation. Statistical analysis underscored this observation and revealed significantly lower *EGFR* gene copy numbers in tumors containing a hypermethylated *SOCS3* promoter (median 0.9, IQR 0.4) when compared to tumors without *SOCS3* promoter hypermethylation (median 1.7, IQR 19.0, Mann-Whitney  $U$  test,  $P = 0.0006$ ; Fig. 2c).

We also compared the semiquantitatively determined EGFR protein expression levels between tumors with and without *SOCS3* promoter hypermethylation. The median EGFR protein expression score was significantly lower in

tumors with *SOCS3* promoter hypermethylation (median 1.5, IQR 3.0) compared to tumors without this aberration (median 8.5, IQR 6.0, Mann–Whitney *U* test,  $P = 0.0004$ ; Fig. 2c). Of note, the single primary glioblastoma (pGB115) with *SOCS3* promoter hypermethylation did show neither a relevant increase in *EGFR* gene copy number (fold change: 1.2) nor EGFR protein expression (Score: 0).

#### Stable shRNA-mediated *SOCS3* knock-down increases STAT3 and FAK activation levels in glioblastoma cells

To further assess the impact of *SOCS3* on potential downstream targets within the EGFR signaling pathway, we used vector-based shRNA-mediated RNAi to generate U251 glioblastoma cells with a stable *SOCS3* knock-down. Two different hairpins (sh1 and sh2) were used to exclude off-target effects of the respective shRNAs and two different subclones of each hairpin (shX-1 and shX-2) to exclude insertion-site-specific side effects. Transfection with both hairpins resulted in a consistent *SOCS3* knock-down in comparison to the negative scrambled (scr) control with a subtotal depletion of *SOCS3* expression in glioblastoma cells transfected with hairpin 1 (86–99%) and a somewhat lesser reduction of *SOCS3* expression in cells transfected with hairpin 2 (62–65%; Fig. 3).

Employing phosphorylation-specific antibodies, we studied the *SOCS3* effect on protein phosphorylation, i.e. activation levels of STAT3 (Tyr705), FAK (Tyr397), MAPK (Thr202/Tyr204) and Akt (Ser473). Of note, *SOCS3* depletion resulted in a strong and unequivocal increase in the phosphorylation levels of STAT3 and FAK in all investigated tumor cell subclones relative to the respective negative control cells (Fig. 4). The phosphorylation pattern of MAPK in dependence on *SOCS3* reduction was less uniform with an increased expression of p-MAPK only in hairpin 1 and a reduction of p-MAPK levels in U251 glioblastoma cells transfected with hairpin 2. For AKT, we did not detect any relevant changes in the protein phosphorylation levels comparing *SOCS3* knock-down and control cells (Fig. 4).

#### Cell-based functional assays reveal a major effect of *SOCS3* depletion on tumor cell invasion

To evaluate the functional consequences of *SOCS3* inactivation in U251 glioblastoma cells we performed cell-based assays for tumor cell proliferation, apoptosis and invasion. Tumor cell proliferation measured by BrdU incorporation exhibited an uneven either minor increase or reduction in individual *SOCS3* knock-down clones in comparison to control cells but none of these changes was statistically significant (Fig. 5a). Using a caspase-3/7 assay

we observed a moderate inhibition of apoptotic activity in the majority (3 out of 4) of the investigated *SOCS3* knock-down clones with statistically significant results for two of the investigated subclones (Students's *t* test; sh1-2,  $P = 0.0476$ ; sh2-2,  $P = 0.0001$ ; Fig. 5b).

The predominant functional effect of *SOCS3* reduction, however, was detected when investigating the differential invasive capacities of U251 glioblastoma cells using a modified Matrigel<sup>TM</sup>-coated Boyden chamber assay. All four investigated *SOCS3*-depleted tumor cell clones showed an unequivocal increase in their invasive capacities relative to the respective control cells with the highest, up to nearly fivefold increase in subclone sh2-2. Using Student's *t* test analyses this increase proved highly significant in three out of the four investigated *SOCS3* knock-down clones (sh1-1,  $P = 0.005$ ; sh2-1,  $P = 0.004$ ; sh2-2,  $P = 0.002$ ; Fig. 5c). To independently corroborate the *SOCS3* effect on tumor cell invasion, we employed siRNA-mediated silencing of *SOCS3* in U251MG and A172 glioblastoma cells. In line with our findings in the stably transfected *SOCS3* knock-down U251MG clones, we observed an uniform and significant increase of glioma cell invasion after *SOCS3* depletion in both cell lines (Suppl. Fig. 1).

## Discussion

*SOCS3* functions as a negative regulator of the JAK/STAT signal transduction pathway and has been described as a tumor suppressor in human cancers. In diffusely infiltrating human gliomas, the role of *SOCS3* was less clear, particularly in regard to the fact that these tumors consist of a number of different subentities that are characterized by distinct patterns of molecular aberrations [11]. As such, primary glioblastomas contain a high frequency of activating *EGFR* aberrations, while secondary glioblastomas and their lower-grade precursor lesions in their majority lack *EGFR* aberrations and are instead characterized by frequent mutations in the *IDH1* and *TP53* genes [15]. These different molecular preconditions may imply to the distribution of *SOCS3* alterations between the different subtypes of gliomas, particularly in regard to the fact that *SOCS3* and EGFR receptor signaling share common downstream signaling targets. In a preceding publication, Martini and colleagues [12] reported on the negative prognostic effect of *SOCS3* promoter hypermethylation in gliomas but restricted their study to primary glioblastomas and did not refer to the EGFR status in their patients.

We investigated a panel of 60 human gliomas of different histological subtypes and WHO grades for *SOCS3* alterations and made the striking observation that *SOCS3* inactivation by promoter hypermethylation was observed



with the lowest frequency in primary glioblastomas (4%), i.e. exactly in those tumors that contain the highest rate of EGFR aberrations. We then more closely assessed the correlation between EGF receptor aberrations and *SOCS3* promoter hypermethylation in our tumor panel. Indeed, tumors with *SOCS3* promoter hypermethylation exhibited both significantly lower relative *EGFR* gene copy numbers as well as significantly lower EGFR protein expression scores than tumors without this epigenetic aberration, suggesting that *SOCS3* inactivation by promoter hypermethylation in gliomas might be an alternative molecular alteration to EGFR amplification/overexpression. Interestingly, two other recent publications add further proof to our hypothesis of an inverse relation between *SOCS3* protein function and EGFR signaling. In human hepatocytes and in human embryonic kidney 293 cells *SOCS3* was found to impair the phosphorylation of STAT3 in response to EGF [22, 29], thus indicating that *SOCS3* differentially regulates EGF receptor signaling. Also, in line with this hypothesis, the single primary glioblastoma (pGB115) in our panel with a hypermethylated *SOCS3* promoter did show neither elevated *EGFR* gene copy numbers nor EGFR protein overexpression. On histological review, the tumor exhibited the classical morphological features of glioblastoma and molecularly the tumor was wild-type for *IDH1* and *IDH2*, as typical for the majority of primary glioblastomas. Thus, our results do not exclude that primary glioblastomas may require a hypermethylated *SOCS3* promoter, nevertheless the low frequency of *SOCS3* hypermethylation (4%) is contrary to the one described by Martini and colleagues [12] who report on up to 35% of primary glioblastomas with *SOCS3* promoter hypermethylation. While in the 25 primary glioblastomas in our panel we had a nearly equal proportion of tumors with (13 cases) and without (12 cases) EGF receptor amplifications, the higher *SOCS3* methylation frequency in the paper by Martini et al. might be due to an overrepresentation of *EGFR* wildtype tumors in their tumor panel that are potentially more susceptible to require *SOCS3* hypermethylation. On the other hand, closer to the low *SOCS3* methylation frequencies observed in our panel, an alternative study by Zhou and colleagues [34] did detect neither *SOCS3* hypermethylation nor reduced *SOCS3* expression levels in 10 glioblastoma cell lines and 12 primary glioblastoma tissues.

Further investigation of the downstream signaling effects of *SOCS3* within the EGF receptor signaling pathway revealed a strong and unequivocal increase in the phosphorylation levels of STAT3 and FAK in all investigated *SOCS3* knock-down clones relative to respective U251MG control cells. The positive effect of *SOCS3* depletion on STAT3 activation is in line with the functional role of *SOCS3* as a negative regulator of JAK/STAT

signaling [3, 8] and also our finding of an increased FAK activation after *SOCS3* knock-down had been concordantly reported in progenitor B lymphocytes and human hepatocellular carcinoma cells. The question on how exactly *SOCS3* depletion increases FAK Y397 phosphorylation remains a matter for further investigation. One study reported that *SOCS3* regulates CXCL12-induced FAK phosphorylation through the ubiquitin–proteasome pathway during B lymphopoiesis. Another report in human hepatocellular carcinoma points to a direct physical interaction of *SOCS3* with phosphorylated FAK and this interaction mediating polyubiquitination of FAK, resulting in proteasome-dependent degradation of FAK [9, 14].

As both STAT3 and FAK can be alternatively phosphorylated by activated growth factor receptors, such as the epidermal growth factor receptor (EGFR), these findings may add further proof to our hypothesis that *SOCS3* inactivation may substitute for the activation of identical intracellular signaling pathways in those types of gliomas that do not bear activated EGF receptors. By means of STAT3 activation *SOCS3*-depleted cancer cells may transactivate multiple target genes impacting such diverse cell conditions as proliferation, differentiation or migration and invasion [6, 32]. The activation of FAK has been described to impact tumor cell growth, cytoskeletal organization and microtubule organization and is functionally even more directly linked to an increase of invasive properties in a variety of human tumors, including gliomas [19].

As two further downstream EGFR signaling intermediates, p44/p42 MAPK (mitogen-activated protein kinase, ERK1/2) and AKT (protein kinase B), had been described to be regulated by *SOCS3* [16, 23, 33], we investigated the activation levels of both molecules in our *SOCS3* knock-down glioma cells. For p44/p42 MAPK we obtained non-uniform results with a moderate increase in activation levels for only one of the transfected hairpins (sh1). This partial lack of a MAPK activation increase may be potentially due to the weaker *SOCS3* knock-down efficiency of hairpin 2 (sh2) but may also argue for a less consistent impact of *SOCS3* on the p44/p42 MAPK activation levels in glioma cells. For AKT there was no increase of phosphorylation levels after *SOCS3* depletion. Thus, our results are not indicative of a close interrelationship between these two molecules in human gliomas.

As to the functional implications of *SOCS3* inactivation in human glioma cells, we found a predominant impact on tumor cell invasion with uniformly and significantly increased invasive capacities in *SOCS3*-depleted glioma cells. Our findings are in line with reports on an increased migratory and invasive potential after *SOCS3* knock-down in tumor entities other than gliomas [14, 16, 30]. Moreover, the above-described specific activation pattern of STAT3

and particularly FAK after SOCS3 knock-down is compatible with a predominant functional impact on tumor cell invasion.

We observed an only moderate reduction of apoptotic cell properties that, however, was statistically significant in two out of the four investigated SOCS3 knock-down clones. The only moderate and uneven effect of SOCS3 reduction on apoptosis may be due to the circumstance that we measured basal apoptosis without stimulation. Also, the detection of a significant dysregulation in two subclones, each from different hairpins, argues against an off-target and for a true anti-apoptotic effect of impaired SOCS3 in glioma cells. We did not observe consistent or significant effects on tumor cell proliferation after SOCS3 inactivation, arguing for a negligible effect of SOCS3 on tumor cell proliferation in glioblastoma cells. Recapitulating that we found a more frequent *SOCS3* promoter hypermethylation and transcriptional downregulation in lower-grade astrocytic and oligodendroglial tumors than in glioblastomas and that these tumors share the diffuse infiltrative pattern but not the high proliferative activity with glioblastomas, it is thus tempting to speculate that SOCS3 inactivation in these tumors may mainly substitute for the stimulating effect of EGFR on invasion but not on proliferation. Nevertheless, the intracellular signaling relationships are certainly more complex and will need further clarification exceeding the one provided in this manuscript.

In summary, our study molecularly dissects the inactivation mechanisms of *SOCS3* in different histological subtypes of human gliomas and functionally investigates the interrelationships of SOCS3 with EGF receptor signaling in gliomas. We conclude that *SOCS3* inactivation by promoter hypermethylation is significantly less frequent in primary glioblastomas than in all other investigated glioma entities and mutually exclusive to *EGFR* amplification/overexpression. Functionally, *SOCS3* silencing preferentially promotes glioma cell invasion through STAT3 and FAK activation.

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