Silencing GFAP isoforms in astrocytoma cells disturbs laminin-dependent motility and cell adhesion

Martina Moeton,¹ Regina Kanski,¹ Oscar M. J. A. Stassen,¹ Jacqueline A. Sluijs,¹⊙ Gerhard Wiche,‖ Miriam E. van Strien,* and Elly M. Hol*¹∥,¶,2

*Astrocyte Biology and Neurodegeneration, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands; †Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, The Netherlands; ‡Department of Pediatric Oncology and Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands; §Hubrecht Institute, Utrecht, The Netherlands; †Department of Biochemistry and Cell Biology, Max F. Perutz Laboratories, University of Vienna, Vienna, Austria; and ¶Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, Amsterdam, The Netherlands

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, on 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 favor of GFAPδ in astrocytoma cells, by selectively silencing the canonical isoform GFAPα with short hairpin RNAs, induced a change in integrins, 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 summary, 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.—Moeton, M., Kanski, R., Stassen, O. M. J. A., Sluijs, J. A., Geerts, D., van Tijn, P., Wiche, G., van Strien, M. E., Hol, E. M. Silencing GFAP isoforms in astrocytoma cells disturbs laminin dependent motility and cell adhesion. FASEB J. 28, 000–000 (2014). www.fasebj.org

Key Words: intermediate filaments · extracellular matrix · glia · glioma · cytoskeleton

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 (1–4). IF expression is cell type-specific (5). For this reason, IF proteins are frequently used to determine the origin of tumors, also in the brain (6). GFAP marks tumors that originate from astrocytes (7–11). The level of GFAP expression in these tumors varies among patients (12), and high-grade glioma subtypes can be discerned based on the correlated expression of the IF proteins nestin, vimentin, synemin, and GFAP (13). 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 (14–16) as well as to a more malignant phenotype (17, 18).

The majority of literature on GFAP in astrocytic tumors does not discriminate among different isoforms, as the antibodies and primers used recognize several GFAP isoforms. To date, 10 different isoforms have been detected in the human brain (19, 20). The

Abbreviations: ACTB, β-actin; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HEK, human embryonic kidney; HPRT, hypoxanthine phosphoribosyltransferase; IF, intermediate filament; KD, knockdown; KO, knockout; MHC, major histocompatibility complex; NSC, neural stem cell; NTC, nontargeting construct; PBS, phosphate-buffered saline; PLL, poly-l-lysine; qPCR, quantitative polymerase chain reaction; RNAi, ribonucleic acid interference; shRNA, short hairpin ribonucleic acid; UTR, untranslated region

¹ These authors contributed equally to this work.
² Correspondence: Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Str. 4.205, P.O. Box 85060, 3508 AB Utrecht, The Netherlands. E-mail: e.m.hol-2@umcutrecht.nl
doi: 10.1096/fj.13-245837
This article includes supplemental data. Please visit http://www.fasebj.org to obtain this information.
alternatively spliced isoform GFAP\(\delta\) is highly expressed in specific human astrocyte subpopulations, including the neurogenic astrocytes of the subventricular zone. Thus, GFAP\(\delta\) can be used as a marker for NSCs of the developing and adult human brain in this niche (2, 20, 21). As findings indicate that cells with NSC characteristics are present in human brain tumors (22), it is important to take the GFAP\(\delta\) splice isoform into account when studying glioma. Indeed, we and others have shown that GFAP\(\delta\) is present in astrocytoma cells (23, 24). In astrocytic tumors, the expression levels of GFAP\(\delta\) indicate a correlation with the malignancy grade (17, 18).

In contrast to GFAP\(\alpha\), and due to a different C-terminal tail, GFAP\(\delta\) by itself is assembly compromised. It requires additional type III IF expression for proper filament formation (2, 24, 25). Hereby, the expression levels of the GFAP\(\delta\) protein are a crucial determinant of proper GFAP network formation. Changing the GFAP IF network has been shown to affect astrocyte physiology. Expression of GFAP\(\delta\) at high concentrations induces a collapse of the IF network (2, 24, 25). Non-isoform-specific silencing or knockout (KO) of GFAP influences astrocyte morphology, proliferation, motility, and adhesion (26–29). Until now, the function of specific GFAP isoforms in these processes has not been studied. As GFAP\(\delta\) is highly expressed in specific astrocyte subpopulations, such as neurogenic astrocytes and astrocytomas, we anticipated that a change in the ratio of GFAP\(\alpha\) to GFAP\(\delta\), in favor of GFAP\(\delta\), leads to functional changes in these cells.

We here demonstrate efficient and specific silencing of GFAP\(\alpha\) using isoform-specific short hairpin ribonucleic acid (shRNA), thereby changing the ratio in favor of GFAP\(\delta\), which results in an endogenous shift of IF network composition. We analyzed the effect of GFAP isoform knockdown (KD) 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 GFAP\(\alpha\)-specific KD to the silencing of all GFAP isoforms.

**MATERIALS AND METHODS**

**Cell culturing**

All cells were cultured at 37°C under a humidified 5% CO\(\text{2}\)/95% air atmosphere. Human embryonic kidney (HEK)-293T cells and the U373 human astrocytoma cell line were cultured in Dulbecco’s modified Eagle medium (DMEM) Glutamax (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotic mixture of 10 U/ml penicillin G and 10 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). For replating, cells were washed with versene (171 mM NaCl, 5.37 mM Na\(\text{2}\)HPO\(\text{4}\) \(\text{3}\) \(\text{5}\) mM KCl, 1.8 mM KH\(\text{2}\)PO\(\text{4}\), and 855 \(\mu\)M EDTA), trypsinized (using 0.25% trypsin), and resuspended in FBS containing medium. Cells were either split for maintaining the line (2 \(\times\) wk) or plated for experiments.

**shRNA constructs**

Lentiviral shRNA expression plasmids from The RNAi Consortium (TRC) Mission library (30) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The plasmids express 52-bp shRNA molecules with 21-nt mRNA specificity, driven by the ubiquitously active U6 snRNA promoter in the pLKO.1 vector backbone. The human GFAP shRNA constructs used were GFAP\(\alpha\): TRCN0000083733 (5’-ccctcttaactacacacaaa-3’), targeting nt 2674–2694, which is in the 3’ untranslated region (UTR) of the NM_002055.4 GFAP\(\alpha\) transcript, and pan-GFAP: TRCN0000083756 (5’-gctattagaaggaagcat-3’), targeting nt 577–597 of NM_002055.4, which is part of exon 2, present in all major GFAP isoforms (20). The SHC002 nontargeting construct (NTC: 5’-aaaaattagagacacacc-3’, Sigma-Aldrich), with no homology to human sequences, was used as control shRNA.

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

Lentiviruses encoding NTC, GFAP\(\alpha\), or pan-GFAP shRNA were produced as described before (31, 32) with some alterations. In short, 10 \(\times\) 10\(\text{5}\) HEK-293T cells were plated in a 15-cm culture dish and transfected with a total of 90 \(\mu\)g of the envelope (pMD2.G), packaging (pCMV-dR8.74), and p1365RRL plasmid. To this end, the total 90 \(\mu\)g of DNA was mixed with PEI (67.5 ng/\mu\)l) and incubated for 15 min at room temperature before adding the mix drop-wise 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, Fullerton, CA, USA) for 2.5 h. The resulting pellet was resuspended in phosphate buffered saline (PBS; pH 7.4), portioned into aliquots, 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 HEK-293T cells were transduced. After 2 d 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) per milliliter.

U373 cells were plated in 24-well plates with 25,000 cells/well. The next day, cells were transduced with lentiviral particles encoding either NTC (negative control), pan-GFAP (pan-GFAP KD), or GFAP\(\alpha\) shRNA (GFAP\(\alpha\) KD) with a multiplicity of infection (MOI) of 0.5. Medium was refreshed after 16 h. At 3 d after transduction, cell medium was replaced by medium containing 1 \(\mu\)g/ml puromycin (Sigma-Aldrich). Cells were grown, split, and kept in puromycin-containing medium to ensure that only transduced cells survived. Puromycin was removed ≥3 d before the functional experiments were performed.

**RNA isolation, cDNA synthesis, and real-time quantitative polymerase chain reaction (qPCR)**

For RNA isolation, cells were harvested, and total RNA was isolated with Trisure (Bioline, Taunton, MA, USA), 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, Willimington, DE, USA). Subsequently, RNA was reverse-transcribed with the Quantitect Reverse Transcription Kit (Qiagen, Valencia, CA, USA), 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 \(\mu\)l/well.
TABLE 1. Primers used for qPCR

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP(\alpha)</td>
<td>CCCACTCTGCTTTGACTGAGC</td>
<td>CTTTCTCCGCGCTTATAGGG</td>
</tr>
<tr>
<td>GFAP(\beta)</td>
<td>TCCAAACCTGCAAGATCTGAGAG</td>
<td>CTAATAGTTGATCTGGTCTTT</td>
</tr>
<tr>
<td>GFAP(K)</td>
<td>TGTCTAGACGAGGCTCCTGAGC</td>
<td>AGAGCCTGATCGGTGTGG</td>
</tr>
<tr>
<td>GFAP(\Delta) exon 6</td>
<td>TGGCGGGCCGAGGATC</td>
<td>CAGGTTCTCCAGCAGATGTT</td>
</tr>
<tr>
<td>GFAP(\Delta) 135</td>
<td>TCTGGCCGGCCGGCAGGTA</td>
<td>CAGGACCTTCCAGCAGATGTT</td>
</tr>
<tr>
<td>GFAP(\Delta) 164</td>
<td>GAGGGGCAGCTTTATCC</td>
<td>CAGGTTCTCCAGCAGATGTT</td>
</tr>
<tr>
<td>Ki67</td>
<td>AGATGCGGAGGTCTCCATGCG</td>
<td>TCCGGTTAACCTCTGACAG</td>
</tr>
<tr>
<td>ITGB1</td>
<td>GACGCGGCGCGGAAAGATGTG</td>
<td>GGGCCCTGGTTGATGATCTCCA</td>
</tr>
<tr>
<td>ITGB4</td>
<td>TACCCCTCAAGGTTGTCAGAGA</td>
<td>CCGGTATATAAGCAGCTTCCC</td>
</tr>
<tr>
<td>ITGA1</td>
<td>CCTGGGAGAAGATGGTTGGAT</td>
<td>TGGCCCAACTAAGGGAAGAA</td>
</tr>
<tr>
<td>ITGA2</td>
<td>AAGATGGTCGGCGCAGACG</td>
<td>TGGCTAGGACGAAATAAGG</td>
</tr>
<tr>
<td>ITGA3</td>
<td>GAGCTGGCCAGGCTTATGGAGA</td>
<td>GAGGAGCTGCAATGGCTGTTT</td>
</tr>
<tr>
<td>ITGA6</td>
<td>CATAATGGACGTCGGGCTTG</td>
<td>TGGCAGGCTGTTAACATC</td>
</tr>
<tr>
<td>ITGA7</td>
<td>GGTTTGTGTCGAAACCTTCC</td>
<td>TGGCTGAGGAGGAGTACC</td>
</tr>
<tr>
<td>LAMA1</td>
<td>GTTCGAGATCCTTCGGCAAGA</td>
<td>CGGCGAGCCTGATGATTA</td>
</tr>
<tr>
<td>MHC I</td>
<td>CACACCTCTTCCTTGTGACTTCAAG</td>
<td>CCGACTCCCCCCATATGCTAACA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGCAGCGCAAACTGCTTGGAC</td>
<td>GGGATGCGCTGTTGCTATCA</td>
</tr>
<tr>
<td>HPRT</td>
<td>ATGGGAGGGCCACTACATTGCTT</td>
<td>ATGTAACCCAGGACTGACCA</td>
</tr>
<tr>
<td>ACTB</td>
<td>GGCTCTCTCTGGCGCAGA</td>
<td>CTGCTGTGGAAGTTGGAC</td>
</tr>
<tr>
<td>PLEC</td>
<td>AGATCGGAGGGGCGGAGG</td>
<td>GAGCGGACCAGGCAGTCAC</td>
</tr>
</tbody>
</table>

using the SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA). 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 mM). 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 among samples, we normalized the target PCR to the geometric mean of PCRs to the reference genes hypoxanthine phosphoribosyltransferase (HPRT), major histocompatibility complex 1 (MHC), β-actin (ACTB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data were corrected for interexperimental differences as described in Ruijter et al. (35). For specific KD samples, significance was tested using a Mann-Whitney test. All other qPCR data were tested using a Kruskal-Wallis test with a ×20 objective or with a Leica SP5 confocal with a ×63 objective (Leica Microsystems, Wetzlar, Germany).

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; and 1 mM ethylenediaminetetraacetic acid (EDTA)] with 1% Triton-X100 and added protease inhibitors [100 μg/ml phe- nylmethanesulfonylfluoride (PMSF); Roche Diagnostics, Indianapolis, IN, USA] and 0.5 μg/ml leupeptin (Roche Diagnostics). Cells were vortexed and incubated on ice for 5 min before centrifugation at 11,700 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, Rockford, IL, USA) according to manufacturer’s descriptions. Proteins were mixed with 2× loading buffer (2×: 100 mM Tris, pH 6.8; 4% SDS; 20% glycerol; 200 mM dithiothreitol; and bromphenol 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

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Catalog no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan-GFAP</td>
<td>Dako (Copenhagen, Denmark)</td>
<td>1:4000 ICC, 1:8000 WB</td>
<td>Z0334</td>
</tr>
<tr>
<td>hGFAP(\beta)</td>
<td>Manufactured in-house (October 5, 2001 bleed)</td>
<td>1:1000 ICC, 1:1300 WB</td>
<td>–</td>
</tr>
<tr>
<td>hGFAP(\alpha)</td>
<td>Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)</td>
<td>1:1300 WB</td>
<td>sc-6170</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Chemicon (Temecula, CA, USA)</td>
<td>1:5000 ICC and WB</td>
<td>AB5733</td>
</tr>
<tr>
<td>Plectin</td>
<td>G.W.</td>
<td>1:5000 WB</td>
<td>Ab 9 (55)</td>
</tr>
</tbody>
</table>

ICC, immunocytochemistry; WB, Western blot.
Whatman Protran membranes (GE Healthcare, Waukesha, WI, USA) using a semidry Trans-Blot system (Bio-Rad, Hercules, CA, USA) for 1 h. 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; and 150 mM NaCl with 0.2% Tween-20) 3 times before secondary antibodies, diluted in SuMi, were incubated for 1 h at room temperature. Blots were washed again 3 times in TBS-T before scanning with an Odyssey scanner (LI-COR, Lincoln, NE, USA). Primary antibodies used are listed in Table 2. Secondary antibodies used are IRdye 800 (1:2000; LI-COR) and Dyelight Cy5 (1:4000; Jackson ImmunoResearch).

Motility assays

Cells were plated on glass dishes coated with poly-(l-lysine) (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 either washed or not dried. Cells were allowed to adhere for 24 h before cells were tracked overnight. Every 10 min, an image was taken with a Zeiss Axiovert 2000 (Zeiss Microscopy, Oberkochen, Germany). Cells were kept at 37°C and 5% CO2 in a preheated and humidified incubation chamber (Okolab, Ottaviano, Italy) during the imaging. Images were compiled into a time-lapse sequence. For motility analysis, 25 cells/condition were followed in 6 independent experiments, which resulted in a total of 150 cells. Cell soma movement was manually tracked using a manual tracking ImageJ plug-in (version 1.46f; W. S. Rasband, U.S. National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/). The average velocity per cell was calculated and then averaged for all cells in 1 condition/experiment. Data were corrected for interexperimental differences as described in Ruijter et al. (33). Differences were tested for significance 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 min. After washing twice with PBS plus 1% BSA, cells were incubated with RNase (0.5 mg/ml; Boehringer, Ridgefield, CT, USA) for 15 min to enzymatically remove RNA, which is necessary for specific staining of nuclear DNA. Subsequently, cells were washed twice with PBS plus 1% BSA, stained with propidium iodide (PE; 50 mg/ml; Sigma-Aldrich) for 15 min, and washed with PBS plus 1% BSA. Flow cytometry was performed on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was done using the FlowJo software (Tree Star, Ashland, OR, USA; 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/well. At stated time points, cells were gently washed with versene 3 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 h, and washed in PBS once, after which phase-contrast and fluorescent images 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 images using ImageJ. The area, perimeter, and form factor were calculated by ImageJ. Form factor was calculated with the formula $A / P^2$, where $A$ is area, and $P$ is perimeter. Form factor is used as a measure for cell morphology where perfect round cells will have a form factor of 1. The perimeter of $\geq30$ cells/condition was measured for 6 independent experiments. Data were corrected for interexperimental differences as described in Ruijter et al. (33). 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 6.02 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant at values of $P < 0.05$ at a 95% confidence interval.

RESULTS

Selection of target sequences for isoform-specific KD of GFAP

To change GFAP network composition in astrocytomas, we aimed at silencing the expression of GFAP using RNA interference (RNAi)-based gene silencing. Based on the sequence information of different GFAP splice variants (20), specific target regions in the GFAP transcript were selected. For an isoform-specific KD, we focused on silencing the canonical isoform GFAPα, which will decrease the GFAPα/GFAPβ ratio. Shifting the ratio in favor of GFAPβ expression mimics the IF network in more malignant forms of astrocytoma cells or neurogenic astrocytes (2, 17, 18) 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 GFAPβ or GFAPκ. mRNA levels of Δexon 6, Δ164, and Δ135 are low in human brain (19, 23, 34). Analysis of GFAP mRNA expression levels in human astrocytoma cell lines revealed that GFAPΔ164 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 $\sim100\times$, and Δexon 6 is $1000\times$ lower expressed. In addition to targeting GFAPα, we selected a sequence to silence all GFAP isoforms. Exon 2 is a constitutive exon, present in all isoforms, and was therefore chosen as a target (Table 3). We obtained lentiviral shRNA expression constructs from the TRC shRNA library (30) 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 KD of GFAP

U373 astrocytoma cells were transduced with an NTC control shRNA or an shRNA targeting pan-GFAP (pan-GFAP-KD cells) or GFAP\(\alpha\) (GFAP\(\alpha\)-KD cells). Transduced cells were selected by puromycin to ensure a stable KD of GFAP. Efficiency and specificity of the KD was validated by analyzing GFAP\(\alpha\) and GFAP\(\delta\) mRNA expression. As expected, GFAP\(\alpha\) was significantly down-regulated in both the pan-GFAP-KD (\(\sim 73\%\)) and GFAP\(\alpha\)-KD cell lines (\(\sim 58\%\)) (Fig. 2A). As expected, GFAP\(\delta\) transcript expression was also down-regulated in pan-GFAP-KD cells (\(\sim 36\%\)). Intriguingly, in cells with a specific KD of GFAP\(\alpha\), expression of GFAP\(\delta\) was significantly up-regulated (\(\sim 600\%\); Fig. 2B). Expression levels of GFAP\(\alpha\) and GFAP\(\alpha\)135 were also up-regulated in GFAP\(\alpha\)-KD cells, but since expression levels are much lower compared to GFAP\(\delta\) expression (10- and 86-fold, respectively) the contribution of GFAP\(\delta\) is much more substantial (Supplemental Fig. S1). GFAP\(\alpha\)exon 6 levels were not significantly changed (Supplemental Fig. S1), and GFAP\(\alpha\)164 levels were too low to detect (data not shown).

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

Previously, it has been demonstrated that GFAP\(\delta\) overexpression induces aggregation of the GFAP fila-

**Figure 1.** Selection of target sequences for isoform-specific KD of GFAP. A) Expression of human GFAP isoforms in the U373 astrocytoma cell line. Table 3 depicts shRNA candidates targeting human GFAP isoforms. B) Schematic representation of the GFAP transcript. 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 down-regulates GFAP\(\alpha\) (GFAP\(\alpha\) KD). Bars represent means + sem (\(n=4\)).

**TABLE 3.** shRNA candidates targeting human GFAP isoforms

<table>
<thead>
<tr>
<th>Name</th>
<th>Target sequence</th>
<th>Exon</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan</td>
<td>GCGTATAGCAGAGAGCAGAT</td>
<td>2</td>
<td>577–597, NM_002055.4</td>
</tr>
<tr>
<td>(\alpha)</td>
<td>CCCCCTCTTCTCACCACACAAA</td>
<td>3' UTR</td>
<td>2674–2694, NM_002055.4</td>
</tr>
<tr>
<td>NTC</td>
<td>CAAACAGATGAGAGCACCACAA</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

See Fig. 1.

GFAP silencing has no effect on cell proliferation

First, we investigated the proliferative capacity of pan-GFAP-KD and GFAP\(\alpha\)-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 GFAP\(\alpha\)-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 GFAP\(\alpha\) signif-
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 (26, 29).

Figure 2. Isoform-specific KD of GFAP. A) qPCR data showed a significant down-regulation of GFAPα transcripts in both pan-GFAP-KD (P=0.007) and GFAPα-KD cells (P=0.03) compared to NTC control cells. B) GFAPδ transcripts were significantly down-regulated in pan-GFAP-KD (P=0.007) and up-regulated in GFAPα-KD cells (P=0.007). All data were normalized to reference genes HPRT, MHCII, ACTB, and GAPDH and are presented as means ± SEM (n=6) *P < 0.05. C, D) We confirmed KD on the protein level by Western blot (C) and immunocytochemistry (D, left panel). Due to an up-regulation of GFAPδ on KD of GFAPα (D, right panel), GFAPα-KD cells did not show a reduction of total GFAP expression when stained with a pan-GFAP antibody detecting all isoforms (D, left panel). NTC, pan-GFAP-KD, and GFAPα-KD cells were stained with a pan-GFAP antibody together with Hoechst (Hst) and vimentin (Vim). Distribution of the IF network was maintained in the KD cells compared to control cells (D, right panel). Scale bars = 100 μm (left panel); 20 μm (right panel).

Figure 3. Silencing of GFAP maintains the proliferation capacity of astrocytoma cells. Silencing of pan-GFAP or GFAPα had no effect on Ki67 expression or the cell cycle of astrocytoma cells. A) qPCR data showed that Ki67 expression remained unchanged in pan-GFAP-KD and GFAPα-KD cells. Data were normalized to reference genes HPRT, MHCII, ACTB, and GAPDH and are presented as means ± SEM (n=6). *P < 0.05. B) Mean relative distribution of pan-GFAP-KD or GFAPα-KD cells across the different cell cycle phases, measured by DNA staining and subsequent FACS analysis (n=3). Error bars = SEM.
Confirming a regulatory role for GFAP on cell morphology, we first determined whether down-regulation of pan-GFAP expression changed cell morphology in human astrocytoma cells on a standard PLL substrate (Fig. 4A, top panel). 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 (26). Cells with a stable KD of all GFAP isoforms (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-

**Figure 4.** Silencing of pan-GFAP, but not GFAPα, alters morphology dependent on the presence of laminin. A) Phase-contrast images of U373 cells with different GFAP network compositions on PLL-coated (top panel) or laminin-coated (bottom panel) coverslips. Scale bars = 100 μm. B) Pan-GFAP-KD cells showed a significant difference in area compared to GFAPα-KD cells (P=0.006) on PLL. Perimeter of pan-GFAP-KD cells was significantly lower compared to both the control and GFAPα-KD cells (P=0.005). 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 cells (P=0.003). 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. *P < 0.05.
GFAP-KD cells (Fig. 4B, right panel, form factor) 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 (35, 36). We observed that the cell morphology, seen on laminin (Fig. 4A, bottom panel), differed from cells on PLL (Fig. 4A, top panel). 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 (Fig. 4C, middle panel). This condition resulted in a significant change in the form factor, reflecting that cells displayed a less round morphology on laminin (Fig. 4C).

In summary, 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 on a specific GFAPα KD, a situation in which an up-regulation of GFAPβ expression occurs (Fig. 2B), indicating that the presence of GFAPβ might compensate for the lack of GFAPα.

Pan-GFAP KD 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 h. 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 KD 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 (37). 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 (38). We investigated the integrin subunits β1 and β4, as well as α2, α3, α6, and α7. These integrin subunits heterodimerize to form different integrin receptors that bind laminin (Fig. 5C). We observed a clear pattern of integrin expression: the pan-GFAP-KD cells had higher integrin expression compared to control or GFAPα-KD cells. For integrins β1, α2, α6, and α7, these differences were significant (Fig. 5D–F).

Taken together, KD 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 down-regulates integrin expression, reduces cell motility, and promotes the expression of laminin

GFAPα-KD cells showed a marked down-regulation 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 (26, 28, 39, 40). Cells with GFAP isoform KD 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 KD, motility was not significantly changed (Fig. 6A). We also assessed motility on PLL and 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 (41, 42). 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 down-regulated in the GFAPα-KD cells only (Fig. 6C). Protein quantification confirmed a down-regulation 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 up-regulation of the LAMA1 transcript in GFAPα-KD cells (Fig. 6D).

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

**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/β ratio in favor of GFAPβ expression, an effect enhanced by up-regulation of endogenous GFAPβ by the cell itself. Modulation of the GFAP isoform ratio revealed that on a specialization of the GFAP network, the expression 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 (2, 21), and its expression level is a crucial determinant of the GFAP IF network assembly (24, 25).

We here demonstrate that decreasing GFAPα expression levels induced an endogenous up-regulation 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 on transgenic GFAPδ overexpression (2, 24). However as shown here, an endogenous, and more subtle, up-regulation 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 (24). A collapse of the IF network, due to GFAPδ expression, is concentration dependent (24, 25, 43). Thus, we can conclude that the endogenous up-regulation due to GFAPα KD 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 GFAPα in the IF network. In a physiological situation in the brain, GFAPδ-expressing cells have not been shown to display a collapsed GFAP network.

**Figure 6.** Specific silencing of GFAPα promotes the expression of laminin and reduces motility in astrocytoma cells. A) GFAPα-KD cells had a reduced cell motility on laminin-coated glass coverslips compared to controls ($P<0.0001$; $n=6$). Single-cell motility was measured as the average velocity in micrometers per minute of a single cell, in a sequence of images, which were taken overnight. Average velocity was significantly reduced in GFAPα-KD cells compared to control cells. B) Reduction in migration in GFAPα-KD cells was less but still significant when cells were plated on PLL-coated coverslips. C) Plectin expression was significantly down-regulated in GFAPα-KD cells only. D) qPCR data on LAMA1 showed a significant increase in laminin expression in GFAPα-KD cells in comparison to pan-GFAP-KD and control cells. A–D) Data were normalized to reference genes HPRT, MHCII, ACTB, and GAPDH and are presented as means ± sem ($n=6$). *$P<0.05$. E) Western blot analysis confirmed a down-regulation of plectin in GFAPα-KD cells on 10% SDS-PAGE gels. Quantification of plectin protein levels show reduced plectin in GFAPα-KD cells (40.6±10.5%) and variable levels of plectin in pan-GFAP-KD cells (60.9±23.2%). Data are means ± sem of 2 experiments.
reflect more physiological expression levels compared to the overexpression studies.

**IF and cell morphology**

The induction of GFAPδ on KD of GFAPα might be sufficient to maintain the morphological characteristics of astrocytoma cells, since GFAPα-KD cells demonstrate none of the morphological changes present in cells with a pan-GFAP KD. 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, KO of all GFAP isoforms was shown to alter cell morphology (26). Taken together, our data highlight that a drastic reorganization of the IF network either by KO or KD 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 KD or in GFAP-KO cells (26, 28). 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 implies 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 down-regulation of integrins β1 and α6. The β1 integrin is localized to the leading edge of migrating astrocytes (44), and blocking the β1-integrin receptor has been shown to inhibit astrocyte migration (39). Moreover in gliomas, blocking integrin β1 inhibited cell migration on laminin as well as invasion into matrigel (40). 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 (45). 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 (46). 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 (17, 18). 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 (47, 48). Our findings that the presence of GFAPδ in cells with low GFAPα expression alters the interaction of astrocytoma cells with laminin, which 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, down-regulation 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-KO mice (49). However, GFAP overexpression in fibroblasts has also been shown to increase laminin production (50). This finding is in agreement with an up-regulation of laminin production in reactive astrocytes, which are characterized by an increase in GFAP expression (51). In epithelial cells, laminin-332 expression was shown to be mediated by transforming growth factor β (TGF-β) through integrin signaling (52). We observe changes in integrin β1 expression in GFAPα-KD cells. Integrin-β1-KO mice decrease laminin 1 secretion (53), and migrating astrocytes up-regulate β1 integrin expression together with GFAP and vimentin (39). The decoupling of vimentin from focal adhesions (FAs) attenuated FA downstream signaling (54). These data suggest that regulatory signals occur among 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 summary, we here demonstrated efficient and isofom-specific silencing of GFAPδ, which resulted in increased GFAPβ and LAMA1 expression, decreased plectin, and integrin-β1 expression and a reduced motility of astrocytoma cells. In contrast, pan-GFAP KD 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 isofrom 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.

This project is funded by The Netherlands Organization for Scientific Research (NWO; VICI grant 865.09.003 to E.M.H.), FOM 09MMC06, and NANONET COST BM1002. The Netherlands Institute for Neuroscience and the Hubrecht Institute are institutes of the Royal Netherlands Academy of Arts and Sciences.

REFERENCES


Received for publication November 14, 2013. Accepted for publication March 17, 2014.
Silencing GFAP isoforms in astrocytoma cells disturbs laminin-dependent motility and cell adhesion

Martina Moeton, Regina Kansi, Oscar M. J. A. Stassen, et al.

FASEB J published online April 2, 2014
Access the most recent version at doi:10.1096/fj.13-245837

Supplemental Material  http://www.fasebj.org/content/suppl/2014/05/16/fj.13-245837.DC1.html

Subscriptions  Information about subscribing to The FASEB Journal is online at http://www.faseb.org/The-FASEB-Journal/Librarian-s-Resources.aspx

Permissions  Submit copyright permission requests at: http://www.fasebj.org/site/misc/copyright.xhtml

Email Alerts  Receive free email alerts when new an article cites this article - sign up at http://www.fasebj.org/cgi/alerts

© FASEB