SP100 reduces malignancy of human glioma cells

JANKA HELD-FEINDT[1], KIRSTEN HATTERMANN[2], FRIEDERIKE KNERLICH-LUKOSCHUS[1], H. MAXIMILIAN MEHDORN[1] and ROLF MENTLEIN[2]

[1]Department of Neurosurgery, University Medical Center Schleswig-Holstein UKSH, Campus Kiel, Arnold-Heller-Str. 3, Building 41, 24105 Kiel; [2]Department of Anatomy, University of Kiel, Olshausenstr. 40, 24098 Kiel, Germany

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Abstract. The nuclear autoantigen SP100 (speckled protein 100) is reported to control cellular gene expression, cell growth and differentiation. To investigate its relevance in brain tumors, we investigated SP100 expression and function in human glioblastomas and meningiomas. SP100 was expressed in both tumors at the mRNA and protein levels *in situ* and *in vitro*, however, expression in meningioma samples and meningioma cells exceeded that in glioblastoma samples and cultivated cells significantly. Moreover, whereas nearly all meningioma cells were SP100-immunopositive, only part of the glioblastoma cells were SP100 stainable. *In vitro*, SP100 was upregulated by interferon-α and -γ in both malignant cell types. To study its functional role, SP100 was overexpressed in glioblastoma cells. This SP100 overexpression reduced considerably the glioblastoma cell proliferation and migration to fetal calf serum. We conclude that SP100 expression reduces malignancy of brain tumors. Since meningiomas show a generally higher SP100 expression, this may be one of the factors explaining their lower malignancy compared to glioblastomas.

Introduction

Gliomas are the most common type of primary brain tumors, and the most malignant form, glioblastoma multiforme (GBM; graded into WHO IV), accounts for more than 50% of all intracranial tumors (1). Current standard treatment of GBM is surgical resection of the tumor, followed by adjuvant radio- and chemotherapy. However, prognosis for GBM patients is still poor, approximating median survival time of 12-15 months even with aggressive upfront treatment (2,3). In contrast, the second most common intracranial tumors with an incidence of 13 to 26% of all primary brain tumors are meningiomas (4), predominantly slowly growing benign tumors that can be graded into WHO grade I-III. Most patients with meningiomas have good quality of life in contrast to the patients with malignant gliomas.

The speckled protein of 100 kDa (SP100) is a constituent member of the nuclear body (NB), a subnuclear organelle, also termed PML (promyelocytic leukemia) oncogenic domain, Kr-body or nuclear domain 10 (5-7). NBs seem to be involved in the pathogenesis of human diseases, including acute promyelocytic leukemia and viral infection, and appear to have physiological roles in the control of cell growth, differentiation and apoptosis (8,9). Additionally to their two major components - SP100 and the PML protein, NBs contain transcription factors, chromosomal proteins, tumor suppressors and proto-oncogenes (7).

SP100 itself was first identified using the sera from patients suffering from Primary Biliary Cirrhosis (10,11). SP100 proteins occur in at least four different splice forms, with SP100A being the most abundant form (12). Expression of different SP100 splice forms is induced by interferon (IFN) (13).

As far as known, SP100 does not bind to the DNA alone but is probably recruited to DNA sites by interaction with specific DNA-proteins (14-16). Interestingly, interaction of SP100 with e.g. hHMG2/DSP1 results in transcriptional repression of specific genes whereas SP100 in cooperation with ETS-1, the founding member of the ets family of transcription factors, stimulates or reduces the expression of ETS-1 target genes (14-16). Especially, in primary endothelial cells and breast cancer cells, SP100 inhibits migration and invasion by ETS-1-dependent mechanisms and mediates the upregulation of genes that have antimigratory or antiangiogenetic properties (15,16). Additionally, Möller *et al* (17) showed that SP100 synergizes with the serine/threonine kinase HIPK2 resulting in p53-dependent gene expression. In contrast, Milovic-Holm *et al* (18) demonstrated that the knock-down of SP100 potentiated CD95-activated apoptosis.

Correspondence to: Dr Janka Held-Feindt, Department of Neurosurgery, University Medical Center Schleswig-Holstein UKSH, Campus Kiel, Arnold-Heller-Str. 3, Building 41, 24105 Kiel, Germany
E-mail: held-feindtj@nch.uni-kiel.de

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbeccos's modified Eagle's medium; EMA, epithelial membrane antigen; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBM, glioblastoma multiforme; GFAP, glial fibrillary acidic protein; IFN, interferon; NB, nuclear body; PBS, phosphate buffered saline; PML, promyelocytic leukemia; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SP100, speckled protein of 100 kDa; WHO, World Health Organization

Key words: glioma, meningioma, proliferation, chemotaxis, nuclear bodies

Despite the fact that SP100 seems to play a relevant role in the development of neoplasia, up to now only few investigations have focused on the relation between SP100 and malignancy. Therefore, we analyzed the expression and functional role of SP100 in different brain tumor cells. We showed that SP100 was expressed in high amounts in low-malignant meningiomas whereas in high malignant gliomas only small SP100 amounts were detectable. Co-staining revealed a clear expression in the tumor cells themselves. After SP100 overexpression in glioma cells these tumor cells changed their biological behavior to a less malignant form as demonstrated by reduced proliferation and migration potential.

Materials and methods

Patients and tumor specimens. Glioblastomas WHO IV and meningiomas WHO I were collected from 38 patients (19 patients each) who underwent surgery between March 1999 and September 2007 at the Department of Neurosurgery, Kiel, Germany. The patients included 8 females and 11 males with glioblastoma, and 14 females and 5 males with meningioma. The mean age at diagnosis was 57.42±14.19 for glioblastomas and 55.74±15.56 for meningiomas, respectively. To confirm the homogeneity and composition of the tumor specimens, hemalum/eosin stainings of all tumor tissue samples were routinely surveyed by a neuropathologist assuring the bulk of tissue samples were of tumor origin. All samples were obtained in accordance with approved ethical standards of the responsible committee of the University of Kiel and with the Helsinki Declaration of 1975, as received in 1983. Where sufficient material was available, matched probes of tumors were used in the different experiments.

Cultivation of cell lines. The human glioblastoma cell lines were obtained from the DKFZ (Heidelberg, Germany; U118, U343, U373, A172, T98G) or were generated in our laboratory (A739, A776, A764, 7/06, 9/99, 19/07, 27/07, 43/07) by dissociation and cultivation in Dulbeccos's modified Eagle's medium (DMEM; Invitrogen, Karlsruhe, Germany) plus 10% fetal calf serum (FCS; Invitrogen) as previously described (19-21). Human meningioma cell lines (M15, M16, M17, M19, M22) were also generated in our laboratory and cultured in DMEM plus 20% FCS (22). The different cell lines were checked for purity by immunostaining with cell type-specific markers and for the absence of Mycoplasma contamination by staining with bisbenzimide as described (21,22).

Quantitative RT-PCR (qRT-PCR). For qRT-PCR 38 solid human tumor samples (19 meningiomas WHO I, 19 glioblastomas WHO IV) and 18 tumor cell cultures (5 meningiomas, 13 gliomas) were chosen. Total RNA from different samples was purified with TRIzol Reagent (Invitrogen), treated with RNase-free DNase (1 U/μl, Promega, Madison, WI), and reverse transcribed by RevertAid™ H Minus M-MuLV Reverse Transcriptase (200 U/μl, Fermentas, Vilnius, Lithuania) as described before (23-26). Quantitative PCR was performed in triplicate using a total reaction volume of 20 μl, containing 1 μl of 20X Assays-on-Demand™ Gene Expression Assay Mix (SP100 Hs00162109_m1; Applied Biosystems, Foster City, CA), 10 μl of 2X TaqMan Universal PCR Master Mix and 100 ng or 10 ng of cDNA template (diluted in RNase-free water to 9 μl). After 2 min at 50˚C and 10 min at 95˚C, 40 cycles of 15 sec at 95˚C and 1 min at 60˚C were performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs99999905_m1; Applied Biosystems) mRNA was amplified in each sample as internal positive control. Each plate included at least three 'No Template Controls'. The reaction was carried out with the MyiQ™ Single Color real-time PCR Detection System (Bio-Rad, Munich, Germany) and fluorescence data were converted into C_T measurements. Normalized ΔCT values of each sample were calculated as $CT_{\text{gene of interest}}$ - CT GAPDH. $\Delta C_T = 3.33$ corresponds to one order of magnitude. Low ΔC_T values indicate high expression.

Stimulation experiments. To analyze regulation of SP100 mRNA expression, meningioma and glioblastoma cells (2-5x10^5) were stimulated overnight with 10 ng/ml of recombinant human interferon (IFN)α (Peprotech, Hamburg, Germany) or 10 ng/ml IFNγ (Peprotech) dissolved in DMEM containing 0.5% FCS, respectively. As controls, unstimulated cells cultured in 10% (glioblastoma cells) or 20% (meningioma cells) FCS or in 0.5% FCS alone (both) were analysed as well. Following extraction of total RNA, qRT-PCR was performed in triplicate with SP100-specific 20X Assays-on-Demand Gene Expression Assay Mixtures (see above). For stimulation experiments gene expression was calculated with $2^{(\text{normalized CT non-stimulated} - \text{normalized CT stimulated})}$ = n-fold of control.

Protein isolation and Western blotting. For SP100-Western blot experiments tumor samples (50-100 mg) were homogenized in 1 ml ice-cold Tris-Triton-buffer (50 mM Tris-HCl, pH 7.8, 140 mM NaCl, 1% Triton X-100, 2 mM EDTA) plus 10 μl/ml Halt™-Protease Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL, USA) as described (27,28). After centrifugation for 10 min at 4˚C and 10,000 x g, 8 μg of protein were dissolved in SDS-PAGE sample buffer, fractionated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond™-P PVDF membrane, Amersham Biosciences, Buckinghamshire, UK). After blocking with 2% casein/Tris-buffered saline plus 0.1% Tween 20 (TBS-T) for 60 min at RT, the membrane was incubated with the SP100 antibody (1:500; goat polyclonal, sc-16328; Santa Cruz Biotechnology, CA, USA) dissolved in 2% casein/TBS-T at 4˚C overnight. After washing, the membrane was incubated with the secondary antibody (1:100,000; peroxidase-conjugated anti-goat immunoglobulins; Santa Cruz Biotechnology) dissolved in 2% casein/TBS-T for 1 h at RT. Horseradish peroxidase activity was detected with the ECL Advance Western Blotting Detection kit (Amersham Biosciences) and exposure to X-ray film (Hyperfilm™ECL™, Amersham Biosciences). Protein integrity was verified by re-probing the membranes with anti-GAPDH (1:300; goat polyclonal; sc-2020; Santa Cruz Biotechnology) after antibody stripping using Re-Blot Plus Stripping Solution (Chemicon, Temecula, CA) (30 min at RT).

Fluorescence microscopy. Cryosections of solid human glioblastoma and meningioma specimens were fixed in acetone/methanol and blocked with Sudan black as described before (28,29). Specimens were incubated overnight with primary

antibody against SP100 (1:500; goat polyclonal, sc-16328; Santa Cruz Biotechnology) in TBS-T at 4˚C. Primary antibodies were omitted for negative controls. Double immunofluorescence staining: After washing (3X TBS-T, 10 min) the first secondary antibody (donkey anti-goat labeled with Alexa Fluor 488; 1:1,000, Invitrogen) was incubated for 1 h at 37˚C in darkness. The second primary antibodies (for meningiomas: mouse monoclonal epithelial membrane antigen (EMA); 1:20; M0613, Dako, Glostrup, Denmark; for glioblastomas: mouse monoclonal glial fibrillary acidic protein (GFAP); 1:500; MAB3402, Chemicon) were applied and incubated overnight at 4˚C. Second primary antibodies were omitted for negative controls. The slides were washed again (3X TBS-T) and incubated with the second secondary antibody (donkey anti-mouse labeled with Alexa Fluor 555; 1:1,000, Invitrogen) for 1 h at 37˚C. After washing, nuclei were stained with diamidino-2-phenylindole (DAPI) as described previously (29). Sections were embedded in Immu-Mount (Shandon, Pittsburgh) and fluorescence microscopy with digital photography was performed using a Zeiss microscope (Zeiss, Oberkochen, Germany). Single immunofluorescence staining: as secondary antibody donkey anti-goat labeled with Alexa Fluor 488 (1:1,000, Invitrogen) was used. Afterwards, slides were washed, nuclei were stained and specimens were analysed as described above.

Plasmid construction and transfection. pcDNA 3.1 (+) SP100 plasmid construction which allows expression of the full-length SP100 protein in mammalian cells was described before by Yordy *et al* (15). pcDNA 3.1 (+) SP100 was kindly provided by Dr J.S. Yordy and Professor D.K. Watson (Medical University of South Carolina, Charleston, USA) and plasmid identity was verified by sequencing (SEQLAB, Göttingen, Germany). Transfections were performed using glioblastoma cell lines and FuGENE® HD transfection reagent (Roche, Mannheim, Germany) on cells cultivated to 80%-confluency. Transfection was performed according to manufacturer's instruction (Roche). For stable clones, cells were transfected with a mixture of 2 μg pcDNA 3.1 (+) SP100 plasmid or as control plasmid pcDNA 3.1 (+), 8 μl FuGENE® (ration 8:2) and 100 μl Opti-MEM I (Invitrogen). Transfection procedure was performed in DMEM containing 10% FCS, after 6 h medium was changed and cells were cultured for additional 48 h in DMEM plus 10% FCS. After trypsinisation, single clones were selected and expanded by addition of Geneticin (G418 sulfate, Invitrogen; 1,000 μg/ml). Different clones were checked for SP100 expression by qRT-PCR and immunofluorescence staining in comparison to untransfected cells and mock controls. For qRT-PCR, total RNA was isolated with TRIzol Reagent (Invitrogen), DNAse digestion, copy DNA synthesis and SP100 qRT-PCR were performed as described above. Normalized ΔC_T values of each sample were calculated as $CT_{\text{gene of interest}} - CT_{GAPDH}$ and gene expression was calculated with $2^{(\text{normalized CT control - normalized CT mock or SP100})}$ = n-fold of control. For immunofluorescence staining, 20,000 transfected cells grown for three days on poly-D-lysine-coated coverslips were washed with phosphate buffered saline (PBS), fixed for 10 min with ice-cold acetone followed by two rinses in PBS. Then, blocking was performed with 0.1% bovine serum albumin (BSA) and 0.2% glycine in PBS for 60 min at room temperature. Coverslips were incubated with goat polyclonal anti-human SP100 antibody dissolved in PBS at 4˚C overnight (1:500; sc-16328; Santa Cruz). Primary antibodies were omitted for negative controls. Slides were rinsed in PBS and donkey anti-goat labeled with Alexa Fluor 488 (1:1,000, Invitrogen) was used as secondary antibody (incubation 1 h at 37˚C in PBS, in darkness), then slides were washed, nuclei were stained with DAPI and specimens were analysed as described above.

Proliferation assays. For proliferation assays stable SP100-expressing clones, corresponding mock and untransfected controls ($1x10^5$ cells) were grown for 0, 2, 4, 6, and 8 days in 10% FCS-supplemented DMEM, washed with PBS and incubated at -20˚C overnight. After being thawed at room temperature, 250 μl of the CyQuant GR dye/cell-lysis buffer and 5 μl RNase [CyQuant® Cell Proliferation Assay kit (C-7026); Molecular Probes] was added to the cells. Then, lysate was scraped off and added to 125 μl lysis buffer and 250 μl 2X CyQuant GR. The sample fluorescence was measured using a fluorescence microplate reader (CM Genios, Tecan, Crailsheim, Germany) with filters appropriate for 480 nm excitation and 520 nm emission maxima. Results were calculated as DNA content in percentage of individual 0 day results.

Migration assays. For migration assays the QCM™ Chemotaxis 96-well cell migration assay (ECM 510; Chemicon, Temecula, CA, USA) was used. Cells of stable SP100-expressing clones, corresponding mock and untransfected controls were grown to 80% confluence, washed three times with DMEM + 0.5% FCS, and incubated for another 24 h in DMEM + 0.5% FCS. Cells were detached, dissolved in DMEM plus 5% BSA (Sigma, Dreisenhofen, Germany) and $1.5x10^5$ cells were placed in the upper chamber of each migration assay well and incubated for 24 h at 37˚C and 5% CO_2 as described before (30). When migrating into direction of the lower chamber, containing DMEM supplemented with 10% FCS, cells had to pass an 8 μM pore size polycarbonate membrane. Migrated cells on the bottom of the insert membrane were dissociated from the membrane when incubated with Cell Detachment Buffer (Chemicon), lysed and detected by CyQuant GR dye (Chemicon) at 480 nm using a fluorescence microplate reader (CM Genios, Tecan). Cell amounts were calculated with a standard curve, which was constructed by plotting absorbance values against known cell amounts. Migrated cells were displayed in % to untransfected controls.

Statistical analysis. Statistical method of unpaired Student's t-test was used in this study. Significance levels are indicated by $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

Results

Expression of SP100 in human brain tumors. SP100 expression in human brain tumors was analyzed by qRT-PCR (quantitative RT-PCR) of solid and cultured human meningiomas and glioblastomas, respectively. If possible, matched probes of the same tumor sample for solid and cultured materials (indicated by filled symbols in Fig. 1) were investigated. As shown in Fig. 1, in relation to solid meningiomas, mRNA expression

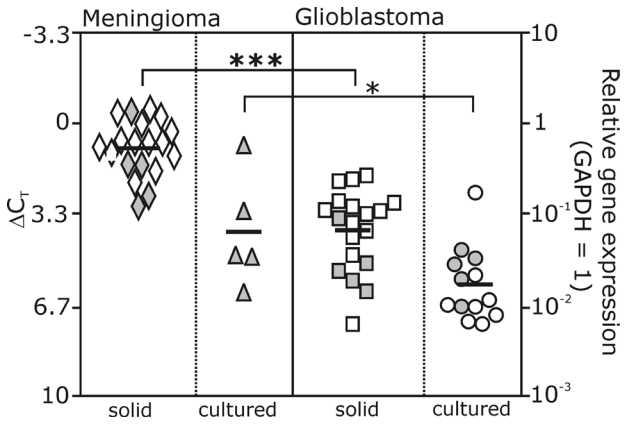

Figure 1. Transcription of SP100 in human solid and cultured meningioma and glioblastoma tissue samples as determined by qRT-PCR. ΔC_T values for GAPDH are displayed [$\Delta C_T = C_T^{(target)} - C_T^{(GAPDH)}$] in logarithmic scale: ΔC_T=3.33 corresponds to one magnitude, low ΔC_T values indicate high expression levels. In solid benign meningiomas (WHO grade I) SP100 mRNA expression was comparably high whereas in solid malignant glioblastomas (WHO grade IV) SP100 expression was about 8-fold lower, which was highly significant ($^{***}p<0.001$). *In vitro* tumor cell cultures showed slightly reduced SP100 transcription. However, SP100 mRNA expression was significantly lower in cultured glioblastoma cells compared to cultured meningioma cells ($^{*}p<0.05$). Grey symbols indicate matched samples of solid tissue and cultivated tumor cells.

levels of SP100 were significantly reduced in solid human glioblastomas [$^{***}p<0.001$ with low ΔC_T (cycle threshold) values indicating high expression and $\Delta C_T = 3.33$ corresponding to one order of magnitude]. When comparing cultured meningioma and glioblastoma cells, it was apparent that in relation to individual solid materials SP100 mRNA expression levels were lower, but nevertheless also a clear reduction of SP100 was measurable in glioblastoma cells ($^{*}p<0.05$). The normalized mean ΔC_T values for SP100 were 0.91±1.03 and 3.92±1.50 in solid meningiomas and glioblastomas, and 3.98±2.10 and 5.91±0.82 in cultured meningioma and glioblastoma cells, respectively.

To confirm the qRT-PCR findings on the protein level, SP100 was detected by Western blotting in solid meningioma and glioblastoma samples. Exemplary results are shown in Fig. 2. While expression of the internal standard GAPDH was very similar between different tumor types, a clear more prominent signal of SP100 protein was detectable in meningioma samples. Moreover, as described, the major isoform SP100A showed a signal at its calculated molecular weight of 54 kDa, and in addition exhibited aberrant electrophoretic motilities with an aberrant molecular weight of 100 kDa in both tumor types (31).

To localize and characterize SP100-expressing cells in solid brain tumor samples *in situ* we performed fluorescence microscopy (Fig. 2). For both tumor types SP100 protein was detectable in the nuclei of the stained cells. Additionally, based on results of double immunofluorescence staining with meningioma/glioblastoma specific cell markers (epithelial membrane antigen (EMA)/glial fibrillary acidic protein (GFAP), respectively), it became obvious that the tumor cells themselves expressed SP100 (Fig. 2, right hand side, inserts). While in glioblastoma samples only few tumor cells were SP100 immunoreactive, in meningioma samples nearly all cells exhibited high SP100 expression (Fig. 2). In summary we showed that both types of tumors express SP100 *in situ* and in *vitro* on both mRNA and protein levels, but in relation to low malignant meningiomas SP100 expression is reduced in high malignant glioblastomas, and here restricted to only part of the tumor cells.

Transcriptional regulation of SP100 in human brain tumor cells. As the expression of SP100 is known to be regulated on the transcriptional level (13), we next examined whether the

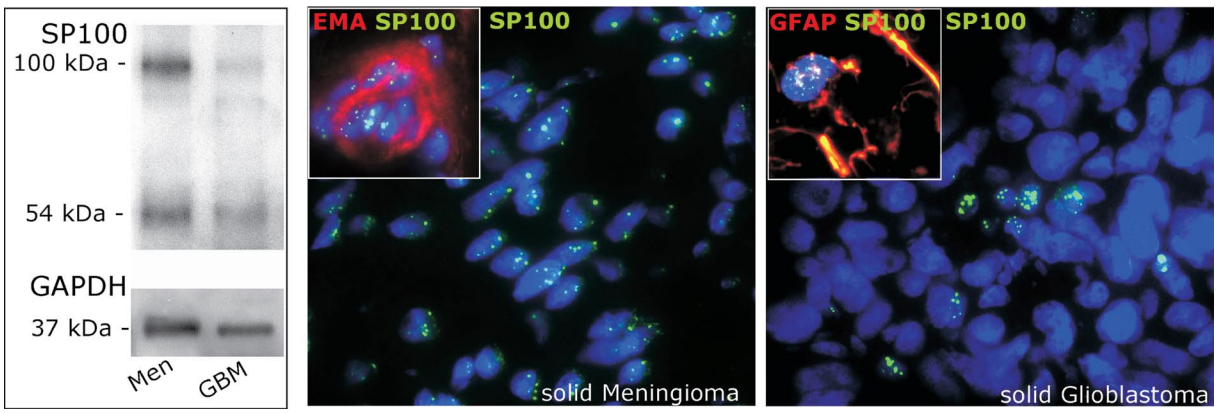

Figure 2. Expression of SP100 in human solid meningioma and glioblastoma samples as shown by Western blotting (left) and fluorescence-immunohistochemistry (right). The Western blot analysis revealed stronger SP100 signals for the meningioma (Men) homogenates compared to glioblastoma (GBM) samples whereas GAPDH as internal control for loading and integrity was almost equal (representative results are shown). Alongside with the band at the predicted molecular weight of 54 kDa a second band was detected at 100 kDa which has been described previously and refers to aberrant electrophoretic mobility. By fluorescence-immunohistochemistry stronger SP100 expression in meningioma samples could be confirmed and SP100 could be localized to the nuclei of tumor marker expressing cells. Thus, in solid meningiomas SP100 (green) was found spot-like in the nuclei of cells stained positive for EMA (epithelial membrane antigen, red), whereas in solid glioblastomas SP100 expression was much more restricted and occurred in the nuclei of few GFAP expressing tumor cells (GFAP, glial fibrillary acidic protein, red). Nuclei were counterstained with DAPI. Examples of different meningioma and glioblastoma samples are shown.

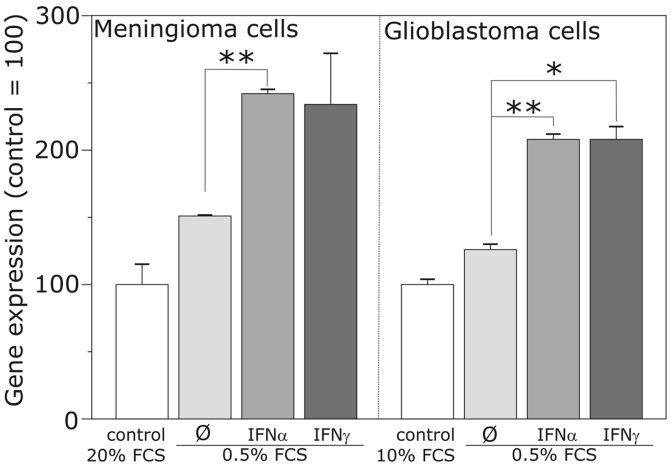

Figure 3. Transcriptional regulation of SP100 in human meningioma (men) and glioblastoma (gbm) cells by interferon (IFN) as determined by qRT-PCR. Cells were incubated in DMEM + 0.5% FCS with or without 10 ng/ml IFNα or IFNγ for 24 h, or in growth medium [DMEM + 20% FCS (men) or 10% FCS (gbm)] prior to RNA isolation and qRT-PCR. ΔC_T values were linearized (linear gene expression = $2^{-\Delta CT}$) and normalized to the respective growth medium control (control = 100). In meningioma cells, IFNα and IFNγ stimulation yielded an induction of SP100 up to 240%. In glioblastoma cells, expression levels were elevated up to about 210%. Reduced serum conditions (DMEM + 0.5% FCS) alone slightly induced the transcription of SP100 to 150% (men) and 125% (gbm). Results from two individual stimulations, significant inductions mediated by IFNα and IFNγ in comparison to serum reduced conditions are marked: $^{*}p<0.05$, $^{**}p<0.01$.

proinflammatory molecules INFα and INFγ would regulate the expression of SP100 in human brain tumor cells. In the cultured human meningioma and glioblastoma cells, both cytokines effectively enhanced the expression of SP100 mRNA (Fig. 3). In relation to unstimulated controls (cultured with 20% fetal calf serum (FCS) and 10% FCS for meningiomas and glioblastomas, respectively; control = 100%) both INFα and INFγ (stimulation with reduced serum conditions) induced SP100 mRNA expression up to 241±6% (INFα) and 238±75% (IFNγ) in meningioma and up to 208±9% (INFα) and 209±19% (IFNγ) in glioblastoma cells, respectively. Interestingly, serum depletion alone (DMEM+ 0.5% FCS) results in an up-regulation of SP100 mRNA of 150±1% and 125±6% for meningioma and glioblastoma cells, respectively. In relation to reduced serum conditions, INFα-triggered induction of SP100 mRNA expression was statistically significant ($^{**}p<0.01$).

Overexpression of SP100 as a tool to study the biological role of SP100 in tumor biology. As a further tool to investigate the biological importance of SP100 in tumor cell biology, we overexpressed SP100 in cultured human glioblastoma cells by stable transfection. In relation to untransfected and mock (transfected with the pcDNA 3.1 (+) without inset) controls different SP100-transfected clones showed a 4- to 6-fold overexpression of SP100 mRNA, measured by qRT-PCR experiments (exemplary results are shown in Fig. 4). In detail, corresponding normalized mean ΔC_T values for SP100 mRNA expression were 5.89 for untransfected cells, 5.83 for mock controls and 3.43/3.93 for SP100-overexpressing clones, respectively. Additionally, immunocytochemistry experiments underlined qRT-PCR results, in relation to untransfected and mock controls in different SP100-overexpressing glioblastoma clones, a marked SP100 positive immunoreactivity was observed on the nuclei of transfected tumor cells (exemplary results are shown in Fig. 4). Summarized, we were able to generate SP100-overexpressing glioblastoma cells, which are a valuable tool for further investigations.

Proliferation and migration potentials of SP100-overexpressing glioblastoma cells. Since SP100 seems to be involved in control of cell growth, differentiation, apoptosis (8,9) and migration/invasion processes (15,16), we analyzed the influence of SP100 overexpression in glioblastoma cells with regard to their proliferative and migratory potentials.

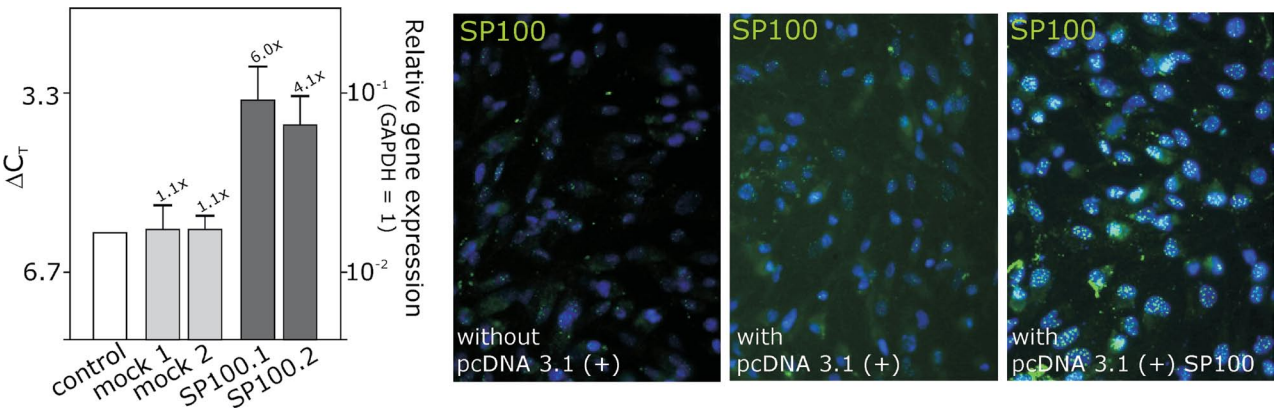

Figure 4. Overexpression of SP100 in glioblastoma cells was confirmed by qRT-PCR (left) and immunocytochemistry (right). Glioblastoma cell cultures were transfected with pcDNA 3.1 (+) with SP100 and pcDNA 3.1 (+) without inset and selected with 1,000 μg/ml G418 to obtain different stable SP100 expressing clones as well as mock controls. Transcription levels of SP100 were analyzed by qRT-PCR yielding up to 6-fold elevated SP100 transcription in different SP100 expressing clones compared to non-transfected cells whereas transcription was not altered in mock controls. Immunostainings of non-transfected, SP100 and mock transfected cells confirmed SP100 expression on protein level with characteristic spot-like stainings in the nuclei that resembled the *in situ* stainings and approved correct expression and localization of the SP100 protein. Exemplary data of different clones are shown, respectively.

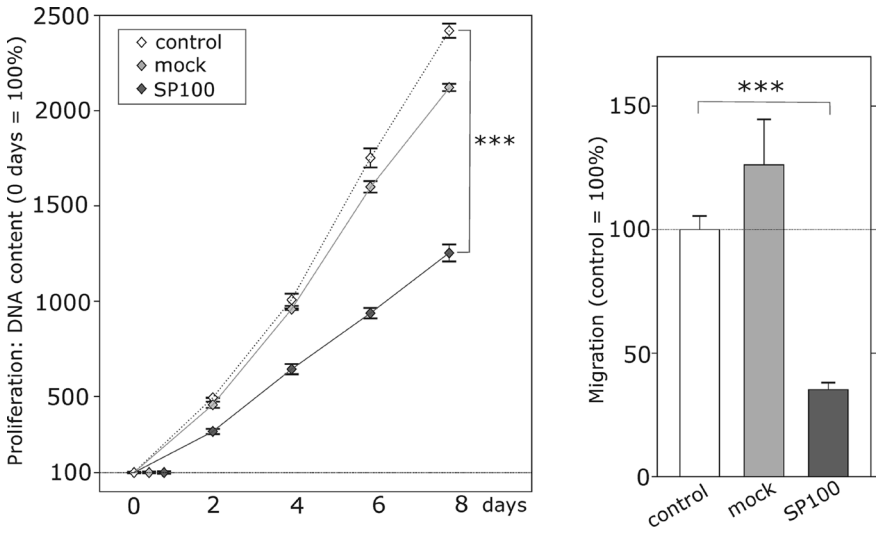

Figure 5. Proliferation (left) and migration (right) of non-transfected glioblastoma cells (control), stable mock and SP100 clones. Proliferation in normal growth medium (DMEM + 10% FCS) was monitored by DNA content measurement and normalized to the respective starting point (day 0 = 100%). Stable SP100 expressing cells grew significantly slower than non-transfected and mock controls (***p<0.001) within the whole time-course reaching just half cell numbers at the endpoint (day 8) compared to controls. Migration potential was analyzed by quantitative DNA-measurement of cells that had migrated from reduced serum conditions through a membrane towards optimum serum concentration. Stable SP100 clones migrated significantly less than non-transfected and mock controls resulting in about 60% less cells at the serum facing side of the membrane compared to controls (***p<0.001). Representative data of different clones are shown, respectively.

With reference to their individual cell number at the starting point (day 0 = 100%), SP100-overexpressing clones showed in relation to untransfected and mock controls over a total time course of 8 days a clearly reduced proliferative potential (***p<0.001 for comparison of untransfected and SP100-overexpressing clones at day 8). Exemplary results are shown in Fig. 5. In detail, for untransfected and mock controls DNA content increased in relation to day 0 up to 2418±75% and 2122±38% at day 8, respectively, whereas SP100-overexpressing clones showed only a DNA content of 1254±90% in relation to their individual controls at day 0 (DNA content = 100%). Interestingly, beside an influence on proliferation SP100 overexpression also yielded a clear reduction of the migratory potential of human glioblastoma cells (Fig. 5). In this case, in relation to untransfected (100±5%) and mock controls (126.2±18.4%), SP100 overexpression resulted in a reduction of migration to 35.2±2.7% in individual glioblastoma clones (***p<0.001 for comparison of untransfected and SP100-overexpressing clones).

In summary, SP100 overexpression influenced biological properties of high malignant glioblastoma cells towards a less malignant phenotype characterized by reduced proliferative and migratory potentials.

Discussion

Promyelocytic leukaemia nuclear bodies (PML NBs) are found within the nucleus of mammalian cells. They are formed from the constituent proteins PML and SP100 and are an obvious feature of the nuclear landscape, although, their functions have still to be unambiguously defined (32), PML NBs appear to be involved in the pathogenesis of human diseases, seem to have physiological roles in the control of cell growth and differentiation, and might be dynamic hubs sensing stress and DNA damage (8,9). To gain more insight into the relevance and functional importance of SP100 for tumor cells in particular in brain tumors, we investigated and compared SP100 expression in human glioblastomas and meningiomas. Although SP100 could be detected in both types of tumors, its expression considerably varied between them; whereas SP100 was constantly and highly expressed in meningiomas *in situ* and *in vitro*, glioblastomas showed a generally lower expression and SP100 was restricted to only a part of the tumor cells.

Despite the importance of nuclear structures for tumorigenesis, little is known about SP100 expression and cancer. A recent study demonstrated a low expression of SP100 in laryngeal cancer, but failed to correlate these findings with malignancy and did not investigate functional implications (33). In breast cancer cells and in HeLa cells SP100 interacts with the transcription factor ETS1 and regulates ETS1 transcriptional activity (14). Thereby, this inhibits the invasion of breast cancer cells (15). A similar interaction could also be demonstrated in non-malignant endothelial cells (16). It has further been shown that SP100 is necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers (34). However, a correlation between SP100 expression and malignancy as done here, but a broader functional analysis of SP100 in cancer cells has not yet been successfully conducted.

In our functional studies, overexpression of SP100 in glioblastoma cells clearly reduced their proliferation and migration. Thus, typical properties of malignant cells are reduced by SP100. Corresponding effects have been reported for MDA-MB-231 breast cancer cells: Here SP100 repressed the transcription of matrix metalloproteinase-1 (MMP1) and inhibited their invasion through Matrigel-coated membranes (15). Thereby, inhibition of migration by IFNα was greater

than that by increased SP100 expression, implying that SP100 contributes to, but does not replace the biological effect of INFα (15). Nevertheless, our results and the investigations of Yordi *et al* (15) are consistent with the observations that IFNs, which strongly induce SP100 expression also in glioma cells, reduce angiogenesis and metastatic spread of certain tumors (35,36). Additionally, since NBs are implicated in apoptosis, specifically they have been shown to be involved in caspase-independent, but also in caspase-dependent mediated processes (37) and recent data suggested that SP100 itself contains a potential caspase recruitment domain in its N-terminal region (38), the NBs with their core proteins PML and SP100 are a interesting tool for analysis of programmed cell death in different tumor cells.

Summarized, since high malignant glioblastomas are characterized by low SP100 expression levels the tumor-suppressive effects of SP100 may account in part for the higher malignancy of glioblastomas as compared to meningiomas. Furthermore, since interferons upregulate SP100 in both types of brain tumor cells, application of these cytokines would be beneficial for their treatment. The functional data presented here, serve as a starting point to identify the function of NBs in high malignant brain tumors, especially for understanding the role of SP100 and PML proteins in these tumor types.

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