

Inhibition of Collagen XVI Expression Reduces Glioma Cell Invasiveness

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Key Words

Collagen XVI • Glioblastoma • Invasion • Protocadherin • Integrin • Kindlin • Activation

Abstract

Glioblastomas are characterized by an intense local invasiveness that limits surgical resection. One mechanism by which glioma cells enforce their migration into brain tissue is reorganization of tumour associated extracellular matrix (ECM). Collagen XVI is a minor component of connective tissues. However, in glioblastoma tissue it is dramatically upregulated compared to the ECM of normal cortex. The aim of this study is to delineate tumour cell invasion and underlying mechanisms involving collagen XVI by using a siRNA mediated collagen XVI knockdown model in U87MG human glioblastoma cells. Knockdown of collagen XVI resulted in decreased invasiveness in Boyden chamber assays, and in a reduction of focal adhesion contact numbers per cell. Gene expression was upregulated for protocadherin 18 and downregulated for kindlin-1 and -2. Proliferation was not affected while flow cytometric analysis demonstrated reduced β 1-integrin activation in collagen XVI knockdown cells. We suggest that in glioblastoma tis-

sue collagen XVI may impair the cell-cell interaction in favour of enhancement of invasion. The modification of the β 1-integrin activation pattern through collagen XVI might be a molecular mechanism to further augment the invasive phenotype of glioma cells. Elucidating the underlying mechanisms of glioma cell invasion promoted by collagen XVI may provide novel cancer therapeutic approaches in neurooncology.

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Introduction

Collagen XVI is a member of the fibril associated collagens with interrupted triple helices (FACIT) collagens and constitutes a minor component of the ECM of skin, cartilage, brain and intestine. It is integrated into distinct fibrillar aggregates such as fibrillin-1 containing microfibrils in skin and D-banded cartilage fibrils [1, 2]. The presence of collagen XVI in the dermal epidermal junction (DEJ) zone of the papillary dermis indicates an active role in anchoring microfibrils to basement membranes. By interconnecting ECM proteins to cells, collagen XVI is likely to be able to move these proteins and hence affects ECM networks, ensuring mechanical an-

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chorage of the cell and outside-inside signal transduction. Cell-matrix interactions are mostly mediated via integrins which connect the ECM with the cytoskeleton and enable ECM-components to induce cellular reactions like cell adhesion, migration or gene activation [3]. Collagen XVI interacts with integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, however with higher affinity to $\alpha 1\beta 1$ [4]. $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins are expressed on glioma cell surfaces and contribute to cell adhesion and migration [5, 6], hence these integrins are likely to be an important link in terms of interaction of cells with the ECM via collagen XVI. A strong expression of collagen XVI was detected in murine dorsal root ganglia during embryogenesis as well as during regeneration after nerve injury [7]. This may indicate that both maturation and regeneration of nerve cells require an interaction between neurons and the extracellular matrix (ECM), in which the specific role of collagen XVI is not known to date.

Glioblastomas or gliomas are a relatively common form of primary central nervous system (CNS) tumours occurring in adults, and glioblastoma multiforme is the most devastating type of glioma. Despite dramatic improvements in neuroimaging and neurosurgical techniques, the prognosis in patients with gliomas has not improved significantly during the past 40 years [8-10]. We demonstrated a strong upregulation of collagen XVI in glioblastoma tissue, which is characterized by aggressive tumour cell invasion into its surrounding tissue. Using glioma cell lines as model system we observed that collagen XVI ameliorates adhesion of these cells to matrix with the glioma cell line U87MG showing strongest adhesion on collagen XVI among all tested glioma cell lines [11].

Two other members of the FACIT collagens are described to be involved in tumorigenesis and tumour cell invasion, collagens XIX and XV. Both collagens are mainly localized in basement membranes of normal tissues. During the development of ductal and lobular carcinomas of the breast basement membranes become fragmented and even lost in the progression of malignancy. In the very beginning of tumour cell invasion, some extracellular proteins, like FACIT collagens may act as attractants for tumour cells. Notably, collagen XIX and XV disappear from the basement membrane zone early in the development of the invasive carcinomas possibly inducing remodelling of the ECM which promotes tumour cell infiltration [12]. Members of the FACIT collagens may act as bridging proteins between collagen fibrils and other components of the ECM and thus contribute to tissue organization and stability. One may suggest that tumour cells utilize these matrix components to identify and to

adhere to basement membranes. They seem to represent the first contact to tumour cells and facilitate their adhesion. The loss of FACIT collagens during progression of the disease may indicate that these proteins, representing the initial adhesion substrate are subsequently digested by invading tumour cells to enable rearrangement of the surrounding matrix.

Here, we hypothesize that collagen XVI affects migration and invasion capacity of glioma cells. We show that collagen XVI gene silencing in the glioblastoma cell line U87MG affects genes involved in cell-cell and cell-matrix associated signal transduction pathways. Reduction of collagen XVI causes a profound decrease of focal adhesion contacts which are crucial for cell-matrix interaction and transduction of outside-inside signalling. Collagen XVI gene silencing affects invasive behaviour of the U87MG cells, implying collagen XVI to be an important player in tumorigenesis and metastasis of glioblastoma. There is strong evidence for collagen XVI to play a crucial role in interaction between cells and the ECM via molecules of the plasma membrane and the cytoskeleton.

Materials and Methods

Cell Culture

The human glioblastoma cell line U87MG (ATCC: HTB-14) was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (Sigma, St. Louis, USA) and 1% penicillin/streptomycin (PAA, Coelbe, Germany) at 37°C in 5% CO₂.

Transfection

Transfection of the U87MG cells was performed by electroporation using the Amaxa Nucleofector system (Lonza, Allendale, USA) with a Cell Line Nucleofector Kit T (VCA-1002, Lonza, Allendale, USA) according to the manufacturer's protocol. 5x10⁵ cells per reaction were incubated with 80 pmol of siRNA (8pmol / μ l) and 100 μ l Nucleofector solution and run on program X-01. The cells were transfected with two collagen XVI-specific siRNAs: siRNA1 (5'-uga gcu cau uga gau caa u dTdT-3') and siRNA2 (5'-gga cuc aaa uug gaa cac a dTdT-3') and the corresponding sequences as double strand; as well as scrambled siRNA (control) as described elsewhere [11]. All siRNAs were purchased from Qiagen, Hilden, Germany. Transfection was completed by incubating the cells for 48 h at 37°C in 5% CO₂ before they were subjected to RNA and protein isolation or functional assays.

RNA isolation and quantitative real-time PCR

RNA was isolated with Qiagen RNeasy Mini Kit according to the manufacturer's protocol. DNA contamination was eliminated by DNase I enzyme (DNAfree, Ambion/Applied

Biosystems, Austin, USA) and up to 3 µg RNA was converted into cDNA with 500 ng/ml Oligo dT primers, 10 mM dNTPs, 0.1 M DTT and 200 units of SuperScript II enzyme (all Invitrogen, Paisley, UK) using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Waldbronn, Germany) according to manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed with SYBR Green Dye I on the MX3005 (Stratagene, Waldbronn, Germany) in triplicates, using cDNA concentrations that referred to 25 ng of RNA per reaction. For quantification, plasmid standard curves for *COL16A1* were generated in copy numbers ranging from 10^2 to 10^7 to calculate *COL16A1* cDNA copy numbers in samples and to determine knockdown efficiency. For PCDH18, KINDLIN-1 and KINDLIN-2, relative quantification was evaluated by the $\Delta\Delta C_t$ method using GAPDH as reference gene for normalization of gene of interest and, the scrambled RNA transfected cells were used as calibrator. The mean relative quantification (RQ) values and absolute copy numbers were calculated using the detection software MxPro v 4.1 (Stratagene, Waldbronn, Germany). Primers for *COL16A1* [13], *PCDH18* (F: 5'-agc atc tgc agc ttt tcc at-3'; R: 5'-agg gaa ttt tcc cca aca tc-3'), *KINDLIN-1* (F: 5'-cca ccg gga ttc cag tga caa c-3'; R: 5'-ccg ccg gtc aat ttg tgg acc-3'), *KINDLIN-2*, (F: 5'-gca aga cca tgg cgg aca gtt c-3'; R: 5'-gat ggg cct cca aga ttc teg c-3') and *GAPDH* (F: 5'-ctg act tca aca geg aca cc-3'; R: 5'-ccc tgt tgc tgt agc caa at-3') were designed with Primer3 software and synthesized from MWG-Biotech, Ebersberg, Germany.

Quantitative gene expression for MMP-genes was done using Brilliant II Fast qPCR Master Mix from Stratagene (Agilent Technologies, Santa Clara, USA) with Taqman upl probes (Roche). Primers for β -ACTIN: 5'-att ggc aat gag cgg ttc-3' and 5'-tga agg tag ttt cgt gga tgc-3'; *MMP-2*: 5'-ttg atg acg atg agc tat gga-3' and 5'-act tgc agt act ccc cat cg-3'; *MT1-MMP*: 5'-gag cct tgg act gtc agg aa-3' and 5'-ggg gtc act gga atg ctc -3'. β -actin mRNA was used for normalisation.

Protein precipitation and western blotting

Cell pellets were lysed in NP40-buffer containing 1% NP40 (Nonidet®P40 substitute, Fluka, Munich, Germany), 150 mM NaCl, 50 mM Tris-HCl, pH 8.0 (AppliChem, Darmstadt, Germany), and protease inhibitor cocktail (Complete Mini, 1 tablet per 10 ml buffer, Roche, Mannheim, Germany) for 30 min on ice with intermittent shaking. Samples were harvested 2 days or 4 days post transfection as indicated.

Total protein concentration was determined using BCA protein quantification (Pierce, Rockford, USA) according to the manufacturer's protocol. For analysis of collagen XVI knock-down 20 µg and for analysis of PCDH18 expression 50 µg of protein was added to 0.1 M Tris/HCl (pH 6.8, AppliChem, Darmstadt, Germany) sample buffer containing 2% SDS (Roth, Karlsruhe, Germany), 20% glycerine (AppliChem, Darmstadt, Germany), 5% β -mercaptoethanol (Sigma, St. Louis, USA) and 0.01% bromphenolblue (Sigma, St. Louis, USA) and, subjected to 4.5% to 15% gradient SDS-PAGE before being transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). 2ml of cell supernatant was collected and concentrated using Amicon® Ultra centrifugal filters (Millipore). Protein quantification was performed as described above. For western

blot analysis 30µg of protein was loaded onto the gel. For collagen XVI western blot a SemiDry-Blotter (MBT, Micro-Bio-Tec Brand, Gießen, Germany) was used for 1 h at 0.48 mA/cm² and for PCDH18 western blot a Tank blot system (customized construction University Hospital Münster) was used for 4 h at 80 mA. As a control for the PCDH18 antibody 20 µg of cell lysate from mouse lung was included. Blocking was performed for 1 h at RT with 5% dry milk (Roth, Karlsruhe, Germany) in T-PBS (0.05% Tween-20 in 1x PBS). The membrane was incubated with the primary antibody (antibodies: gp-anti-Col XVI, protein A purified, dilution, 1:1000 [14] or rabbit-anti-PCDH18 (dilution, 1:250; Sigma-Aldrich Inc., St. Louis, USA), polyclonal anti-Kindlin-1 (rabbit IgG: 5 µg/ml, Abcam, Cambridge, UK), polyclonal anti-Kindlin-2 (rabbit IgG: 3 µg/ml, Sigma-Aldrich) and rabbit- anti- β -actin (dilution, 1:5000; Abcam) over night at 4°C. After washing off excess antibody solution, the membrane was incubated with horseradish peroxidase conjugated secondary antibody (goat-anti-guinea pig peroxidase conjugated; dilution, 1:30.000; Sigma-Aldrich Inc., St. Louis, USA) for collagen XVI antibody or donkey-anti-rabbit IgG (Dilution, 1:5000; Jackson Immuno Laboratories Inc., West Grove, USA) for PCDH18, Kindlin-1, Kindlin-2 and β -actin antibodies for 1 h at RT. Immunoreactive bands were detected with chemiluminescence (ECL Western Blotting Substrates, Pierce, Rockford, USA).

Proliferation assay

96-well plates (MaxiSorb, BD Falcon, Franklin Lakes, USA) were coated separately with poly-L-lysine (5 µg/ml; Sigma, St. Louis, USA), collagen I (10 µg/ml; Sigma, St. Louis, USA), recombinant collagen XVI [14] (10 µg/ml and 30 µg/ml) over night at 4°C. This was followed by two washing steps with PBS and a coating with 1 % BSA solution (w/v) (Biomol, Hamburg, Germany) for 2 h at RT to block unspecific binding sites. The plates were washed two times with PBS and stored at 4°C until usage. Transfected U87MG cells were trypsinized, seeded into the coated 96-well-plates at a density of 7.500 cells/well and cultivated over night at 37°C and 5% CO₂. EZ4U proliferation assay (Biomedica, Vienna, Austria), based on conversion of tetrazolium salt to formazan by active succinatdehydrogenase from living cells, was performed according to the manufacturers protocol for 2 h, 4 h and 6 h. Absorption was determined at 450 nm and a reference wavelength of 600 nm in a microplate reader (Tecan, Maennedorf, Switzerland). The assay was performed in triplicates and as 3 independent experiments.

Cell morphology and focal adhesion contacts

For examination of the cell morphology, transfected cells were grown on uncoated chamber slides (BD Falcon, Franklin Lakes, USA, 40.000 cells/chamber) in normal growth medium. After incubation over night the cells were fixed with 4% PFA in PBST (0.1% Tween) for 5 min before washing in PBS, adding 0.1 M NH₄Cl (AppliChem, Darmstadt, Germany) for 10 min and washing again. The cells were then blocked over night at 4°C with 1% NGS (normal goat serum, Dako, Glostrup, Denmark) in PBS. For visualization of focal adhesion contacts, the cells were incubated with the primary antibody mouse-anti-human vinculin (Sigma, St. Louis, USA, 1:500 in 1% NGS in PBS) for 1 h at 37°C, followed by the secondary Alexa 488 conjugated

goat-anti-mouse antibody (Invitrogen, Paisley, UK, 1:500 in 1% NGS in PBS) for another hour at 37°C. The cell nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Paisley, UK, 300 nM) for 10 min. Finally the slides were mounted in fluorescent mounting medium (Dako, Glostrup, Denmark) and evaluated with laser scanning microscopy (C1 confocal microscope, Nikon) and Nikon C4 camera and software.

Evaluation of focal adhesion contact number (FACs)

Determination of FACs numbers in U87MG cells was carried out by counting the number of FACs represented by positive vinculin fluorescence staining in five randomly chosen fields of view and then determining the average number of FACs per cell.

Cell invasion assay

Differences in the invasiveness of collagen XVI-silenced cells to control cells were investigated by using a Boyden Chamber Invasion assay (BD Biosciences, San Jose, USA). The system is based on two medium-filled compartments with a well diameter according to a 24 well plate, separated by an 8 µm microporous membrane coated with Matrigel, diluted 1:3 with serum-free medium (BD Biosciences, San Jose, USA). The lower compartment contains fibroblast conditioned culture supernatant (from 80% confluent fibroblasts cultured in DMEM, 10% FCS, 1% Pen/Strep) in order to stimulate cell migration. Cells (150.000/ml) were placed in the upper compartment and were allowed to migrate through the pores of the membrane into the lower compartment. After an incubation time of 4 h, the membrane separating the two compartments was fixed (Hemacolor solution 1, #111957, Merck, Darmstadt, Germany) and stained to visualize cytoplasm and nuclei (Hemacolor solution 2, #111956, Hemacolor solution 3, # 111955, Merck, Darmstadt, Germany). The number of cells migrated to the lower side of the membrane was determined with light microscopy choosing five independent fields of view for each independent experiment (N = 4).

Adhesion Assay

U87MG cells were seeded at a density of 7.500 cells/well into a 96-well plate without additional coating. Cells were allowed to adhere for 4 h and 6 h at 37 °C at 5 % CO₂. Subsequently, plates were washed with PBS in order to remove non-adhered cells. Adhered cells were fixed with 1 % (v/v) glutaraldehyde solution (Merck, Darmstadt, Germany) for 30 min. Glutaraldehyde was removed and cells were repeatedly washed with PBS. Fixed cells were stained with 0.02 % (w/v) crystal-violet solution (Sigma, Munic, Germany) for 15 min at rt and washed with tap water. Bound crystal violet was removed by incubation for 3 h on a horizontal shaker at 100 rpm with 70 % (v/v) ethanol (Roth, Karlsruhe, Germany). Crystal violet adsorption was measured at 595 nm and each sample was analysed in triplicates from three independent experiments.

FACS analysis for β1-integrin activation

Flow cytometry was performed using a FACSCanto II flow cytometer (BD Biosciences, Heidelberg, Germany) equipped with 488 nm blue and 633 nm red diode lasers. Cells

were fixed for 15 min on ice in methanol:acetone (3:1), washed twice in PBS (+ 2% BSA). First antibodies were incubated for 1.5 h at 37 °C (Monoclonal anti- integrin β1 (C-terminus, mouse IgG: 10 µg/ml, Millipore MAB2259Z, Monoclonal anti- integrin β1D (N-terminus mouse IgG: 10 µg/ml, Millipore MAB1900). After two washing steps in PBS (+ 2% BSA) cells were treated for 1 h at room temperature with secondary antibodies linked to AlexaFluor 488 (Invitrogen, Darmstadt, Germany). Data evaluation was performed with FACSdiva software (BD Biosciences, San Jose, USA). The SFI value was calculated by dividing the geometric mean values of the fluorescence units of scrambled, si1 and si2 samples by the correspondent value of the isotype control, in each channel.

Statistical analysis

Results are shown in average values ±standard error of mean. For each siRNA at least 5 knockdown experiments were analyzed in triplicates. Significances were determined with assistance of GraphPadPrism v.5 by one-way Anova analysis of variance and as posthoc test with unpaired student's t-test.

Results

Knockdown efficiency

Knockdown efficiency of siRNA1 and siRNA2 was evaluated 48 hours (2 days) after transfection in comparison to scrambled (scr) RNA by determination of gene and protein expression level of collagen XVI.

The knockdown effect was evaluated on protein level with western blotting 2 days post transfection. A profound reduction of the full-length collagen XVI chain band for both siRNAs (1+2) was observed in cell lysates (Fig. 1A) and cell supernatant (Fig. 1B) compared to the scrRNA samples and the untreated U87MG cells.

Gene expression level of *COL16A1* was determined in absolute cDNA copy numbers using a plasmid standard curve. It is displayed separately for siRNA1 and siRNA2 in % of controls which are U87MG cells transfected with scrambled RNA (Fig. 1C). GAPDH gene expression served as endogenous control for siRNA specificity and remained unaffected (data not shown). In general, a *COL16A1* transcript reduction of ~60 % was obtained in comparison to the control cells (scr RNA).

Cell morphology and focal adhesion contacts

Morphology of transfected cells, both control and collagen XVI-knockdown cells, was analyzed by fluorescence microscopy. At that level of investigation no morphological differences concerning cell size and shape were detected between control cells and knockdown cells (Fig. 2A). However, cellular distribution and in particular numbers of focal adhesion contacts (FACs) represented

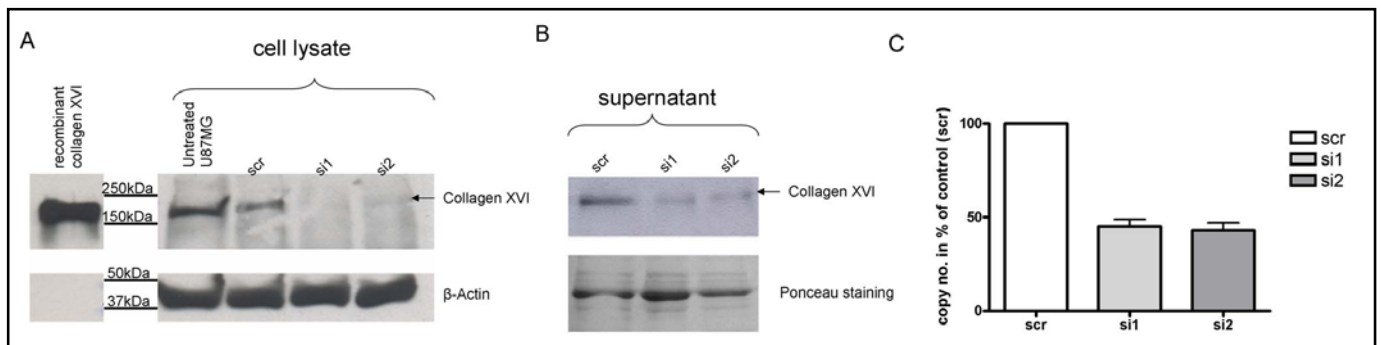


Fig. 1. Analysis of *COL16A1* knockdown efficiency. (A) The knockdown was translated to the protein level as shown for the full-length collagen XVI chain in cell lysates in comparison to the scr control and untreated U87MG cells. β -actin. protein expression is unaffected which indicates a specific knockdown of collagen XVI. N = 4 (B) Collagen XVI inhibited cells secreted less collagen XVI into the supernatant in comparison to scr controls. Ponceau Red stained blot membrane served as loading control. N=3 (C) Knockdown efficiency of *COL16A1* was analyzed with qRT-PCR and shows an average of 60% lower *COL16A1* gene expression in transfected U87MG cells (si1 and si2) compared to control cells transfected with scrambled RNA (scr). N = 19, si1= siRNA 1, si2 = siRNA 2, scr = scrambled RNA = controls.

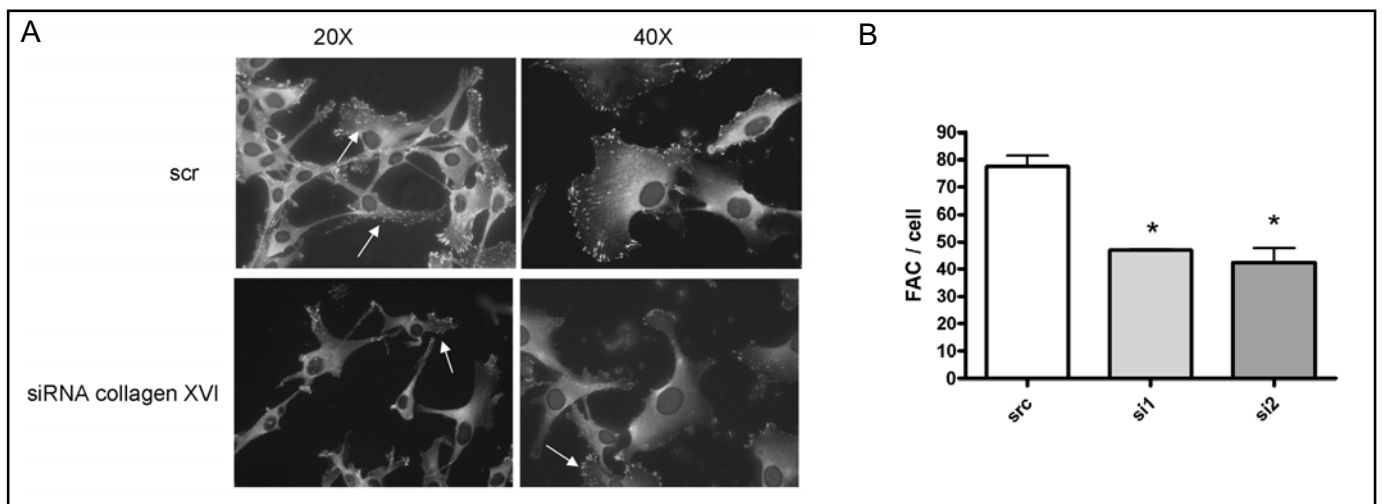


Fig. 2. Cell morphology and number of focal adhesion contacts. (A) U87MG cells were stained using vinculin antibodies to visualize cell morphology and focal adhesion contacts. Representative images (magnification 200x and 400x) demonstrate that in collagen XVI-knockdown cells FACs were found more concentrated in the cellular periphery while in control cells FACs (white arrows) appeared to be more evenly distributed. N = 4. (B) The number of FACs per cell is significantly decreased in collagen XVI knockdown cells. The evaluation of the number of focal adhesion contacts yielded a significant decrease of 40-45% of FACs per cell after inhibition of collagen XVI. A similar reduction of focal adhesion contacts was observed for both siRNAs. N = 4; * = $p < 0.05$.

by vinculin staining were affected in knockdown cells. Evaluation of the number of FACs per cell by counting the positive vinculin stained spots yielded a significant decrease of 40-45% of FAC number per cell in case of collagen XVI knockdown (Fig. 2B).

Proliferation properties

Analysis of cell proliferation assays revealed no differences between collagen XVI-knockdown U87MG cells and controls on various surface coatings (BSA and PLL as controls, 10 μ g and 30 μ g of recombinant collagen XVI) during the time line of the assay (2, 4 and 6 hours), (data not shown).

Cell invasion and adhesion potential

Collagen XVI-knockdown cells revealed a significantly reduced invasive potential in the Boyden Chamber assay compared to control cells. Around 50% less U87MG cells migrated through the microporous membrane coated with Matrigel after *COL16A1* gene expression was suppressed (Fig. 3A). Here, si1RNA mediated collagen XVI knock down reduced invasion more effectively compared to si2RNA. Adhesion of collagen XVI-knockdown cells to cell culture plastic revealed no differences compared to control cells after 4 hours and 6 hours of incubation (Fig. 3B). Therefore, decreased invasion rate is not a

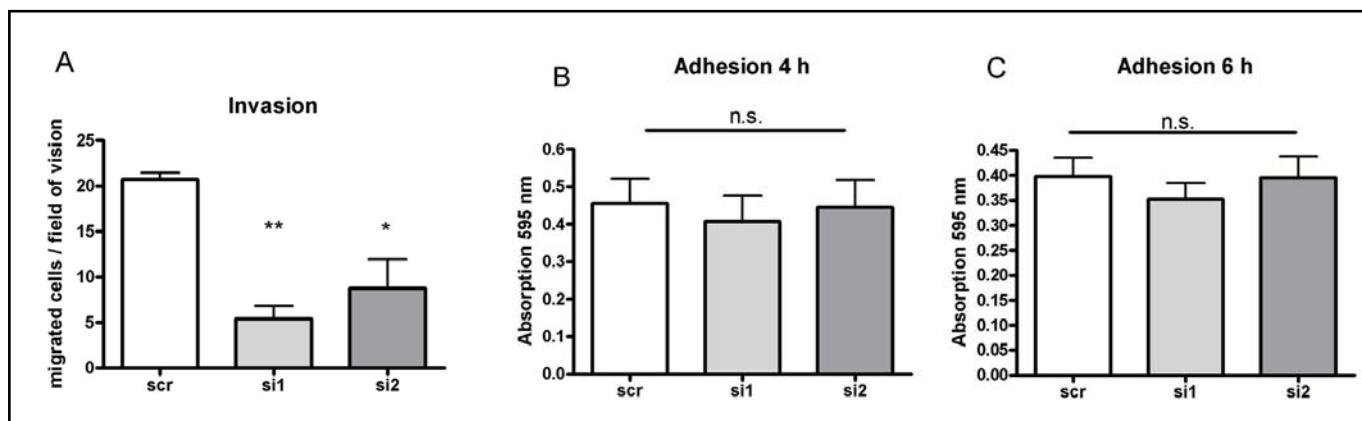
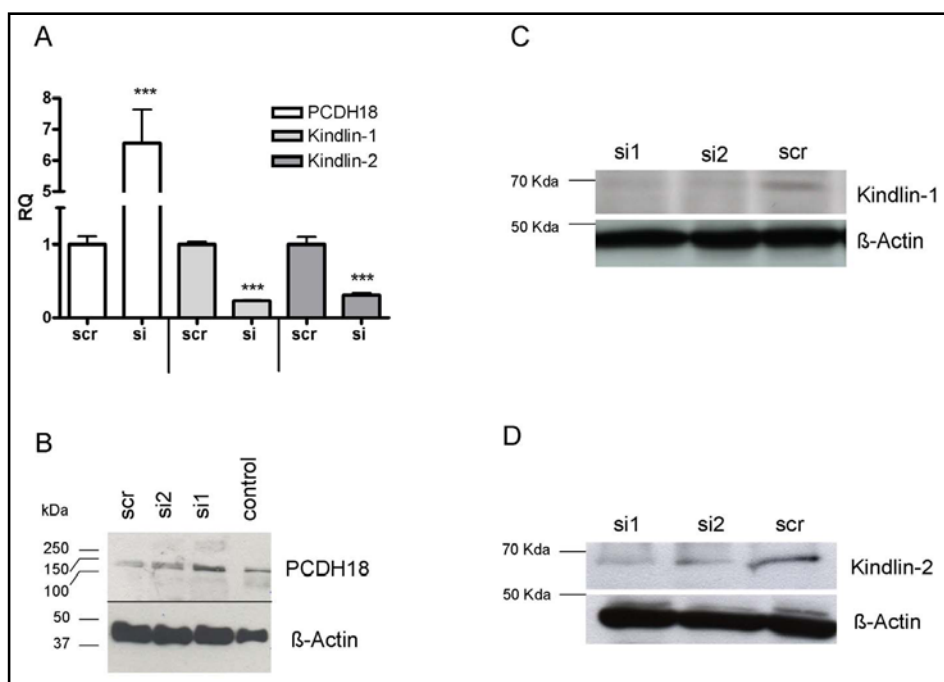


Fig. 3. Invasive potential of collagen XVI inhibited cells. (A) Downregulation of collagen XVI expression resulted in a reduced invasion of U87MG cells in a Boyden Chamber assay compared to scr-control cells. The number of cells migrated to the lower compartment after 4 hours was significantly decreased in knockdown cells compared to the control. N = 4; * = $p < 0.05$; ** = $p < 0.01$. (B, C) Adhesion capacity after 4 hours and 6 hours was not influenced by reduced collagen XVI expression. N = 6.

Fig. 4. Downstream gene expression analysis. (A) Gene expression of *PCDH18*, *kindlin-1* and *kindlin-2* was determined by qRT-PCR and evaluated using the $\Delta\Delta C_t$ method with GAPDH as endogenous loading control set to 0 and U87MG cells transfected with scrambled RNA as calibrator. *PCDH18* gene expression was profoundly upregulated whereas gene expression of *kindlin-1* and *kindlin-2* was clearly downregulated. N = 6. *** = $p < 0.001$. Protein expression of PCDH 18 (B), Kindlin-1 (C) and Kindlin-2 (D) was determined with western blotting analysis of cell lysates. β -Actin was used as internal loading control. N=3. Control: cell lysate from mouse lung.



consequence of increased cellular adhesion.

Analysis of downstream gene and protein expression

Quantitative real-time RT-PCR was carried out for analysis of gene expression of protocadherin 18 (PCDH18), kindlin-1 and kindlin-2. Relative gene expression analysis with the $\Delta\Delta C_t$ method demonstrated a profound upregulation of *PCDH18*. In addition to this, *kindlin-1* and *kindlin-2* mRNA levels were significantly reduced in collagen XVI-silenced cells (Fig. 4A). Analysis of protocadherin 18 protein expression in cell lysates revealed one clear band of the protein at about 126 kDa. Samples which were lysed 2

days post transfection generated a stronger signal for PCDH18 in collagen XVI-knockdown U87MG cell lysates compared to the scr controls (Fig. 4B).

Western blot analysis of Kindlin-1 (Fig. 4C) and Kindlin-2 (Fig. 4D) protein expression using cell lysates have shown that both molecules were inhibited on protein level in addition to mRNA level.

Analysis of matrix degrading enzymes MMP-2 and MT1-MMP

As MMP-2 and MT1-MMP are important proteases for cell metastasis and thus migration of cells we have analysed mRNA level of both enzymes (Fig. 5). Knock-down of collagen XVI did not affect gene expression

Fig. 5. Gene expression analysis for MMP-2 and MT1-MMP. mRNA level for MMP-2 (A) and MT1-MMP (B) were analyzed with specific Taqman probes for both enzymes. N=3.

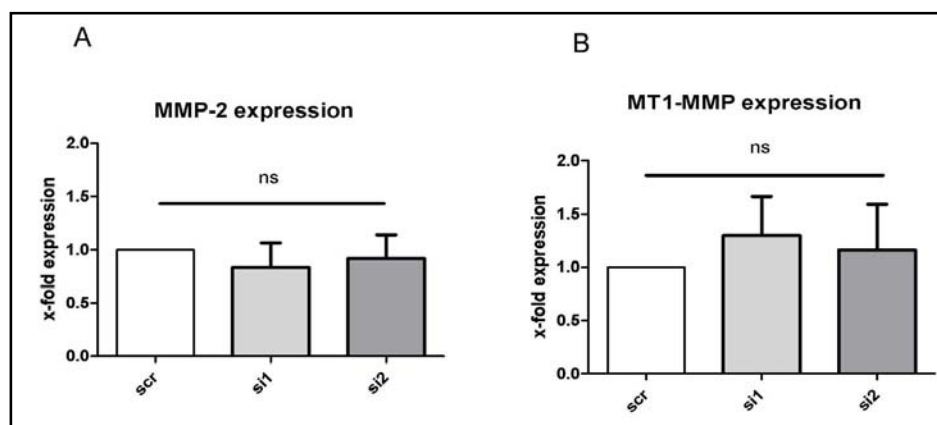


Fig. 6. Flow cytometric analysis of β 1-integrin expression and activation status. FACS analysis was performed to determine β 1-integrin expression and activation on collagen XVI-knock-down U87MG cells and scr controls 96 hours post transfection. (A) The antibody integrin beta1D (MAB2259Z) recognizes activated β 1-integrin only. Compared to controls β 1-integrin was about 50% less activated on collagen XVI-knockdown cells. (B) The antibody integrin beta1 (MAB1900) binds to total β 1-integrins (both activated and inactivated forms). Therefore, the result demonstrates that total expression of β 1-integrin was not altered on collagen XVI-knockdown cells. black line: Isotype control which is the secondary antibody without first antibody. si1= siRNA 1, si2 = siRNA 2, scr = scrambled RNA.

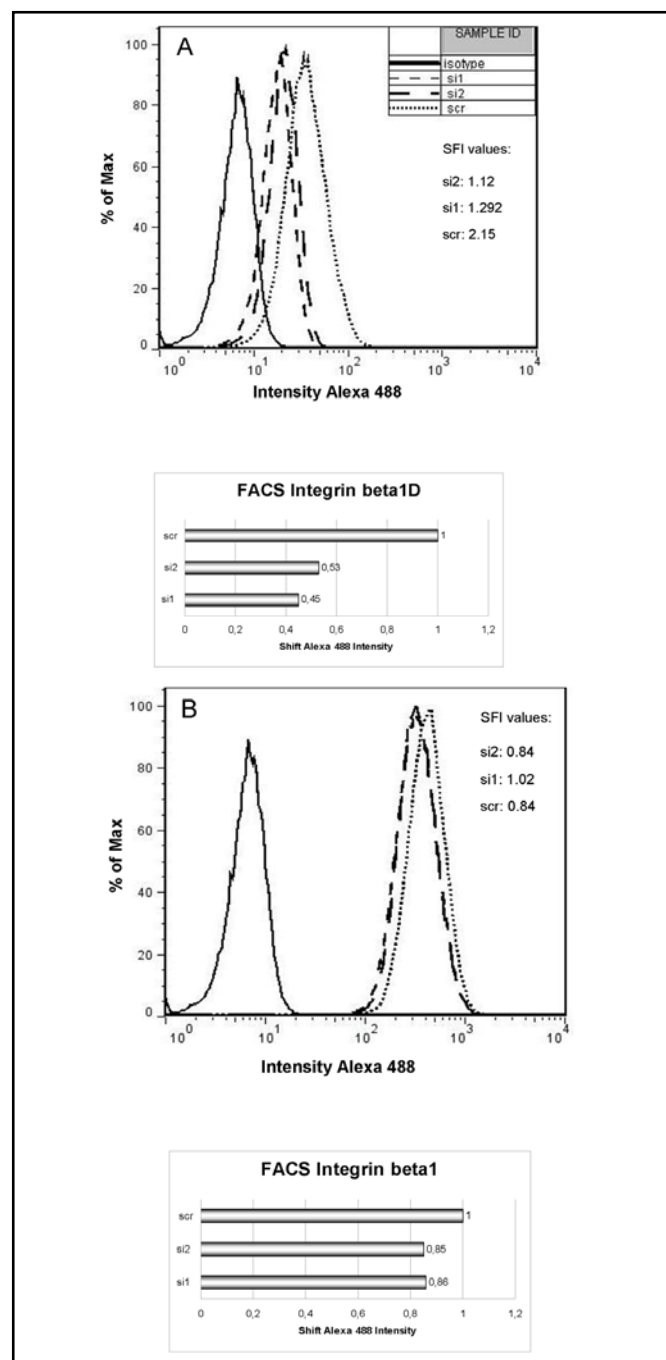
of MMP-2 (Fig. 5A) and MT1-MMP (Fig. 5B).

Analysis of β 1-integrin activation status

Flow cytometric analysis of collagen XVI-knock-down and control U87MG cells 96 hours post transfection revealed a clear reduction of the signal count for activated β 1 (antibody integrin beta1D recognizes the activated β 1-integrin form only) compared to control cells. Specific antibody binding signals to activated β 1-integrins were reduced of about 50% in collagen XVI-knockdown cells for both siRNAs (Fig. 6A). Notably, protein expression of total β 1-integrin was mainly unaltered (Fig. 6B).

Discussion

It is of great interest to unravel details of how a cancerous cell of the CNS interacts with the surrounding ECM. Here, the matrix bears a dual role as a physical barrier and as anchoring point for tractional forces. Delineating mechanisms of cell-to-cell and cell-to-matrix interactions is imperative in understanding the invasiveness of tumour cells in order to develop new therapeutical strategies in neurooncology. Thus, inhibition of collagen XVI



via RNAi in the tumour cell line U87MG provides a potent tool to analyze the role of this collagen in tumour development and metastasis. In a previous study this cell line has revealed high levels of collagen XVI gene and protein expression and also a strong interaction with recombinant collagen XVI in adhesion and migration assays [11].

ECM in the central nervous system is composed of molecules synthesized and secreted by neurons and glial cells. Normal human brain is largely free of a well-defined ECM [15], except for the perivascular space that represents a frequent site for glioma cell invasion [16]. Glioblastomas are defined by an accumulation of several large ECM-components, i.e. collagens I, IV, VI, and fibronectin, laminin, versican, decorin, and tenascin-C [17, 18]. It has been demonstrated that increasing concentrations of collagen I correlate positively with tumour invasiveness, but negatively with tumour growth. Functional studies have shown that migrating glioma cells are able to influence and alter their surrounding matrix by alignment, strain stiffening and breaking of specific components [19]. In contrast to normal cortex, expression of collagen XVI is highly upregulated in glioblastoma tissue mainly pericellular and around cerebral vessels adjacent to the basement membrane. Profound upregulation of collagen XVI expression in glioblastomas suggests that collagen XVI within the tumour-specific ECM is of crucial importance for the development and/or establishment of tumours. As there is evidence that collagen XVI is involved in signal transduction and cell-matrix interaction [4], it may be a prerequisite for ensuring tumour invasion and metastasis. Major matrix components like fibrillar collagens occur in high quantities compared to minor components like FACIT-collagens. However, members of this collagen family act as linker proteins between fibrillar structures and extra-fibrillar matrix via other adapter molecules like COMP or matrilins [2, 20] and thus are crucial for formation and stability of large fibrillar networks.

In vitro studies have shown the importance of fibronectin, laminin, collagen IV and other ECM proteins in stimulating a migratory phenotype in glioblastoma cell lines [21]. In this line, knockdown of collagen XVI in U87MG cells did result in a reduced invasive potential. Of note, overexpression of collagen XVI in PCI13, a representative oral squamous cell carcinoma (OSCC) cell line, strongly induced cellular invasiveness [22]. Decreased invasiveness of collagen XVI-inhibited U87MG cells is not due to increased matrix adhesion as this is not changed during the time line (4-6 hours) of the invasion

assay. Glioma cell invasion is regarded to occur along ECM protein-containing structures, such as along tracts of myelinated fibres [23-25]. Collagen XVI seems to be a preferred substrate for glioma cells and might serve as a migratory tract in a rearranged matrix micro-environment. Cellular migration involves initial protrusion of the leading edge, formation of adhesive sites, contraction of the cell, and release of adhesive sites at the cell rear [26]. Adhesive sites are variable in size and molecular composition. In focal adhesion contacts (FACs), integrins, actin stress fibers, other structural proteins, and signalling molecules accumulate to form a multi-protein complex [27]. Immunofluorescence staining displays a clear reduction in the number and alteration in distribution of FACs in collagen XVI-knockdown cells. Adhesion of cells is a prerequisite for cellular migration and invasion, including creation of space and the ability to move into this space. The process of cell migration involves dynamic interactions between the cell and its ECM. The $\alpha\beta$ -integrin heterodimers are able to bind non-covalently to structures of many ECM proteins and serve as transmembrane linker. For collagen XVI high affinity binding sites to $\alpha1\beta1$ - and $\alpha2\beta1$ - integrins have been identified in the C-terminal region of the molecule [4]. The $\beta1$ chain appears to be a critical component, as it is capable of hetero-dimerization with collagen XVI binding α -subunits. Of note, in PCI13 collagen XVI overexpressing OSCC cells $\beta1$ -integrin activity is induced together with an increased $\alpha1$ -integrin expression [22]. Additionally, we demonstrated that myofibroblasts isolated from inflamed colon regions of Crohns Disease patients induced expression of collagen XVI together with $\alpha1$ -integrin expression while $\alpha2$ -integrin was unaffected [13].

Intracellularly, the $\beta1$ -integrin subunit interacts with kindlins in a multiprotein complex which is part of the cytoskeleton [28]. Thereby contractile tension forces are generated that induce morphological changes and cellular motility [16]. On the other hand, kindlins are able to induce integrin-ECM interactions by activating integrins and the subsequent generation and induction of the formation of FACs. An Affimetrix cDNA microarray approach (human genome U133 Plus 2.0 Array performed for two knockdown cDNAs and two non-interfering cDNA samples of U87MG; data not shown) revealed a significant down-regulation of kindlins among other genes in collagen XVI-knockdown cells. Comparison of collagen XVI-knockdown and control cells by quantitative PCR confirmed this observation for *kindlin-1* and *kindlin-2*. These results are in line with a clearly decreased $\beta1$

integrin activity but unchanged total expression of the $\beta 1$ -integrin subunit on the cell surface in collagen XVI-knockdown U87MG cells.

Of note, cell-matrix interactions are inverse regulated to cell-cell interactions which are mediated via cadherins among other cell adhesion molecules. Cadherins are cell-cell contact molecules that mediate Ca^{2+} -dependent cell-cell adhesion and are localized in adherence-type junctions. They can form bridges to the actin cytoskeleton and play an important role in the organization of tissues. Protocadherin 18 (PCDH18), a member of the delta-protocadherin family in brain [29, 30] belongs to the cell adhesion molecule family which is frequently downregulated in cancers [31], proved to be strongly up-regulated in collagen XVI knockdown cells. Members of the protocadherin family as PCDH8, -10, -17 and -20 are suggested to functioning as tumour suppressor genes as silencing in carcinomas induces cell proliferation and migration/ invasion of tumour cells. A recent study demonstrated a tumour suppressor role of PCDH10 with inhibition of the growth, migration, and invasion of nasopharyngeal cancer cells upon overexpression [32-34]. In line with this observation is that in high-grade glioblastomas an upregulation of N-cadherin correlates with a dramatic decrease in invasive behaviour [35]. Here, we show that after inhibition of collagen XVI expression protocadherin 18 upregulation correlates with a significant decrease in invasive potential of U87MG cells.

Tumour cell growth and invasion in the CNS needs complex interactions between malignant cells, glial cells, neuronal cells and endothelial cells. It is discussed whether an invasive cell utilizes constitutively pre-existing brain ECM for migration, or if it is actively producing and secreting its own matrix for subsequent invasion [36]. The enzymatic profile of tumour cells may also have an enormous impact on the integrity of ECM components. Here, we were unable to detect an impact of collagen XVI on

regulation of gene expression level of the two important MMPs, MMP-2 and MT1-MMP, in tumour metastasis. However, it has been demonstrated for several FACIT collagens, that single isolated collagen domains can exhibit anti-tumour activity [37]. As recombinant [14] and authentic [38] collagen XVI might be proteolytically cleaved into fragments with distinct molecular weights, it should be the focus of follow up studies to investigate the effect of tumour associated enzymes, i.e. MMPs, on the cleavage of collagen XVI and the effect of singular domains of collagen XVI on tumorigenesis.

Our results suggest that glioma cells in fact create their own microenvironment by upregulating expression and biosynthesis of specific macromolecules as collagen XVI. We demonstrated that loss of collagen XVI alters glioma cell invasion. Reduced invasiveness of collagen XVI-knockdown U87MG cells might be correlated with increased PCDH18 expression which ensures increased cell-to-cell contact. Increased cell-to-cell interaction presumes reduced cell-matrix interaction as demonstrated by reduced invasion. This observation is supported by reduced kindlin-1/-2 gene and protein expression which results in reduced integrin $\beta 1$ activation without alteration of total $\beta 1$ -integrin protein expression

Further functional studies on the invasive glioblastoma phenotype in order to characterize the link between collagen XVI and downstream signalling molecules which directly mediate cell-to-cell and cell-to-matrix interactions are necessary to improve strategies of anti-invasive cancer therapies.

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