The astrocytic cytoskeleton: Unravelling the role of GFAP
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Silencing GFAP isoforms in astrocytoma cells disturbs laminin dependent motility and cell adhesion


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Abstract

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed in astrocytes and neural stem cells. The GFAP gene is alternatively spliced and expression of GFAP is highly regulated during development, upon brain damage, and in neurodegenerative diseases. GFAPα is the canonical splice variant and is expressed in all GFAP-positive cells. In the human brain, the alternatively spliced transcript GFAPδ marks specialized astrocyte populations, such as subpial astrocytes and the neurogenic astrocytes in the human subventricular zone. We here show that shifting the GFAP isoform ratio in favour of GFAPδ in astrocytoma cells, by selectively silencing the canonical isoform GFAPα with short hairpin RNAs, induced a change in integrin, a decrease in plectin, and an increase in expression of the extracellular matrix component laminin. Together, this did not affect cell proliferation, but resulted in a significantly decreased motility of astrocytoma cells. In contrast, a downregulation of all GFAP isoforms led to less cell spreading, increased integrin expression, and a 100-fold difference in the adhesion of astrocytoma cells to laminin. In conclusion, isoform-specific silencing of GFAP revealed distinct roles of a specialized GFAP network in regulating the interaction of astrocytoma cells with the extracellular matrix through laminin.
Introduction

Glial Fibrillary Acidic Protein (GFAP) is a type III intermediate filament (IF) protein, which is widely used as a marker for mature astrocytes. GFAP expression is highly regulated during development and in pathology. Besides in mature gray and white matter astrocytes, GFAP expression is also found in radial glia during development, adult neural stem cells (NSCs), and reactive astrocytes (Middeldorp et al., 2010; Roelofs et al., 2005; Sanai et al., 2004; Sofroniew and Vinters, 2010). IF expression is cell type specific (Chung et al., 2013). For this reason, IF proteins are frequently used to determine the origin of tumors, also in the brain (Ho and Liem, 1996). GFAP marks tumors that originate from astrocytes (Deck et al., 1978; Louis et al., 2007; Rousseau et al., 2006; Rutka et al., 1997; Yung et al., 1985). The level of GFAP expression in these tumors varies between patients (Rebetz et al., 2008) and high grade glioma subtypes can be discerned based on the correlated expression of the IF proteins nestin, vimentin, synemin and GFAP (Skalli et al., 2013). The total level of GFAP expression does not seem to be associated with the tumor malignancy grade, as high GFAP expression has been correlated both to a less malignant (Jacque et al., 1978; Niu, 2011; Velasco et al., 1980) as well as to a more malignant phenotype (Choi et al., 2009; Heo et al., 2012).

The majority of literature on GFAP in astrocytic tumors does not discriminate between different isoforms, as the antibodies and primers used recognize several GFAP isoforms. To date, 10 different isoforms have been detected in the human brain (Kamphuis et al., 2014; Middeldorp and Hol, 2011). The alternatively spliced isoform GFAPδ is highly expressed in specific human astrocyte subpopulations, including the neurogenic astrocytes of the subventricular zone. Thus, GFAPδ can be used as a marker for NSCs of the developing and adult human brain in this niche (van den Berge et al., 2010; Middeldorp and Hol, 2011; Roelofs et al., 2005). As there are indications that cells with NSC characteristics are present in human brain tumors (Vescovi et al., 2006), it is important to take the GFAPδ splice isoform into account when studying glioma. Indeed, we and other have shown that GFAPδ is present in astrocytoma cells (Middeldorp et al., 2009; Perng et al., 2008). In astrocytic tumors, there are indications that the expression levels of GFAPδ correlate with the malignancy grade (Choi et al., 2009; Heo et al., 2012).

In contrast to GFAPα, and due to a different C-terminal tail, GFAPδ by itself is assembly-compromised. It requires additional type III IF expression for proper filament formation (Nielsen and Jørgensen, 2004; Perng et al., 2008; Roelofs et al., 2005). Hereby, the expression levels of the GFAPδ protein are a crucial determinant of proper GFAP network formation.
Changing the GFAP IF network has been shown to affect astrocyte physiology. Expression of GFAPδ at high concentrations induces a collapse of the IF network (Nielsen and Jorgensen, 2004; Perng et al., 2008; Roelofs et al., 2005). Non isoform specific silencing or knockout of GFAP influences astrocyte morphology, proliferation, motility and adhesion (Lepekhin et al., 2001; Rutka and Smith, 1993; Rutka et al., 1994; Weinstein et al., 1991). Up till now the function of specific GFAP isoforms in these processes has not been studied. As GFAPδ is highly expressed in specific astrocyte subpopulations, such as neurogenic astrocytes and astrocytomas, we anticipated that a change in the ratio of GFAPα to GFAPδ, in favor of GFAPδ, leads to functional changes in these cells.

We here demonstrate efficient and specific silencing of GFAPα using isoform-specific short hairpin (sh) RNA, thereby changing the ratio in favor of GFAPδ, which results in an endogenous shift of IF network composition. We analyzed the effect of GFAP isoform knockdown on cell morphology, motility, cell adhesion, and extracellular matrix (ECM) protein expression, astrocyte functions that are intimately linked to IF protein expression. To identify the function of a specialized IF network, we compared the GFAPα specific knockdown to the silencing of all GFAP isoforms.

Material and Methods

Cell culturing

All cells were cultured at 37°C under a humidified 5% CO2 / 95% air atmosphere. HEK-293T cells and the U373 human astrocytoma cell line were cultured in Dulbecco’s modified Eagle medium (DMEM) Glutamax (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotic mixture of 10 U/ml penicillin G and 10 mg/ml streptomycin (Invitrogen). For replating, cells were washed with versene (171 mM NaCl, 5.37 mM Na2HPO4, 3.35 mM KCl, 1.8 mM KH2PO4, 855 μM EDTA), trypsinized (using 0.25% trypsin) and resuspended in FBS containing medium. Cells were either split for maintaining the line (twice per week) or plated for experiments.

shRNA constructs

Lentiviral shRNA expression plasmids from the The RNAi Consortium (TRC) Mission library (Root et al., 2006) were obtained from Sigma-Aldrich. The plasmids express 52 basepair shRNA molecules with 21-nucleotide (nt) mRNA specificity, driven by the ubiquitously active
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U6 snRNA promoter in the pLKO.1 vector backbone. The human GFAP shRNA constructs used were: GFAPα: TRCN0000083733 (5’-cccttcttactcacacacaaa-3’, targeting nt 2674-2694 which is in the 3’ UTR of the NM_002055.4 GFAPα transcript, and pan GFAP: TRCN0000083736 (5’-gcctatagacaggaagcagat-3’, targeting nt 577-597 of NM_002055.4 which is part of exon 2, present in all major GFAP isoforms (Middeldorp and Hol, 2011). The SHC002 non-targeting shRNA construct (NTC; 5’-caacaagatgaagagcaccaa-3’; Sigma-Aldrich), with no homology to human sequences, was used as control shRNA.

**Lentiviral vector production and creation of stable knockdown lines**

Lentiviruses encoding NTC, GFAPα, or pan GFAP shRNA were produced as described before (Naldini et al., 1996a, 1996b) with some alterations. In short, 10x106 HEK 293T cells were plated in a 15-cm culture dish and transfected with a total of 90 μg of the envelope (pMD2.G), packaging (pCMV-dR8.74) and p156RRL plasmid. To this end, the total 90 μg of DNA were mixed with PEI (67.5 ng/μl), and incubated for 15 min at RT, before adding the mix dropwise to the cell culture. The culture medium was replaced 16 h after transfection and the medium containing viral particles was collected 24 h after transfection. Supernatants were ultracentrifuged at 22,000 rpm (rotor SW28, Beckman-Coulter) for 2.5 h. The resulting pellet was resuspended in Phosphate Buffered Saline (PBS) (pH 7.4), aliquoted and stored at -80 °C until further use.

To measure viral titers, a dilution series across five orders of magnitude of the viral stock solutions was made and HEK293T cells were transduced. After 2 days of incubation at 37°C, the number of transduced fluorescent cells at the different viral dilutions was counted and the viral titer was estimated in transducing units (TU) /ml.

U373 cells were plated in 24-well plate with 25,000 cells per well. The next day cells were transduced with lentiviral particles encoding either NTC (negative control), pan GFAP (pan GFAP KD), or GFAPα shRNA (GFAPα KD) with a multiplicity of infection (MOI) of 0.5. Medium was refreshed after 16 hours. Three days after transduction, cell medium was replaced by medium containing 1 ug/ml puromycin (Sigma Aldrich). Cells were grown, split and kept in puromycin-containing medium, to ensure that only transduced cells survive. Puromycin was removed at least 3 days before the functional experiments were performed.

RNA isolation, cDNA synthesis and quantitative real-time PCR

For RNA isolation, cells were harvested and total RNA was isolated with Trisure (Bioline) according to the manufacturer’s protocol. The resulting RNA pellet was dissolved
in RNase-free water. The RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Subsequently, RNA was reverse-transcribed with Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer’s protocol. The cDNA was stored at -20°C for later use in the qPCR reaction. qPCR was performed in 96-well plates, with a final volume of 10 µl/well using the SYBR Green PCR kit (Applied Biosystems). Each reaction volume contained 5 µl of SYBR Green mix, 3.5 µl of H2O, 1 µl of cDNA sample, and 0.5 µl of primer mix (sense and antisense primers, each 2 pmol/µl). The plate was sealed before the qPCR program was started with the following cycling conditions: 2 min at 50°C; 10 min at 95°C; 15 s at 95°C, and 1 min at 60°C for 40 cycles. After the amplification protocol, a dissociation curve was constructed by ramping the temperature from 60 to 90°C.

To correct for differences in cDNA amounts between samples, we normalized the target PCR to the geomean values of PCRs to the reference genes hypoxanthine phosphoribosyltransferase (HPRT), major histocompatibility complex 1 (MHC1), β-actin (ACTB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data was corrected for interexperimental differences.

Table 1: Primers used for qPCR

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>GFAPα</td>
<td>CCCACTCTGCTTTTGACTGAGC</td>
<td>CTTCTTTCCGGCCCTTAGAGGG</td>
</tr>
<tr>
<td>GFAPδ</td>
<td>TCAACCTCGAGATTCCCCGAG</td>
<td>GGGAATGTTGATCCGGTTCT</td>
</tr>
<tr>
<td>GFAPκ</td>
<td>GTCAGTACACGAGGACCTCG</td>
<td>ACGACGCTGCAGTGCACAG</td>
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<tr>
<td>GFAPΔexon 6</td>
<td>TGGCGGCGCAGGATC</td>
<td>CACGGTCTTTCACCACAGGTT</td>
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<td>GFAPΔ135</td>
<td>TCTCGCGCGGCACGGGAGTA</td>
<td>GGGAATGTTGATCCGGTTCT</td>
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<tr>
<td>GFAPΔ164</td>
<td>GAGGCCGCGCGGAAAGATG</td>
<td>CACGGTCTTCACCACAGGTT</td>
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<td>Ki67</td>
<td>AGAGTGCGAAGTGTCTCATGC</td>
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<td>ITGB1</td>
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<td>ITGA2</td>
<td>ACAAGTGTGCCCCGAGCAC</td>
<td>TAGCCACGCAAATCCAAAAG</td>
</tr>
<tr>
<td>ITGA3</td>
<td>GGGCGCAGCAGCTAATGGAGA</td>
<td>GAGCGAGTCATCCTGCTGTT</td>
</tr>
<tr>
<td>ITGA6</td>
<td>CTATTTTGAGATCCGCCGCTG</td>
<td>TGGGCGGAGGTCAATTCTGT</td>
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<tr>
<td>ITGA7</td>
<td>GGGTGCTGCCAAACCACCTC</td>
<td>TGCCGAGGAGGGATGCC</td>
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<tr>
<td>LAMA1</td>
<td>GTTTCGAAACTCTCGCAGA</td>
<td>CTGCCAGCACCATTGTGAGC</td>
</tr>
<tr>
<td>MHC I</td>
<td>CCACTGCCACACCTCTGACTTCA</td>
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<td>GAPDH</td>
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<tr>
<td>HPRT</td>
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<td>ATGTAATCCAGGAGTCCAGC</td>
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<tr>
<td>Beta Actin</td>
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<td>CACCCGCCGAGGCTCAG</td>
</tr>
<tr>
<td>Plectin</td>
<td>AGATCGAGCGCGCCAGGAGT</td>
<td>TGGCGGAGGTCAATTCTG</td>
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as described in Ruijter et al. 2006 (Ruijter et al., 2006). For specific knockdown samples, significance was tested using a Mann-Whitney test. All other qPCR data was tested using a Kruskal-Wallis with a Dunn’s post hoc test. The primers used are listed in Table 1.

**Immunocytochemistry**

Cells were incubated in SuMi buffer (50 mM Tris, 150 mM NaCl, 0.25 % gelatine and 0.5% Triton X-100, pH 7.4) for 10 min permeabilizing cells and blocking aspecific binding sites. Cells were incubated with primary antibodies diluted in SuMi as stated in Table 2 overnight at 4°C. Cells were washed 3 times in Phosphate Buffered Saline (PBS) before incubated in secondary antibodies diluted in SuMi together with the nuclear dye Hoechst (Sigma). Cells were washed and either stored in PBS or embedded in Mowiol (100 mM Tris Buffered Saline (TBS) pH 8.5, 25% Glycerol, 10% Mowiol) and stored at 4°C. All secondary antibodies were from Jackson Immune Research and diluted 1:1400 in SuMi. Micrographs were taken with a Leica (Leica microsystems) epifluorescent microscope with a 20x objective or with a Leica SP5 confocal with a 63x objective.

**Protein measurements and western blots**

Cells were washed with versene before being scraped with a cell scraper into 100 µl of cold lysis buffer consisting of suspension buffer (100 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM Ethylenediaminetetraacetic acid (EDTA)) with 1% Triton-x100 and added protease inhibitors (100 µg/ml phenylmethanesulfonylfluoride (PMSF, Roche Diagnostics) and 0.5 µg/ml Leupeptin (Roche Diagnostics). Cells were vortexed and incubated on ice for 5 min before centrifugation at 11.7 x g for 1 min. The supernatant was taken off and stored at -20°C in a fresh tube. Protein concentrations were measured using a BCA kit (Pierce, Thermo Scientific) according to manufacturer’s descriptions. Proteins were mixed with 2X loading buffer (2X:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Cat #</th>
</tr>
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<tbody>
<tr>
<td>panGFAP</td>
<td>Dako</td>
<td>1:4000 (1:8000 WB)</td>
<td>Z0334</td>
</tr>
<tr>
<td>hGFAPδ</td>
<td>Manufactured in house (10-05-2001 Bleed)</td>
<td>1:1000 (1:1300 WB)</td>
<td>-</td>
</tr>
<tr>
<td>hGFAPα</td>
<td>Santa Cruz biotechnology inc.</td>
<td>1:1300 WB</td>
<td>sc-6170</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Chemicon</td>
<td>1:5000</td>
<td>AB5733</td>
</tr>
<tr>
<td>Plectin</td>
<td>G. Wiche, Univ. Vienna, Austria</td>
<td>1:5000 WB</td>
<td>Ab#9 (Andrà et al., 2003) we identified plectin 1a (&gt; 500 kDa)</td>
</tr>
</tbody>
</table>
100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 200 mM dithiothreitol and bromophenol blue) and boiled for 5 min at 95°C before loading on a 7.5% or 10% SDS-PAGE reducing gel. After electrophoresis, proteins were blotted on Whatman Protran membranes (GE Healthcare) using a semi-dry Trans-Blot system (Biorad) for 1 hour. Blots were incubated with SuMi for 10 min before incubation overnight at 4°C with primary antibodies. Blots were subsequently washed in TBS-T (100 mM Tris-Hcl pH 7.4, 150 mM NaCl with 0.2% Tween-20) 3 times before secondary antibodies, diluted in SuMi, were incubated for 1 hour at room temperature (RT). Blots were washed again 3 times in TBS-T before scanned with an Odyssey scanner (LI-COR). Primary antibodies used are listed in Table 2. Secondary antibodies used are IRdye 800 (1:2000) (LI-COR, NE, USA) and Dyelight Cy5 (1:4000) (Jackson Immune Research).

**Motility assays**

Cells were plated on glass dishes coated with PLL (20 μg/ml) or laminin (10 μg/ml). PLL and laminin coatings were incubated at 37°C for 1 h. PLL-coated dishes were washed in versene and air dried before cells were seeded, while laminin coating was not washed or dried. Cells were allowed to adhere for 24 hours before cells were tracked overnight. Every 10 min a picture was taken with a Zeiss Axiovert 2000 (Zeiss microscopy). Cells were kept at 37°C and 5% CO2 in a pre-heated and humidified incubation chamber (OKO labs) during the imaging. Images were compiled into a timelapse sequence. For motility analysis, 25 cells were followed per condition in 6 independent experiments resulting in a total of 150 cells. Cell soma movement was manually tracked using a manual tracking Image J plugin (W.S. Rasband, NIH, Bethesda, MD, version 1.46f, http://imagej.nih.gov/ij/). The average velocity per cell was calculated and then averaged for all cells in one condition per experiment. Data was corrected for interexperimental differences as described in Ruijter et al. 2006 (Ruijter et al., 2006). The difference in cell velocity was tested using a non parametric Kruskal-Wallis test with a Dunn’s post hoc test.

**Cell Cycle analysis**

For cell cycle analysis, cells were washed with versene, trypsinized, and fixed in ice cold 70% ethanol for 30 minutes. After washing twice with PBS + 1% BSA, cells were incubated with RNAsse (Boehringer, 0.5 mg/ml) for 15 minutes to enzymatically remove RNA, which is necessary for specific staining of nuclear DNA. Subsequently, cells were washed with PBS + 1% BSA, stained with propidium iodide (PI, Sigma, 50 mg/ml) for 15 minutes, and washed with PBS + 1% BSA. Flow cytometry was performed on a FACS Canto II flow cytometer.
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(BD Biosciences). Data analysis was done using the FlowJo software (Tree Star, http://www.treestar.com).

Adhesion assays

Glass coverslips were coated with PLL or laminin for 1 h at 37°C. PLL coating was washed once with sterile water and air dried. Coatings were blocked with 1% BSA solution for 1 h at 37°C. Cells were trypsinized, resuspended in medium containing serum to inhibit the trypsin, counted and replated in serum-free medium with 30,000 cells per well. At stated time points, cells were gently washed with versene three times, subsequently fixed with 4% PFA for 10 min, and stored in PBS. Cells were stained with Hoechst (diluted 1:1000 in SuMi) for 1 hour, washed in PBS once, after which phase contrast and fluorescent pictures were taken using a Zeiss Axiovert 2000.

Cell morphology measurements

Cells plated for single cell motility assays were used for morphology measurements. The perimeter was drawn manually from phase contrast pictures using ImageJ. The area, perimeter and form factor was calculated by ImageJ. Form factor was calculated with the following formula: . Form factor is used as a measure for cell morphology where perfect round cells will have a form factor of 1. The perimeter of at least 30 cells per condition was measured for 6 independent experiments. Data was corrected for interexperimental differences as described in Ruijter et al. 2006 (Ruijter et al., 2006). Differences were tested for significance using a non parametric Kruskal-Wallis test with a Dunn's post hoc test.

Statistics

All statistical tests were performed using Graphpad Prism 6 (version 6.02) (Graphpad Software Inc.). Differences were considered significant when p<0.05 at a 95% confidence interval.

Results

Selection of target sequences for isoform-specific knockdown of GFAP

To change GFAP network composition in astrocytomas, we aimed at silencing the expression of GFAP using RNAi-based gene silencing. Based on the sequence information of different GFAP splice-variants (Middeldorp and Hol, 2011), specific target regions in the
GFAP transcript were selected. For an isoform-specific knockdown, we focused on silencing the canonical isoform GFAPα, which will decrease the GFAPα/δ ratio. Shifting the ratio in favor of GFAPδ expression mimics the IF network in more malignant forms of astrocytoma cells or neurogenic astrocytes (Choi et al., 2009; Heo et al., 2012; Roelofs et al., 2005) and allows for the investigation of such a specialized GFAP network. To silence GFAPα, we targeted the 3’ UTR sequence, which is shared by minor GFAP isoforms such as GFAPΔexon 6, Δ164 and Δ135, but not by the more abundant isoforms GFAPδ or κ. mRNA levels of Δexon 6, Δ164 and Δ135 are low in human brain (Hol et al., 2003; Kamphuis et al., 2014; Middeldorp et al., 2009). Analysis of GFAP mRNA expression levels in human astrocytoma cell lines revealed that GFAP Δ164 mRNA was undetectable in U373 astrocytoma cells using specific primers (Fig 1A). Δ135 and Δexon 6 transcripts were present, but their expression levels were significantly lower than that of GFAPα (Fig 1A). For this reason, we selected the 3’ UTR as target sequence, which in astrocytoma cell lines will mainly reflect the modulation of GFAPα, as Δ135 is approximately 100x, and Δexon 6 is 1000x times lower expressed. In addition to targeting GFAPα, we selected a sequence to silence all GFAP isoforms. Exon 2 is a

![Fig. 1. Selection of target sequences for isoform-specific knockdown of GFAP. A) Expression of human GFAP isoforms in the U373 astrocytoma cell line. B) Table depicting shRNA candidates targeting human GFAP isoforms. C) Schematic representation of the GFAP transcript. The red bars indicate the target sites of the two shRNA candidates. Targeting exon 2 silences all GFAP transcripts (pan GFAP KD), whereas targeting the 3’UTR encoded in exon 9 mainly downregulates GFAPα (GFAPα KD). Graph bars show mean with standard error of the mean (SEM), n=4.](image-url)
constitutive exon, present in all isoforms, and was therefore chosen as target (Fig 1B and 1C).

We obtained lentiviral shRNA expression constructs from the TRC shRNA library (Root et al., 2006) encoding either a shRNA targeting the 3’ UTR or exon 2 of the GFAP transcript and lentiviral particles were produced.

**Validation of an isoform specific knockdown of GFAP**

U373 astrocytoma cells were transduced with a non-targeting control shRNA (NTC), an shRNA targeting pan GFAP (pan GFAP KD) or GFAPα (GFAPα KD). Transduced cells were selected by puromycin to ensure a stable knockdown of GFAP. Efficiency and specificity of the knockdown was validated by analyzing GFAPα and GFAPδ mRNA expression. As expected, GFAPα was significantly downregulated in both the pan GFAP KD (~73%) and GFAPα KD cell lines (~58%) (Fig. 2A). As expected, GFAPδ transcript expression was also downregulated in pan GFAP KD cells (~36%). Intriguingly, in cells with a specific knockdown of GFAPα, expression of GFAPδ was significantly upregulated (~600%) (Fig. 2B). Expression levels of GFAPκ and GFAPΔ135 were also upregulated in GFAPαKD cells but since expression levels are much lower compared to GFAPδ expression (10 fold and 86 fold respectively) the contribution of GFAPδ is much more substantial (Supplementary Fig.1). GFAPΔexon 6 levels were not significantly changed (Supplementary Fig.1) and GFAPΔ164 levels were too low to detect (data not shown).

We confirmed the GFAPα and GFAPδ expression data at the protein level using both western blot and immunocytochemistry analysis. Knockdown of GFAP for 30 days or longer resulted in silencing of GFAPα expression as confirmed by a pan GFAP and a specific GFAPα antibody, respectively (Fig. 2C). Consistent with the effect on the transcript level, the pan GFAP KD reduces GFAPδ expression, whereas a GFAPα-specific knockdown resulted in an increased expression of GFAPδ (Fig. 2C).

Previously, it has been demonstrated that GFAPδ overexpression induces aggregation of the GFAP filament network (Nielsen and Jorgensen, 2004; Perng et al., 2008; Roelofs et al., 2005). We therefore investigated whether the increase of endogenous GFAPδ, induced by knockdown of GFAPα, resulted in a similar re-organization of the IF network. To this end, U373 cells were stained for pan GFAP or GFAPδ. Surprisingly, no aggregation of the GFAP network was observed. GFAPα KD cells showed a GFAP network, which is present throughout the whole cytoplasm similar as in control cells (Fig. 2D left panels). In addition, the distribution as well as the expression levels of the type III IF protein vimentin remained unchanged (Fig.
Taken together, we here demonstrated efficient and specific silencing of GFAPα or pan GFAP in human astrocytoma cells. Intriguingly, isoform-specific knockdown of GFAPα induced GFAPδ mRNA and protein expression, indicating a compensatory upregulation of the non-targeted isoform. In contrast to transgene-mediated overexpression, endogenous upregulation of GFAPδ in the presence of a GFAPα knockdown did not result in an aggregation of the GFAP network.
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GFAP silencing has no effect on cell proliferation

First, we investigated the proliferative capacity of pan GFAP KD and GFAPα KD cells in comparison to control cells. We studied the expression levels of Ki67, a proliferation marker present in all cell cycle phases except G0. No differences in Ki67 expression levels were found in pan GFAP KD and GFAPα KD cells in comparison to control cells (Fig. 3A). To assess whether silencing of GFAP is associated with changes in cell cycle progression of U373 cells, we performed cell cycle analysis using flow cytometry. Neither silencing of pan GFAP nor of GFAPα significantly altered the percentage of cells in the G0/G1, S, or G2/M phase (Fig. 3B). Taken together, two independent assays revealed no significant effect of isoform-specific or pan GFAP silencing on cell proliferation.

Pan GFAP, but not GFAPα, silencing alters cell morphology dependent on the presence of laminin

Modification of the IF network was previously associated with changes in astrocyte morphology (Lepekhin et al., 2001; Weinstein et al., 1991). Confirming a regulatory role for GFAP on cell morphology, we first determined whether downregulation of pan GFAP expression changed cell morphology in human astrocytoma cells on a standard PLL substrate (Fig. 4A upper row). To this end, cells were cultured on PLL and the cell area and perimeter were measured. From these parameters the form factor was calculated, which is a measure of cell morphology (Lepekhin et al., 2001). Cells with a stable knockdown of all GFAP isoforms...
Fig. 4. Silencing of pan GFAP, but not GFAPα alters morphology dependent on the presence of laminin. A) Phase contrast pictures of U373 cells with different GFAP network compositions on PLL (upper panel) or laminin (lower panel) coated coverslips. Scale bars represent 100 μm. B) Pan GFAP KD cells showed a significant difference in area compared to GFAPα KD cells (p= 0.006) on PLL. The perimeter of pan GFAP KD cells was significantly lower compared to both the control and GFAPα KD cells (p=0.005). The form factor, which is a measure of the shape of the cell, was not altered between conditions on PLL. C) When cells were plated on laminin coated coverslips the area of the pan GFAP KD cells was still lower compared to control and GFAPα KD (p=0.003). The change in perimeter seen on PLL was absent on a laminin substrate indicating that the cells were spread out more on laminin. This was also reflected in the reduction in the form factor between control cells and pan GFAP KD cells (p=0.007). Box plots show median with 25 and 75 percentiles. Whiskers go from minimum to maximum value.
Silencing GFAP isoforms in astrocytoma cells (pan GFAP KD) showed a trend in the reduction of the area (Fig. 4B, left panel) and a significant reduction in cell perimeter (middle panel). However, the shape of the pan GFAP KD cells (form factor in right panel) was not significantly different compared to control. Hence, we conclude that pan GFAP KD cells display an unchanged general shape but were less spread out on PLL compared to control cells. GFAPα KD cells displayed a significantly different area and perimeter compared to pan GFAP KD cells with no differences in comparison to control cells (Fig. 4B).

Next, we studied whether these effects on morphology also occurred when the cells were cultured on laminin, a natural ECM component that is highly abundant in the brain (Colognato et al., 2005; Franco and Müller, 2011). We observed that the cell morphology, seen on laminin (Fig. 4A lower row), differed from cells on PLL (Fig. 4A top row). The pan GFAP KD cells still displayed a smaller area on laminin (Fig. 4C left panel), but the perimeter was no longer different from control (middle panel). This resulted in a significant change in the form factor, reflecting that cells displayed a less round morphology on laminin (Fig. 4C).

In conclusion, our data revealed that silencing of pan GFAP expression reduces the cell perimeter of human astrocytomas, and this effect is abolished when the cells are cultured on laminin. In contrast, cells displayed no changes in cell morphology upon a specific GFAPα knockdown, a situation in which there is an upregulation of GFAPδ expression (Fig. 2B), indicating that the presence of GFAPδ might compensate for the lack of GFAPα.

**Pan GFAP knockdown leads to higher cell adhesion to laminin and enhanced integrin expression**

Cell morphology is in part influenced by cell adhesion, mediated through interactions between integrins and the ECM. To assess whether the differences we observed in cell morphology are due to differences in cell adhesion, we performed adhesion assays. Cells were plated on PLL- or laminin-coated glass coverslips, washed and fixed after 0.5 and 3 hours. The number of cells that adhered to the coverslips was quantified by counting the Hoechst stained nuclei. Pan GFAP KD cells adhered slightly better to PLL than control cells (Fig. 5A). This difference was dramatically increased on laminin-coated coverslips (Fig. 5B). These data show that cells with a pan GFAP knockdown significantly increased their ability to bind to laminin, which could explain that the cells are less round when plated on laminin (Fig. 4A).

Cell adhesion molecules, such as integrins, are main factors in controlling cell shape and adhesion (Lauffenburger and Horwitz, 1996). Since pan GFAP KD cells required the
presence of laminin to elongate and showed a significantly enhanced adherence to laminin, the expression of laminin-associated integrins was investigated. Integrins need both α and β subunits to form a stable dimer (Hynes, 2002). We investigated the integrin subunits β1 and β4, as well as α2, α3, α6, and α7. These integrin subunits hetero-dimerize to form different

Fig. 5. The expression profile of laminin associated integrins correlates with adhesion properties of GFAP KD cells. A) Adhesion of pan GFAP KD cells was significantly higher on PLL after 0.5h (p=0.01). B) A marked increase in adhesion was observed in the pan GFAP KD in comparison to GFAPα KD cells on laminin after 0.5h (p=0.003) and 3h (p=0.01). C) Integrin α and β subunits together form a dimer. A schematic representation is shown of the integrin subunits that can interact with laminin. D-I) qPCR data showing integrin expression profiles. β1 integrin expression was significantly downregulated in GFAPα KD cells compared to pan GFAP KD or control cells (p=0.003). β4 showed a trend for an upregulation in pan GFAP KD cells compared to GFAPα KD cells. The same pattern of expression between pan GFAP KD cells and GFAPα KD cells was seen for α2 (p=0.01), α6 (p=0.0003) and α7 (p=0.0003). Data were normalized to reference genes HPRT, MHC I, beta actin, and GAPDH and are presented as mean + SEM (n=6), * p < 0.05.
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Integrin receptors that bind laminin (Fig. 5C). We observed a clear pattern of integrin expression: the pan GFAP KD cells had higher expression integrin expression compared to control or GFAPα KD cells. For integrins β1, α2, α6, and α7, these differences were significant (Fig. 5D-I).

Taken together, knockdown of pan GFAP enhanced the expression of integrins associated with binding to the ECM substrate laminin. Consistently, adhesion to laminin was increased in pan GFAP cells in comparison to control or GFAPα KD cells, and this might underlie the
fact that the cells are less round on a laminin substrate. The adhesion capacity of GFAPα KD cells remained unchanged.

**Specific silencing of GFAPα downregulates integrin expression, reduces cell motility, and promotes the expression of laminin**

GFAPα KD cells showed a marked downregulation of the laminin-binding β1 integrin compared to controls (Fig. 5D). Since reduced β1 integrin expression did not lead to a significant decrease in cell adhesion to laminin (Fig. 5B), we investigated whether cell migration was altered. The presence of an IF network as well as the β1 integrin have been linked to cell motility in earlier studies (Lepekhin et al., 2001; Nishio et al., 2005; Rooprai et al., 1999; Rutka et al., 1994). Cells with GFAP isoform knockdown were plated on laminin coated glass and imaged overnight. The cell nucleus was manually tracked through every frame and the average velocity was calculated over all frames. We observed significant lower motility in GFAPα KD cells in comparison to control cells. In cells with a pan GFAP knockdown, motility was not significantly changed (Fig. 6A). We also assessed motility on PLL and we observed that the reduction of cell motility in GFAPα KD cells was not as pronounced on PLL as on laminin. The pan GFAP KD cells did not migrate significantly slower than control cells (Fig. 6B). IFs connect with integrins via linker proteins. Plectin is a linker protein that can bind to integrin subunits as well as IFs (Steinböck and Wiche, 1999; Wiche and Winter, 2011). To relate the differences seen in migration of GFAPα KD cells to IF networks through integrins, we assessed differences in plectin expression. qPCR data showed that plectin transcript levels are indeed significantly downregulated in the GFAPα KD cells only (Fig. 6C). Protein quantification confirmed a downregulation of plectin in GFAPα KD cells (Fig. 6E).

Astrocytes are the main producers of ECM proteins in the brain. GFAPα KD cells showed a decrease in laminin-specific integrin expression, but these cells not only migrated slower on laminin-, but also on PLL-coated glass. We therefore investigated whether the cells themselves produce laminin to create their own in vitro ECM. To this end, we measured the mRNA expression of LAMA1, an α subunit of laminin. No changes in the expression of the LAMA1 transcripts were observed in pan GFAP KD cells in comparison to control cells. Instead, we observed a significant upregulation of the LAMA1 transcript in GFAPα KD cells (Fig. 6D).

In conclusion, our results showed that the GFAPα KD cells, still expressing GFAPδ, displayed lower cell motility. This was associated with an increase in laminin expression and a decrease in plectin and integrin expression.
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Discussion

We here present that RNAi-mediated silencing of a distinct GFAP isoform in human astrocytoma cells results in changes in cell motility, integrin, plectin, and laminin expression. Efficient and specific silencing of GFAPα, using an isoform-specific shRNA, decreased the endogenous GFAPα/δ ratio in favor of GFAPδ expression, an effect which was enhanced by upregulation of endogenous GFAPδ by the cell itself. Modulation of the GFAP isoform ratio revealed that upon a specialization of the GFAP network, the expressed of the ECM protein laminin is increased while cell motility is decreased. In contrast, silencing of pan GFAP expression influenced cell morphology and cell adhesion. This comparative approach revealed that different IF network compositions influence distinct cellular functions and possibly react to ECM stimuli differently.

GFAP filament assembly

Isoform-specific modulation is essential to understand the functional consequences of alternative splicing of GFAP and its regulation in specific astrocyte subtypes. The alternatively spliced isoform GFAPδ is highly expressed in neurogenic astrocytes (van den Berge et al., 2010; Roelofs et al., 2005), and its expression level is a crucial determinant of the GFAP IF network assembly (Nielsen and Jorgensen, 2004; Perng et al., 2008).

We here demonstrate that decreasing GFAPα expression levels induced an endogenous upregulation of GFAPδ, which might represent an intrinsic regulatory mechanism to compensate for the loss of GFAPα. However, the total amount of GFAP expression remained low in the cells. An induction of GFAPδ expression in cells with low GFAPα expression did not influence the distribution of the IF network. We previously reported on a collapse of the IF network upon transgenic GFAPδ overexpression (Perng et al., 2008; Roelofs et al., 2005). However as shown here, an endogenous, and more subtle, upregulation of GFAPδ expression in the presence of GFAPα silencing did not result in an IF network collapse. The endogenous induction of GFAPδ represents a 6.4-fold increase on mRNA level whereas CMV promoter transgene expression induces a dramatic overexpression (Perng et al., 2008). A collapse of the IF network, due to GFAPδ expression, is concentration-dependent (Kamphuis et al., 2012; Nielsen and Jorgensen, 2004; Perng et al., 2008). Thus, we can conclude that the endogenous upregulation due to GFAPα knockdown is not high enough to cause a collapse of the network. The presence of the endogenous IF proteins vimentin (Fig. 2D) and nestin (not shown) ensures proper incorporation of GFAPδ in the IF network. In a physiological situation in the brain,
GFAPδ expressing cells have not been shown to display a collapsed GFAP network. Hence, modulation of the ratio of GFAP isoforms presented here might reflect more physiological expression levels compared to the overexpression studies.

**IF and cell morphology**

The induction of GFAPδ upon knockdown of GFAPα might be sufficient to maintain the morphological characteristics of astrocytoma cells, since GFAPα knockdown cells demonstrate none of the morphological changes present in cells with a pan GFAP knockdown. The ability of the GFAPδ isoform to take over the function of GFAPα suggests that the role of GFAP in cell morphology does not depend on these specific isoforms but is rather determined by the presence of GFAP filaments within the IF network.

In agreement with our findings in pan GFAP KD cells, knockout of all GFAP isoforms was shown to alter cell morphology (Lepekhin et al., 2001). Taken together our data highlight that a drastic re-organization of the IF network either by knockout or knockdown of all GFAP isoforms is needed to alter the morphology of astrocytoma cells.

**Integrin expression and cell motility**

In contrast to normal cell morphology, cells expressing a low GFAPα/δ ratio demonstrated significantly reduced cell motility. Changes in cell motility were observed before in astrocytes with a pan GFAP knockdown or in GFAP knockout cells (Lepekhin et al., 2001; Rutka et al., 1994). We also see a clear but not significant trend for lower motility in cells with a pan GFAP KD on laminin. The significantly lower motility in GFAPα KD cells on laminin and PLL implicates that the presence of GFAPδ cannot compensate for the lack of GFAPα and actually exaggerates the effect on motility in comparison to pan GFAP KD cells. A possible explanation for the reduced motility in GFAPα KD cells is the significant downregulation of integrins β1 and α6. The β1 integrin is localized to the leading edge of migrating astrocytes (Ogier et al., 2006), and blocking the β1 integrin receptor has been shown to inhibit astrocyte migration (Nishio et al., 2005). Moreover in gliomas, blocking integrin β1 inhibited cell migration on laminin as well as invasion into matrigel (Rooprai et al., 1999). Similarly, the α6 integrin subunit has been shown to increase migration in glioma cell lines. Although α6 can form a dimer with both β1 and β4, migration could be inhibited with antibodies against β1, but not with antibodies against β4 (Delamarre et al., 2009). The hypothesis that cell motility is decreased in GFAPα KD cells due to altered laminin binding integrins is strengthened by the higher motility observed on a PLL substrate compared to the laminin substrate. The reason
that we do not observe a full recovery of motility on PLL, could be due to increased secretion of laminin by GFAPα KD cells. Reduced plectin expression in GFAPα KD cells supports an involvement of integrins in the reduced cell motility, since plectin links the IF network to the integrins. Plectin expression itself has been linked to cell motility as well (Katada et al., 2012). Altering the GFAP network therefore influences the expression of multiple proteins involved in the link between the ECM and IFs. Finally, the hypothesis that the decreased ability of GFAPα KD cells to migrate on laminin is integrin-dependent is corroborated by the observed decrease in adhesion of GFAPα KD cells to a laminin substrate. We show that altering the GFAP isoform expression changes the interaction of U373 cells with the ECM. GFAP expression has been used for glioma diagnostics and GFAPδ expression has been linked to higher malignancy (Choi et al., 2009; Heo et al., 2012). However, it is still elusive whether and via which cellular mechanisms GFAPδ expression can influence tumor aggressiveness. The interaction of tumor cells with their ECM has significant consequences for invasion and tumor growth, and indeed glioma cells preferably migrate along blood vessels, which contain laminin in their basal membranes (Farin et al., 2006; Goldbrunner et al., 1999). Our findings that the presence of GFAPδ in cells with low GFAPα expression alters the interaction of astrocytoma cells with laminin, suggests an important function in glioma invasiveness. Our data show that it is important to include GFAP isoform expression, especially that of GFAPδ in relation to other IF proteins, in glioma diagnostics and classification.

**Laminin expression**

Interestingly, specifically GFAPα KD cells showed increased expression of the ECM component laminin itself. In contrast, downregulation of GFAP in pan GFAP KD cells did not have such an effect on laminin. Consequently, the increased laminin expression in the GFAPα KD cells appears to result from the simultaneous loss of GFAPα and an increase in GFAPδ expression, resulting in a specialized IF network composition. Laminin expression has been linked to GFAP expression before. Laminin expression is increased in GFAP knockout mice (Menet et al., 2001). On the other hand, GFAP overexpression in fibroblasts has also been shown to increase laminin production (Toda et al., 1994). This finding is in agreement with an upregulation of laminin production in reactive astrocytes, which are characterized by an increase in GFAP expression (Palu and Liesi, 2002). In epithelial cells, laminin-332 expression was shown to be mediated by transforming growth factor β (TGF-β) through integrin signaling (Moyano et al., 2010). We observe changes in integrin β1 expression in GFAPα KD cells. Integrin β1 knockout mice decrease laminin 1 secretion (Aumailley et al., 2000)
and migrating astrocytes upregulate β1 integrin expression together with GFAP and vimentin (Nishio et al., 2005). The decoupling of vimentin from focal adhesions (FA) attenuated FA downstream signaling (Gregor et al., 2013). These data suggests that there are regulatory signals between intermediate filaments, integrin expression and ECM component secretion. The laminin expression we observe in GFAPα KD cells could therefore be regulated by altered integrin signaling due to changes in the IF network. Whether the increase in LAMA1 expression is a reaction to changes in integrin expression or directly driven by changes in GFAP isoform expression requires further investigation.

In conclusion, we here demonstrated efficient and isoform-specific silencing of GFAPα, which resulted in increased GFAPδ and LAMA1 expression, decreased plectin and integrin β1 expression, and a reduced motility of astrocytoma cells. In contrast, pan GFAP knockdown changed cell morphology, increased integrin expression, and altered adhesion of astrocytes. Taken together, these data emphasize that astrocyte morphology and motility are associated to GFAP protein expression. Moreover, the precise GFAP isoform composition of the IF network is intimately linked to integrin, plectin and laminin expression. Altering IF network composition in astrocytoma cells influences important determinants of tumor invasiveness such as cell migration and adhesion, and thus presents a potential target for diminishing tumor infiltration.

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Supplementary Figures

Supplementary Fig. 1 Regulation of GFAP isoforms after knockdown. qPCR data showed a significant upregulation of A) GFAPκ and B) GFAPΔ135 transcripts in GFAPα KD cells compared to control cells (NTC). C) Expression levels of GFAPΔ Exon 6 remained unchanged. All data were normalized to reference genes HPRT, MHC I, beta actin, and GAPDH and are presented as mean + SEM (n=6), * < p 0.05.

Supplementary Fig. 2 Knockdown of GFAP isoforms does not alter vimentin expression. A) mRNA expression levels of vimentin are not significantly altered in cells with a pan GFAP or GFAPα KD. B) Western blot analysis shows no changes in vimentin protein expression in cells with GFAP KD.
References


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