FKZ 2734 : In vitro gene silencing von Kollagenen in Nerven- und Gliazellen

Antragstellerin: Prof. Dr. Susanne Grässel

Stipendiatin: Dr. Sabine Ratzinger, Dipl.-Biol. Lynn Wales

Orthopädische Klinik für die Universität Regensburg, Abt. Experimentelle Orthopädie, ZMB im BioPark 1, Josef-Engert-Str. 9, D-93053 Regensburg

Introduction

Collagen XVI is a member of FACIT collagens and constitutes a minor component of the ECM of skin and cartilage. It is integrated into distinct fibrillar aggregates such as fibrillin-1 containing microfibrils in skin and D-banded cartilage fibrils (Grassel et al., 1999; Kassner et al., 2003). The presence in the dermal epidermal junction (DEJ) zone of papillary dermis may indicate its active role in anchoring microfibrils to basement membranes. By connecting to ECM proteins as well as to the cytoskeleton, collagen XVI is likely to be able to move these proteins and hence affect ECM networks, ensuring mechanical anchorage of the cell and signal transfer.

A strong expression of collagen XVI was detected in human dorsal root ganglia during the developmental phase as well as during regeneration after nerve injury (Hubert et al., 2006). This may indicate that both maturation and regeneration of nerve cells require an interaction between neurons and the ECM, in which the specific role of collagen XVI is not known to date. In the same line, we have demonstrated a strong upregulation of collagen XVI in several glioma cell lines *in vitro* and also *in situ* in glioblastoma tissue, the most common malignant brain tumour which is characterized by aggressive tumour cell invasion into its surrounding tissue. Compared to other cell lines, the glioma cell line U87MG showed strongest adhesion on collagen XVI as substrate (Senner et al., 2008). These glioma cell lines possibly use collagen XVI to ameliorate their adhesion. Cell-matrix interactions are widely 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 (Hynes, 2002). The major collagen binding integrins, $\alpha1\beta1$ and $\alpha2\beta1$, are expressed on glioma cell surfaces and contribute to cell adhesion and migration (Paulus et al., 1993; Paulus et al., 1996), hence these integrins are likely to be an important link in terms of interaction of cells with the ECM via collagen XVI which binds to both integrins (Eble et al., 2006).

Here, we show that collagen XVI gene silencing in the glioblastoma cell line U87MG regulates a range of other genes involved in cell metabolism, signal transduction or cell-

matrix adhesion, furthermore it causes a substantive decrease of focal adhesion contacts, which are vital for cell interaction and intracellular processes. Collagen XVI gene silencing influences the invasive behaviour of the cells, implying this collagen to be an important player in tumorigenesis and metastasis of glioblastoma. Our data provide evidence for collagen XVI to play a crucial role in interaction between the ECM, the plasma membrane and the cytoskeleton during tumorigenesis.

Results report

Knockdown efficiency

Transfection of the U87MG cells was performed by electroporation. The cells were transfected with two collagen XVI-specific siRNAs (Senner et al., 2008). Transfection efficiency was evaluated 48 hours after transfection. Gene expression level was determined by quantitative real-time PCR with SYBR Green Dye I on the MX3005 Taqman (Stratagene) in triplicates, using cDNA that referred to 25ng 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 cDNA copy numbers and to determine knockdown efficiency. In general, a knockdown of ~60 % was obtained in comparison to the control cells which were transfected with scrambled siRNA (Fig. 1 A).

The knockdown was also evaluated on protein level with immunoblotting loading 20 µg total protein per gel lane. After completing SDS-PAGE, separated proteins were transferred to nitrocellulose membranes and hybridized with a collagen specific antibody (Kassner et al. 2004) and a ß- actin specific antibody as loading control. Immunoreactive bands were detected with a chemiluminescence ECL reagent. A reduction of the full-length collagen XVI chain was detected in the cells transfected with the siRNAs (Fig. 1B).

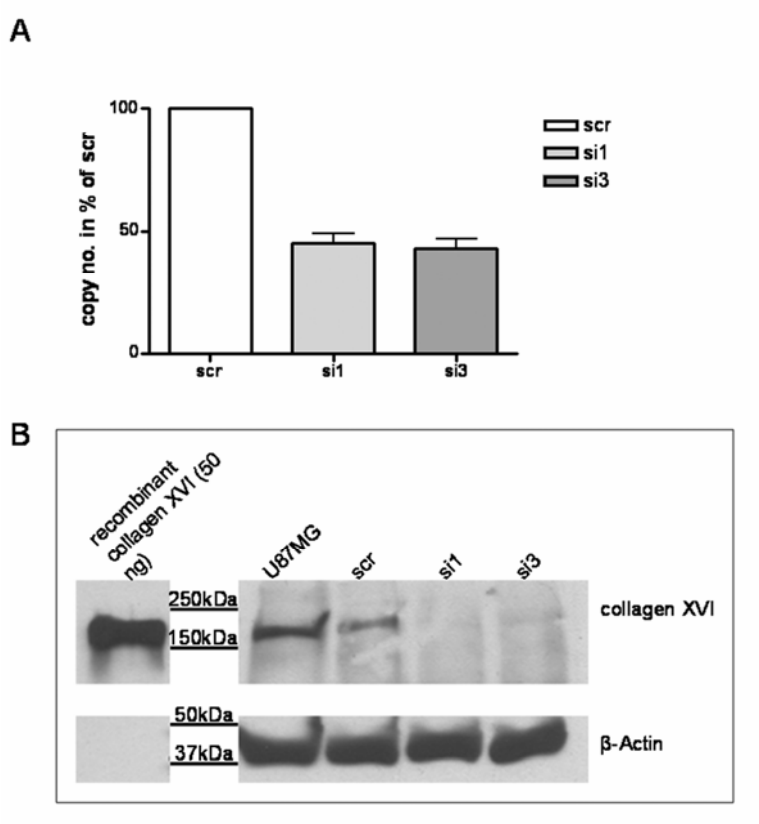

Figure 1: Analysis of knockdown efficiency of *COL16A1*

(A) Knockdown efficiency of *COL16A1* was performed at mRNA level with quantitative PCR and shows ~60% lower *COL16A1* gene expression in transfected cells compared to control cells which were transfected with scrambled RNA.
(B) The knockdown was also manifest on protein level with respect to the full-length collagen XVI protein. ß-actin expression remained unaffected which indicates a specific knockdown of collagen XVI.
scr = scrambled RNA (control)
si1 = Col16A1 specific siRNA 1
si3 = Col16A1 specific siRNA 3

Cell morphology

For examination of the cell morphology, transfected cells were grown on chamber slides over night. For immunohistological staining, both control and collagen XVI-silenced cells were fixed with PFA and stained with anti vinculin antibodies and phalloidin (actin cytoskeleton). Fluorescence microscopy using a confocal laser scanning microscope, revealed no morphological differences between control cells and knockdown cells (Fig. 2A). However, focal contacts (FACs) represented by vinculin staining showed a variation in their distribution. Knockdown cells (si1 and si3) revealed a more pronounced accumulation around the cell nucleus, while the staining in control cells (scr) was more scattered and spread out towards the cellular periphery. The number of FACs was obtained by counting the vinculin

stained structures in different visual fields chosen at random on the slides and then determining the average number of FACs per cell. This evaluation yielded a significant decrease of 40-45% of FACs per cell after silencing of collagen XVI (Fig. 2B). The reduction of FACs in collagen XVI knockdown cells suggests a decrease of cell contacts to their surrounding matrix environment.

Apart from this observation the overall morphology of the cells as well as the arrangement and composition of the actin filaments, visible as red fluorescence, appeared to be unaltered between control cells and collagen XVI silenced cells.

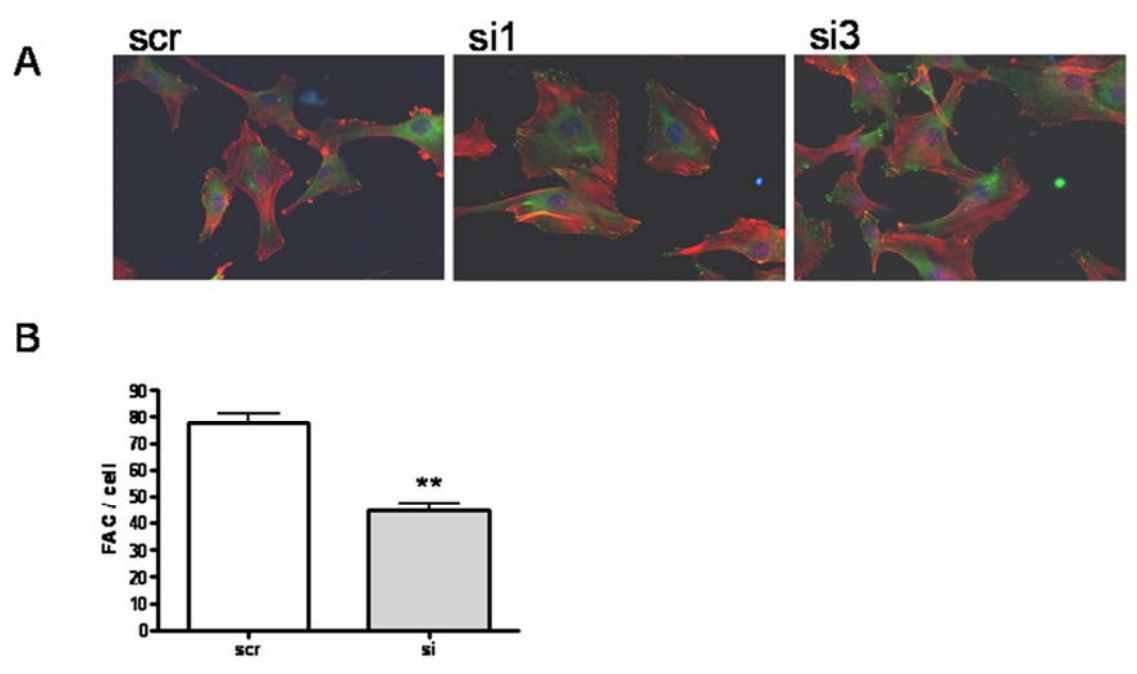

Figure 2: Cell morphology and number of focal adhesion contacts (FACs)

(A) Cells were stained using an anti-vinculin antibody and phalloidin to visualize focal adhesion contacts and actin stress fibres. Collagen XVI silenced cells contained more focal adhesion contacts around the cell nucleus compared to the cellular periphery. In contrast, vinculin staining in control cells was more diffuse and preferentially found in the cellular periphery. Magnification: 40x

(B) The evaluation of the number of focal adhesion contacts yielded a significant decrease of 40-45% of FACs per cell after silencing of collagen XVI. Data of both siRNAs have been combined.

Proliferation properties

96-well plates were coated with poly-L-lysine, collagen I and recombinant collagen XVI over night and U87MG cells were cultivated on the different substrates over night in a density of 7,500 cells/well. EZ4U proliferation assay, based on conversion of tetrazolium salt, was

performed according to the manufacturers' protocol for 2 hours. Tetrazolium is reduced to formazan by active succinatdehdrogenase of living cells and absorption was determined at 450 nm and a reference wavelength of 600 nm. The assay was performed in triplicates.
Analysis of proliferation potential did not show any differences irrespective of the knockdown or of the culture substrates (data not shown).

Cell invasion potential

Differences in the invasiveness of collagen XVI-silenced cells to control cells were investigated by means of a Boyden Chamber Invasion assay. The system is based on two medium-filled compartments separated by a microporous membrane. U87MG 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 hours, the membrane between the two compartments was fixed and stained to visualize cytoplasm and nuclei. The number of cells that were migrated to the lower side of the membrane was determined with light microscopy in five independent fields of view for every experiment.
Collagen XVI-silenced cells revealed a reduced invasive potential in the Boyden Chamber Assay compared to control cells. About 50% less U87MG cells migrated through the microporous membrane when collagen XVI was silenced (Fig. 3A).

Cellular adhesion

Knockdown and control cells were seeded at a density of 7,500 cells/well into a 96-well plate and were allowed to adhere for 4 hours and 6 hours. Fixed cells were stained with 0.02 % (w/v) crystal-violet solution. Bound crystal violet was removed by incubation for 3 hours at a horizontal shaker at 100 rpm with 70 % ethanol. Crystal violet adsorption was measured at 595 nm and each sample was performed in triplicates. Adhesion potential of knockdown cells revealed no differences compared to control cells after 4 hours and 6 hours of incubation (Fig. 3B and C).

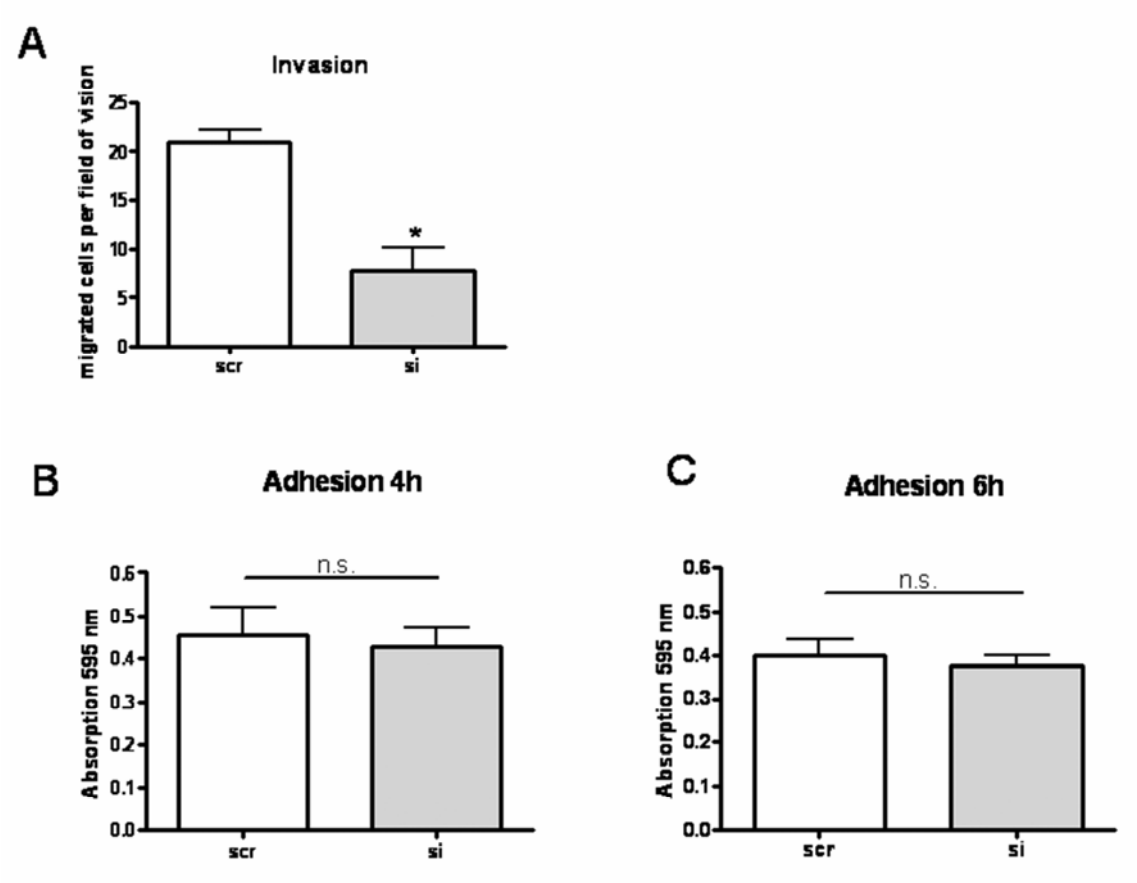

Figure 3: Invasive potential of collagen XVI - silenced U87MG cells

(A) Silencing of collagen XVI resulted in a reduced migration of cells in a Boyden Chamber Assay compared to control cells. The number of migrated cells was significantly decreased in siRNA transfected cells after 4 hours.
Adhesion assay of U87MG cells on cell culture plastic surfaces. Adhesion was evaluated after 4 hours (B) and after 6 hours (C).

Quantification of selected genes

Quantitative RT-PCR was carried out for protocadherin 18 (PCDH18), kindlin-1 and kindlin-2. Relative quantification was evaluated by the ΔΔCt method using an internal calibrator (GAPDH) as endogenous control. The mean relative quantification (RQ) values and absolute copy numbers were calculated using the detection software MxPro v 4.1.

Relative gene expression analysis with the ΔΔCt method resulted in a clear upregulation of *pcdh18.* In addition, the examination of the genes *kindlin-1* and *kindlin-2* showed reduced levels of gene expression in collagen XVI-knockdown cells (Fig. 4).

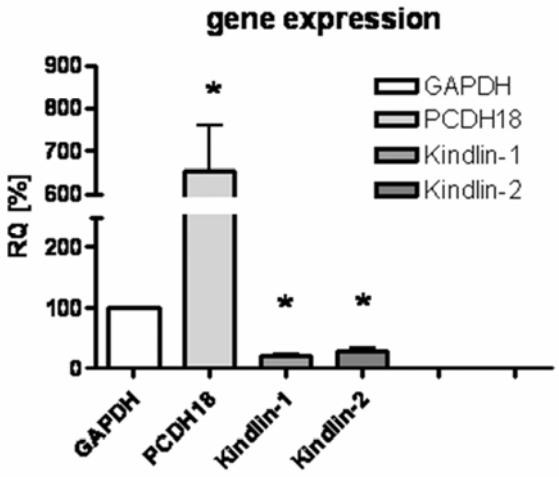

Figure 4: Downstream gene expression analysis

Gene expression of *PCDH18, kindlin-1* and *kindlin-2* was evaluated by quantitative PCR using the relative ΔΔCt method with GAPDH as endogenous control and U87MG cells transfected with scrambled RNA as calibrator. Gene expression was upregulated for *PCDH18* whereas mRNA levels of *kindlin-1* and *kindlin-2* were clearly decreased.

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 with Tukey's multiple comparison test for gene expression results and unpaired student's t-test for number of focal adhesion contacts and invasion and adhesion assay.

Discussion

It is of great interest in cell biology to unravel details of how a cancerous cell interacts with the surrounding extracellular matrix (ECM). The matrix bears a dual role as a physical barrier as well as anchoring point for tractional forces and the understanding of the invasiveness of tumour cells is important for developing new strategies in neurooncology. ECM in the brain is composed of molecules synthesized and secreted by neurons and glial cells. The protein production is cell-type-specific and activity-dependent and determines proliferation, migration, and differentiation of neuronal cells. Normal human brain is largely free of a well-defined ECM (Rutka et al., 1988), except for the perivascular space that represents a frequent site for glioma cell invasion (Uhm et al., 1999). Mechanistical studies have proven, that migrating glioma cells are able to change the surrounding matrix and align it, strain stiffen it and break certain components in it (Kaufman et al., 2005). In contrast to normal cortex, the FACIT collagen type XVI was strongly upregulated in glioblastoma tissue and glioma cell

lines and was demonstrated to promote tumour cell adhesion (Senner et al., 2008). Upregulation of collagen XVI in tumour tissue suggests that collagen XVI within the tumour-specific ECM is of importance for the development and establishment of tumours.

So far it is not clear whether an invasive cell utilizes constitutive brain ECM for migration, or if it is secreting its own matrix for subsequent invasion (Tysnes and Mahesparan, 2001), however, our results suggest that glioma cells are synthesizing their own environment (Senner et al., 2008) and data in this report. Knockdown of collagen XVI in the tumour cell line U87MG provides a potent *in vitro* tool to analyze the role of this collagen in tumour development and metastasis.

We demonstrate that a knockdown of collagen XVI in U87MG cells resulted in a decrease of FACs that way suggesting that collagen XVI promotes matrix adhesion of glioma cells. Additionally, silencing of collagen XVI leads to a reduced invasive potential suggesting that collagen XVI supports migration of tumour cells. *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 (Mahesparan et al., 1999). Glioma invasion is regarded to occur along ECM protein-containing structures, such as along tracts of myelinated fibres (Bernstein et al., 1990) (Laws, Jr. et al., 1993) (Giese et al., 1996). Possibly, collagen XVI as an adhesion substrate for glioma cells, facilitates rearrangement of ECM and serves at migratory tract. Adhesion of cells is a prerequisite of 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. In so-called focal adhesion contacts (FAC), integrins, actin stress fibers and other structural proteins, and signalling molecules accumulate (Burridge and Chrzanowska-Wodnicka, 1996). Intracellularly, the β-integrin subunit interacts with kindlins and other proteins of the cytoskeleton (Harburger et al., 2009). Comparison of knock-down and scrambled cells via qRT-PCR revealed profound down-regulation of *kindlin-1* and *kindlin-2.* We assume kindlins to activate integrins and induce the formation of FAC in the presence of collagen XVI. Notably, cell-matrix interaction is inverse to cell-cell interaction. Protocadherin-18 (*Pcdh18)* proved to be up-regulated in collagen XVI knockdown cells. Cadherins are cell adhesion molecules that mediate Ca^{2+}-dependent cell-cell adhesion and are localized in adherence-type junctions. They form bridges to the actin-containing cytoskeleton and play an important role in tissue organization. A loss of cadherin expression in epithelial tumours is associated with an increased invasive and malignant phenotype (Rosales et al., 1995) (Asano et al., 1997). In

highly malignent gliomas upregulation of N-cadherin correlates with a dramatic decrease in invasive behaviour (Duntsch et al., 2004).

In this study, we could demonstrate that collagen XVI is engaged in tumour cell invasion and adhesion, possibly by modulating integrin activity through down regulation of kindling gene expression. The molecular basis of how collagen XVI regulates gene expression of kindlins and protocadherin remains to be elucidated. Further functional studies on the invasive glioma phenotype, characterized by an altered collagen XVI expression will help to develop alternative strategies of anti-invasive therapies.

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